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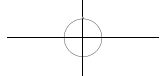
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Table of Contents

- 1 Analysis of Influencing Factors of Moderate to Severe Malnutrition in Elderly Patients with Lung Cancer during Chemotherapy**
Ying Li, Jiaying Qin, Ying Gao, Junying Nie

- 7 Primary Malignant Melanoma of the Larynx: Case Report and Literature Review**
Pan Wang, Yashan Wu, Pei Zhang, Yanan Wang

- 11 Exploring Novel Therapeutic Breakthroughs for Cancers: Potential Roles of miRNAs**
Muhammad Abubakar, Rooma Ayyoub, Baqaur Rehman, Muhammad Naeem Kiani, Usama Ahmed, Jawaria Farooq, Areeb Anwar Usmani

- 25 Effect of Jianpi Wenshen Granules on the Nutritional Control Status Score in Elderly Patients Undergoing Maintenance Hemodialysis**
Hailiang Ye, Teng Ma, Yan Zhou, Taoli Li, Hong Zhu

- 32 Advancing Cancer Stem Cell-Targeted Therapeutic Applications**
Muhammad Abubakar, Rooma Ayyoub, Baqaur Rehman, Muhammad Zubair, Usama Ahmed

- 46 The Effect of Immunotherapy on the Gut Microbiota, Intestinal Barrier, and Immune Function in Patients with Gastric Cancer**
Kui Chen, Yan Hu

- 52 Exploring the Pivotal Association of AI in Cancer Stem Cells Detection and Treatment**
Muhammad Abubakar

- 64 Advancements and Challenges in Biomarkers for Colorectal Cancer Detection: A Comprehensive Review**
Yasir Hameed

- 76 Clinical Analysis of Acupuncture Combined with Acupoint Injection for the Treatment of Chronic Nonbacterial Prostatitis**
Xizhe Sun, Xiwen Yu, Chen Fan, Meixun Chen, Na Dao, Chengxin Hao, Jiamei Wu

- 82 Immunoregulatory Effect and Mechanism of Epigallocatechin-3-Gallate in A Mouse Oral Cancer Model**
Yizhen Li, Siyi Huang, Yanzi Ling, Liyan Fu, Ruyue Zheng, Xinwei Duan, Yueji Luo

- 89 Ultrasonic-Assisted Extraction of Fucoxanthin from Marine Macroalga *Padina australis*: Optimization, Bioactivity, and Structural Characterization**
L. Antony Catherine Flora, Kirubanandan Shanmugam, Renganathan Sahadevan
- 105 A Pan-Cancer Analysis of GAPDH as a Common Biomarker for Various Cancers**
Yasir Hameed
- 121 ATM is a Prognostic Biomarker of Survival in Head and Neck Squamous Cell Carcinoma Patients**
Muhammad Umair Abid
- 136 A Case of *Lautropia mirabilis* Infection in a Lung Transplant Patient and a Review of the Literature**
Kaijin Wang, Jinqian Liu, Qingdi Xia, Bicui Liu, Yishan Dong, Haiyan Cen, Shujun Yi
- 141 Clinical Application Study of Super-Mini Percutaneous Nephrolithotomy in the Treatment of Kidney Stones**
Zehong You, Wenyong Lian
- 146 Expression and Prognostic Potential of *ESR1* in Breast Cancer**
Muhammad Akram
- 159 Study on the Expression, Prognosis, and Clinical Features of *HOXA6* in Liver Cancer**
Huiqiong Zhu
- 165 Roles of Mutant *TP53* Gene in Cancer Development and Progression**
Muhammad Abubakar, Baqaur Rehman
- 182 The Role of MSCT in Evaluating Tumor Size, Density, Immunohistochemical Classification, and Pathological Risk in GIST Patients**
Yi Luo, Jiaman Li, Pengman Chen, Junmao Huang, Shuisheng Yi, Zhenfeng Wen, Xinliang Chen, Xiangtuan Kong
- 189 Corrigendum: EGFR Mutation and FHIT Methylation: Inverse Relationship in Patients with Lung Adenocarcinoma and Tuberculosis**
Mireguli Abudurehman, Xiyou Yan, Baidurula Ainitu

Analysis of Influencing Factors of Moderate to Severe Malnutrition in Elderly Patients with Lung Cancer during Chemotherapy

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Abstract: *Objective:* This study aims to identify the factors influencing moderate to severe malnutrition in elderly lung cancer patients undergoing chemotherapy and to provide a basis for developing clinical prevention and intervention strategies. *Methods:* The clinical data of 150 elderly lung cancer patients who received chemotherapy were retrospectively analyzed. Demographic data, clinical characteristics, laboratory indicators, treatment plans, and other relevant information were collected. Independent risk factors for moderate to severe malnutrition during chemotherapy were identified. *Results:* During chemotherapy, 50 patients (33.33%) developed moderate to severe malnutrition. The BMI of patients with moderate to severe malnutrition was significantly lower than that of patients with no or mild malnutrition (21.20 ± 1.60 vs. 26.14 ± 2.31 , $P < 0.001$), and the proportion of patients with stage IV tumors was significantly higher (60.00% vs. 27.00%, $P < 0.001$). Serum pre-albumin (pre-ALB) and hemoglobin (Hb) levels in patients with moderate to severe malnutrition were significantly lower than those in patients with no or mild malnutrition (152.67 ± 30.41 g/L vs. 252.47 ± 51.24 g/L and 102.44 ± 10.09 g/L vs. 154.21 ± 15.18 g/L, respectively, $P < 0.001$ for all). *Conclusion:* Low BMI, decreased serum pre-ALB levels, and decreased serum Hb levels before chemotherapy are independent risk factors for moderate to severe malnutrition in elderly lung cancer patients during chemotherapy. Close clinical attention should be given to these patients, with early intervention measures such as nutritional support to reduce the incidence of malnutrition and improve patients' quality of life and prognosis.

Keywords: Lung cancer; Chemotherapy; Moderate to severe malnutrition; Influencing factors

Online publication: September 10, 2024

1. Introduction

Lung cancer is one of the most prevalent malignant tumors globally, with the highest morbidity and mortality rates, posing a severe threat to human life and health. Each year, approximately 2.2 million new cases and 1.8 million deaths from lung cancer are reported worldwide, with elderly patients making up a significant proportion of these numbers ^[1,2]. As the global population continues to age, the number of elderly lung cancer

patients is steadily increasing. Chemotherapy, a critical treatment for advanced lung cancer, plays a vital role in inhibiting tumor cell proliferation and spread, thereby prolonging patient survival. Chemotherapy drugs achieve this by inhibiting DNA replication, interfering with cell division, and other mechanisms to control tumor growth and metastasis. However, while targeting tumor cells, chemotherapy drugs inevitably damage normal cells, leading to a range of toxic side effects, including bone marrow suppression, nausea and vomiting, hair loss, and decreased immune function, with malnutrition being one of the most common complications ^[3].

Elderly lung cancer patients are particularly susceptible to malnutrition due to factors such as physiological degeneration, multiple comorbidities, decreased appetite, and the gastrointestinal toxicity of chemotherapy drugs ^[4]. The aging digestive system reduces the capacity for nutrient digestion and absorption, making elderly patients more prone to nutrient deficiencies. Additionally, these patients often suffer from other chronic conditions, such as hypertension, diabetes, and coronary heart disease, which, along with their respective treatments, can further negatively impact their nutritional status. Research has shown that moderate to severe malnutrition not only diminishes the quality of life in elderly lung cancer patients but also exacerbates the toxic side effects of chemotherapy, reduces chemotherapy tolerance, and diminishes its efficacy, ultimately affecting patient survival and prognosis ^[5]. Malnutrition can weaken the immune system, increasing susceptibility to infections and other complications, thereby hindering the smooth administration of chemotherapy. Furthermore, malnutrition can affect the metabolism and excretion of chemotherapy drugs, intensifying toxic side effects and further reducing chemotherapy tolerance.

Therefore, early identification of risk factors for moderate to severe malnutrition in elderly lung cancer patients during chemotherapy and the implementation of targeted interventions are crucial for improving nutritional status, enhancing chemotherapy efficacy, and extending patient survival. This study retrospectively analyzed the clinical data of 150 elderly lung cancer patients to identify the factors influencing moderate to severe malnutrition during chemotherapy and to provide a reference for early clinical intervention and improved patient prognosis.

2. Materials and methods

2.1. General information

A total of 150 elderly lung cancer patients who received chemotherapy at our hospital from October 2022 to October 2023 were selected for this study. All patients had been diagnosed with lung cancer through pathology or cytology and had undergone at least three cycles of chemotherapy. The inclusion criteria were as follows: (1) Patients aged ≥ 65 years; (2) Patients diagnosed with lung cancer via pathology or cytology ^[6]; (3) Patients who had completed at least three cycles of chemotherapy; (4) Patients with complete clinical data. The exclusion criteria were: (1) Patients with other malignant tumors; (2) Patients with severe liver or kidney dysfunction; (3) Patients with mental disorders or impaired consciousness who were unable to cooperate with the study.

2.2. Methods

The following patient data were collected:

- (1) General information: age, gender, BMI, smoking history (yes or no), comorbidities (hypertension, diabetes, coronary heart disease, etc.);
- (2) Clinical characteristics: tumor stage (TNM staging, classified as stages I–IV), pathological type (adenocarcinoma, squamous cell carcinoma, small cell carcinoma, etc.), chemotherapy regimen (chemotherapy drug type, dosage, number of cycles, etc.);

- (3) Laboratory indicators: blood routine, including hemoglobin (Hb) level, white blood cell count, platelet count, etc.;
- (4) Biochemical indicators: serum albumin (ALB) level, serum prealbumin (pre-ALB) level, alanine aminotransferase, aspartate aminotransferase, total bilirubin, creatinine, etc.;
- (5) Nutritional status assessment: Nutritional status before chemotherapy was assessed using the NRS2002 score ^[7], and patients were divided into three groups based on the scoring results: no malnutrition (NRS2002 score ≤ 2 points), mild malnutrition (NRS2002 score = 3 points), and moderate to severe malnutrition (NRS2002 score ≥ 4 points). The NRS 2002 nutritional risk screening scale was used to assess the nutritional risk of patients, categorizing them into no/mild malnutrition (0–2 points) and moderate to severe malnutrition (≥ 3 points).

2.3. Observation indicators

- (1) The incidence of moderate to severe malnutrition in elderly lung cancer patients during chemotherapy. The diagnostic criteria for moderate to severe malnutrition were based on a comprehensive assessment of the patient's weight change, BMI index, and laboratory indicators. Moderate to severe malnutrition was diagnosed if any of the following criteria were met: (i) Weight loss $\geq 5\%$; (ii) BMI $< 18.5 \text{ kg/m}^2$; (iii) Serum albumin $< 30 \text{ g/L}$; (iv) Hemoglobin $< 100 \text{ g/L}$.
- (2) Factors influencing the occurrence of moderate to severe malnutrition in elderly lung cancer patients during chemotherapy.

2.4. Statistical analysis

SPSS 27.0 software was used for statistical analysis of the research data. Measurement data were described as mean \pm standard deviation (SD), and group comparisons were made using the independent sample *t*-test. Enumeration data were expressed as the number of cases and percentage (%), with group comparisons made using the χ^2 test. A *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. Incidence of moderate to severe malnutrition in elderly lung cancer patients during chemotherapy

A total of 150 elderly lung cancer patients were included in this study, comprising 80 males and 70 females aged 65–85 years, with an average age of 70.72 ± 4.21 years. During chemotherapy, 50 patients developed moderate to severe malnutrition, resulting in an incidence rate of 33.33%.

3.2. Factors affecting moderate to severe malnutrition in elderly lung cancer patients during chemotherapy

Significant differences were observed in BMI, tumor stage, serum pre-ALB, and serum Hb levels between patients with moderate to severe malnutrition and those with no/mild malnutrition. Specifically, the BMI of patients with moderate to severe malnutrition was significantly lower than that of patients with no/mild malnutrition (21.20 ± 1.60 vs. 26.14 ± 2.31 , $P < 0.001$), and the proportion of patients with stage IV tumors was significantly higher (60.00% vs. 27.00%, $P < 0.001$). Additionally, serum pre-ALB and serum Hb levels in patients with moderate to severe malnutrition were significantly lower than in those with no/mild malnutrition ($152.67 \pm 30.41 \text{ g/L}$ vs. $252.47 \pm 51.24 \text{ g/L}$ and $102.44 \pm 10.09 \text{ g/L}$ vs. $154.21 \pm 15.18 \text{ g/L}$, respectively, $P <$

0.001 for all). In contrast, there were no significant differences in age, gender, or smoking history between the two groups. See **Table 1** for details.

Table 1. Factors affecting moderate to severe malnutrition in elderly patients with lung cancer during chemotherapy

Factor	Moderate to severe malnutrition (<i>n</i> = 50)	No/mild malnutrition (<i>n</i> = 100)	<i>t</i> / χ^2	<i>P</i>
Age (years)	70.12 ± 4.22	71.02 ± 4.20	1.235	0.219
Gender [<i>n</i> (%)]				
Male	24	55	0.121	0.728
Female	26	45		
BMI (kg/m ²)	21.20 ± 1.60	26.14 ± 2.31	13.571	< 0.001
Smoking history [<i>n</i> (%)]				
Yes	15 (30.00%)	20 (20.00%)	1.893	0.172
No	35 (70.00%)	80 (80.00%)		
Tumor stage [<i>n</i> (%)]				
Stage II–III	20 (40.00%)	73 (73.00%)	15.408	< 0.001
Stage IV	30 (60.00%)	27 (27.00%)		
Serum pre-ALB (g/L)	152.67 ± 30.41	252.47 ± 51.24	12.688	< 0.001
Serum Hb (g/L)	102.44 ± 10.09	154.21 ± 15.18	21.808	< 0.001

Abbreviations: BMI, Body mass index; Hb, Hemoglobin; Pre-ALB, Serum albumin before chemotherapy.

4. Discussion

Nutrition plays an indispensable role in maintaining normal bodily functions, especially in elderly patients with lung cancer, where its importance is even more pronounced. Maintaining good nutritional status is crucial for enhancing a patient's immunity and improving their tolerance to chemotherapy, which in turn can effectively prolong survival and improve overall prognosis^[8,9]. This study indicates that patients with moderate to severe malnutrition have significantly lower BMI compared to those with better nutritional status. Additionally, stage IV lung cancer is more prevalent among patients with moderate to severe malnutrition. Key indicators such as serum pre-ALB and Hb levels are also significantly lower in these patients. However, no significant differences were found between the two groups regarding age, gender, or smoking history. The data analysis suggests that low BMI before chemotherapy, decreased serum pre-ALB levels, and decreased serum Hb levels are independent risk factors for moderate to severe malnutrition in elderly lung cancer patients undergoing chemotherapy.

Generally, patients with lower BMI may face issues such as insufficient fat reserves, weak physical constitution, and low immune function, resulting in poor tolerance to chemotherapy drugs. This makes them more susceptible to the toxic side effects of chemotherapy, such as loss of appetite and difficulty eating, significantly increasing the risk of malnutrition^[10,11]. In terms of tumor staging, stage IV patients are often in a state of high metabolism and energy consumption, commonly associated with severe cancer pain. This condition can lead to changes in dietary habits, with many chemotherapy patients resorting to liquid foods, which often results in inadequate nutrition and a heightened risk of malnutrition^[12,13].

Serum pre-ALB levels are considered a sensitive indicator of nutritional status, and a decrease in this

marker suggests insufficient protein intake or increased protein consumption. A negative nitrogen balance can occur when serum pre-ALB levels decline, leading to an inadequate nutrient supply for the body. During chemotherapy, the body's nutritional demands significantly increase, and the risk of malnutrition rises when the balance between nutritional intake and consumption is disrupted^[14,15]. Additionally, a decrease in serum hemoglobin (Hb) levels can lead to hypoxia and malnutrition. If uncorrected for an extended period, this can cause anemia and impair the function of the hematopoietic system, exacerbating the toxicity of chemotherapy drugs and increasing the risk of moderate to severe malnutrition during chemotherapy^[16].

While this study provides important insights, it is retrospective in nature, which may introduce selection bias, and the sample size is relatively small. Future research should involve large-scale, multicenter prospective studies to further verify and expand upon these conclusions. In clinical practice, healthcare providers should closely monitor patients' BMI, serum pre-ALB, and Hb levels, identify high-risk groups for malnutrition as early as possible, and implement individualized nutritional interventions for these patients.

In summary, low BMI, decreased serum pre-ALB levels, and decreased serum Hb levels are independent risk factors for moderate to severe malnutrition in elderly lung cancer patients undergoing chemotherapy. Therefore, clinicians should pay special attention to these patients and promptly implement intervention measures such as nutritional support to reduce the incidence of malnutrition, thereby improving patients' quality of life and prognosis.

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Disclosure statement

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Primary Malignant Melanoma of the Larynx: Case Report and Literature Review

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Abstract: Primary malignant melanoma of the larynx is extremely rare. This paper reports a case of a patient with primary malignant melanoma of the larynx. Preoperative laryngoscopy revealed a cauliflower-like mass in the supraglottic region, and a CT scan of the pharynx suggested laryngeal cancer with cervical lymph node metastasis. The patient underwent a total laryngectomy with lymph node dissection, and postoperative pathology confirmed a malignant melanoma in the supraglottic region of the larynx.

Keywords: Larynx; Primary; Malignant melanoma

Online publication: September 25, 2024

1. Basic information

In February 2023, the patient, male, 70 years old, was admitted to the hospital due to a sore throat accompanied by hoarseness for half a month. Laryngoscopy showed a grayish-white mass on the right edge of the epiglottis, extending to the laryngeal surface, with a cauliflower-like growth on the right side of the laryngeal surface. A CT scan of the pharynx suggested laryngeal cancer with cervical lymph node metastasis (**Figure 1A**). The preoperative diagnosis was a laryngeal tumor. Under general anesthesia, the patient underwent a total laryngectomy with lymph node dissection. Postoperative pathology revealed a cauliflower-like mass measuring $4.0 \times 3.0 \times 2.5$ cm at the base of the supraglottic epiglottis. The cut surface was gray-white and reddish-brown, with a soft texture (**Figure 1B**).

Microscopic examination showed invasive tumor growth with necrosis and an unclear boundary with surrounding tissues. Under high magnification, the tumor cells exhibited marked atypia, with slightly basophilic cytoplasm, prominent nucleoli, and frequent mitotic figures (**Figures 1C–E**).

Immunohistochemical staining results: S-100, Melan-A, HMB-45 (positive); CK, P40, CK5/6, CgA, Syn (negative); Ki-67 (70% positive) (**Figures 1F–I**). After excluding other possible primary lesions in other parts of the body, the final diagnosis was primary malignant melanoma of the larynx with lymph node metastasis.

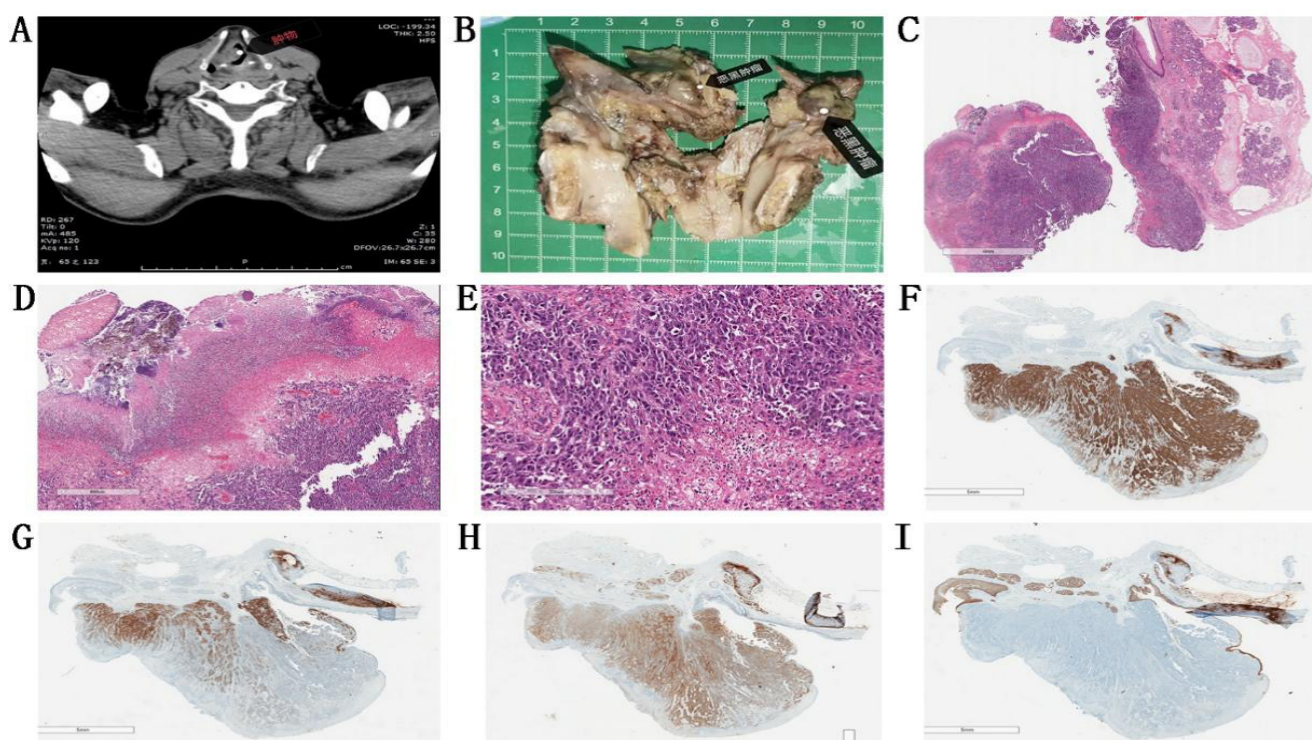


Figure 1. CT and pathological examination images of primary laryngeal melanoma. **(A)** CT scan shows a mass on the right edge of the epiglottis; **(B)** Gross examination reveals a cauliflower-like mass, $4.0 \times 3.0 \times 2.5$ cm in size, at the base of the supraglottic epiglottis, with a gray-white and reddish-brown cut surface and soft texture; **(C)** Low-power microscopic view shows invasive tumor growth (HE $\times 40$); **(D)** Medium-power microscopic view shows abundant sinusoids in tumor cells, with some pigmentation and extensive necrosis (HE $\times 200$); **(E)** High-power microscopic view reveals marked tumor cell atypia, frequent mitotic figures, and prominent nucleoli (HE $\times 400$); **(F–H)** Immunohistochemical staining shows tumor cells positive for HMB-45, Melan-A, and S-100 ($\times 40$); **(I)** Tumor cells negative for CK ($\times 40$).

2. Discussion

Malignant melanoma is a type of cancer that originates from melanocytes derived from the neural crest and accounts for 1% to 3% of all malignant tumors. It can occur in various tissues or areas, such as the skin and mucous membranes^[1]. Mucosal melanoma refers to melanoma occurring in mucous membrane sites such as the nasal cavity, sinuses, oral cavity, esophagus, gastrointestinal tract, anal canal, and genitourinary system. Compared to cutaneous melanoma and other subtypes, mucosal melanoma has a poorer prognosis, with more patients presenting with distant metastases at initial diagnosis and a significantly lower 5-year survival rate. Terada *et al.*^[2] indicated that primary mucosal malignant melanoma of the head and neck might originate from dispersed melanocytes in the mucosa. These cells may be present in the epithelial basal layer of the nasal cavity, oral cavity, oropharynx, and esophagus, while melanocytes are rarely detected in the larynx, which could explain why primary laryngeal melanoma is so rare. This article presents a case of primary malignant melanoma originating from the larynx.

The histology of primary laryngeal malignant melanoma is consistent with that of malignant melanomas at other sites: the tumor grows invasively, melanin is visible, cells exhibit significant atypia, nuclear membranes are clear, nuclear grooves and intranuclear inclusions are observed, prominent nucleoli are visible, and abundant mitotic figures are present. Immunohistochemical staining is positive for S-100, HMB-45, and Melan-A. It is necessary to distinguish it from metastatic malignant melanoma and other laryngeal tumors.

(1) Metastatic malignant melanoma is often multifocal, with lymphatic or vascular invasion, and patients

usually have evidence of tumors outside the larynx.

- (2) Poorly differentiated squamous cell carcinoma, the most common laryngeal tumor, often presents as a well-defined sheet-like structure, with positive immunohistochemical markers for CK, P40, and P63.
- (3) Neuroendocrine tumors, the second most common tumors in the larynx, are composed of uniform round or small polygonal cells, arranged in nests, rosettes, or glandular patterns, with abundant sinusoids in between. Neuroendocrine markers (CgA, Syn, CD56) are positive. These tumors include typical carcinoids, atypical carcinoids, neuroendocrine-type small cell carcinoma, and mixed neuroendocrine-type small cell carcinoma.
- (4) Undifferentiated carcinoma is highly aggressive, with a nested, lobular, or sheet-like distribution under the microscope. It lacks squamous or glandular differentiation, has a high nuclear-to-cytoplasmic ratio, and necrosis and mitotic figures are common. Ki-67 is highly positive on immunohistochemical staining.

This case exhibited typical histomorphological features, and after thoroughly excluding metastasis and other tumors, the diagnosis was confirmed as primary malignant melanoma of the larynx.

The pathogenesis of mucosal melanoma has not been fully elucidated. Some scholars believe that melanocytes originate from the neuroectoderm and can migrate to mucous membranes of the endoderm (such as skin, uvea, and retina) and other ectodermal tissues, but they rarely migrate to the mucosa of the nasopharynx, larynx, or trachea-esophageal tract ^[3]. Melanoma is usually associated with alterations in signaling pathways such as Ras/MAPK, INK4A/ARF, and PTEN/AKT ^[4], with abnormal activation of the Ras/MAPK pathway playing a critical role in melanoma development. Approximately 50% of melanomas are associated with activating mutations of the *BRAF* gene in this pathway ^[5]. A retrospective study in China found that the mutation rate of the *BRAF* gene in Chinese melanoma patients is about 23.7% ^[6]. The molecular characteristics of mucosal melanoma differ significantly from those of cutaneous melanoma, primarily showing a low mutation burden, fewer point mutations, and higher genomic instability ^[7]. The mutation frequency of *BRAF* and *NRAS* is much lower than that of cutaneous melanoma, and most of the mutations are of low activity. In contrast, mutations in *KIT*, *SF3B1*, and *SPRED1* are more frequent than in cutaneous melanoma ^[8], indicating that primary laryngeal malignant melanoma may have a different pathogenesis, leading to differences in treatment strategies.

The treatment for mucosal melanoma primarily involves surgical resection combined with neoadjuvant therapy ^[1]. Research has shown that dual immunotherapy with PD-1 antibodies and CTLA-4 antibodies is superior to monotherapy. The combination of toripalimab and axitinib as a first-line treatment is promising. Toripalimab is an anti-PD-1 monoclonal antibody, and axitinib is a VEGFR1-3 inhibitor. VEGF exerts an immunosuppressive effect by inhibiting dendritic cell maturation. It also reduces the number of regulatory T cells and myeloid-derived suppressor cells, enhancing the efficacy of PD-1 therapy ^[9].

Prognostic factors for malignant melanoma include gender, age, location, tumor Breslow thickness, and tumor Clark invasion depth ^[10]. Although mucosal melanoma cannot be evaluated using Breslow thickness or Clark invasion depth, pathological examination, in this case, revealed that the tumor invaded the lamina propria and submucosa but did not invade the laryngeal cartilage or bone tissue. Local excision of the tumor in melanoma patients can clear the local lesion and reduce recurrence rates, but the prognosis for malignant melanoma of the larynx remains poor. Studies have shown that, following surgical excision, the recurrence rate of laryngeal malignant melanoma is lower than that of other head and neck melanomas ^[11], though the cause remains unclear. The cure rate for primary malignant melanoma of the larynx is still not ideal, with a 5-year survival rate below 20% ^[12], and most patients die from distant metastases. Due to the rarity of primary malignant melanoma of the larynx, there is a lack of data on prognostic indicators. In this case, the patient remained disease-free for 2 months postoperatively, with no recurrence or metastasis observed to date.

Primary malignant melanoma of the larynx is a rare disease with an extremely low incidence. Diagnosis relies mainly on clinical history, imaging studies, histopathology, and immunohistochemical staining. The main treatment for this disease is surgical excision combined with postoperative targeted therapy. The extent of surgical excision is determined by the size, location of the tumor, and the presence or absence of lymph node metastasis. Primary malignant melanoma of the larynx is highly invasive, has a poor prognosis, and is characterized by high postoperative recurrence and systemic metastasis rates. Clinicians should aim for early diagnosis, early surgery, and combined treatment with postoperative radiotherapy and chemotherapy.

Disclosure statement

The authors declare no conflict of interest.

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Exploring Novel Therapeutic Breakthroughs for Cancers: Potential Roles of miRNAs

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Abstract: MicroRNAs (miRNAs) are small non-coding RNAs, 19–25 nucleotides in length, that regulate gene expression. miRNAs are involved in various cellular, biological, and pathological activities, including the development and progression of malignant tumors. Numerous studies have highlighted the significant roles of miRNA deregulation in various cancer types, involving critical pathways such as gene deletions and amplifications, abnormal epigenetic changes, and dysfunctional miRNA regulatory systems. miRNAs can function both as oncogenes and tumor suppressor genes in different contexts. Several cancer hallmarks are associated with deregulated miRNAs, including resistance to cell death, metastasis, sustained proliferative signaling, promotion of angiogenesis, and the suppression of growth factors. Recent research has demonstrated that miRNAs contribute to drug resistance in tumor cells by targeting genes linked to drug resistance or by influencing genes that regulate the cell cycle, apoptosis, and proliferation. A single miRNA often has tissue-specific regulatory functions and can affect multiple genes. Various miRNA types have been identified as potential biomarkers for tumor diagnosis and critical targets for novel therapeutic approaches. This review aims to explore the role of miRNAs in cancer progression, metastasis, and potential therapeutic interventions.

Keywords: MicroRNAs; Cancer; Treatment

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1. Introduction

Cancer is one of the deadliest diseases globally, posing a serious threat to human health. It remains one of the leading causes of death worldwide. According to WHO data, nearly 14.1 million new cancer cases were reported globally in 2012, resulting in 8.2 million deaths. Despite advancements in treatment methods, cancer

incidence continues to rise, with new cases projected to reach 23.6 million by 2030 ^[1]. Multiple factors contribute to cancer development and progression, including genetic and epigenetic alterations. This review summarizes the association between miRNAs and cancer proliferation, mechanisms of miRNA deregulation, and possible therapeutic strategies ^[2].

MicroRNAs (miRNAs) are small non-coding RNAs, consisting of 19–25 nucleotides, that regulate crucial biological functions and control gene expression ^[3]. miRNAs modulate the expression of target genes, either upregulating or downregulating them depending on the regulation mechanism involved. miRNAs are expressed at their genomic loci in either an intragenic manner (when located within an exon or intron of a protein-coding gene) or an intergenic manner (with their promoter controlling their expression). Like protein-coding genes, miRNA genes are transcriptionally regulated by factors such as transcription factors, DNA methylation, mutations, copy number variations, RNA stability, and cleavages ^[4]. The unique regulatory mechanisms of miRNAs often involve miRNA biogenesis, which results in global alterations in miRNA profiles linked to human malignancies.

Intracellular miRNA modulation has emerged as a novel feature of tumor microenvironment growth promotion. Recent studies have identified miRNAs as key mediators in communication between tumor cells and cancer-associated fibroblasts (CAFs), transported through extracellular vesicles. These extracellular miRNAs, originating from CAFs, promote development and survival in various tumor types, particularly colorectal, breast, and pancreatic cancers ^[5].

2. MicroRNAs and Cancers

Research has demonstrated that a single miRNA can target up to approximately 200 distinct elements, such as transcription factors, receptors, and vectors, each with a unique function. Around 30% of human mRNA expression related to cell division, development, and apoptosis may be regulated by miRNAs. Moreover, studies have shown that the expression of certain miRNA types varies significantly between normal and malignant tissues, indicating a critical role for these miRNAs in tumor initiation, growth, invasion, and metastasis. Based on their differential regulation of mRNA in cancer cells, miRNAs are classified as tumor suppressor miRNAs and oncogenic miRNAs, also known as oncomiRs ^[6].

The earliest known tumor-suppressive miRNAs that negatively regulate *bcl-2* are *miR-15a* and *miR-16*. The anti-apoptotic gene *Bcl-2* is elevated in leukemia, lymphoma, and other cancers. Consequently, the downregulation or deletion of these two miRNAs increases the expression of *bcl-2* and promotes the development of lymphoma and leukemia ^[7]. One of the earliest miRNAs discovered, *Let-7*, has been shown to repress the production of the oncogene *Ras*. *Ras* mutations are present in 15%–30% of human malignancies, and when induced by elevated *bcl-2* protein levels, they result in cell transformation. Another study revealed that although *let-7* expression was only slightly reduced in some tumor patients, it decreased significantly in lung cancer patients. The observed increase in *let-7* in human lung cancer cells may inhibit malignant cell growth, suggesting that *let-7* may function as a tumor suppressor gene in lung tissue. It is also notable that, on average, cancer cells produce less miRNA than normal cells ^[8]. This suggests that a subset of miRNAs inhibits tumor growth, and their depletion may increase the likelihood of tumor development.

One study found that in both *in vivo* and *in vitro* experiments involving p53-mediated cell cycle stress or DNA damage, three miRNAs encoded by *miR-34* were identified in human cancers. Among these, *miR-34a* was found in a variety of tumor types. When these miRNAs are abnormally expressed, several cell cycle-regulating genes, including the anti-apoptotic protein *Bcl-2*, are downregulated. This can lead to cell cycle arrest or even cell death. Thus, *miR-34*

functions as a tumor suppressor gene and is essential for regulating cell division and apoptosis^[9].

Oncogenic miRNAs have also been identified, characterized by their significant overexpression in tumor tissues and their role in inducing carcinogenesis. The first functional evidence for an oncomiR came with the identification of *miR-17-92*. The mature miRNAs and pri-miRNAs of *miR-17-92* were found to be significantly overexpressed in B-cell lymphoma and related cell lines. An *in vivo* analysis confirmed that the overexpression of *miR-17-92* promotes the development of myc-induced B-cell lymphoma and increases the frequency of lymphoma occurrence^[10]. Additionally, it was discovered that the myc protein regulates the expression of *miR-17-92*. By directly binding to the *miR-17-92* locus, Myc stimulates its expression and suppresses the production of the transcription factor E2F1 protein, potentially inducing apoptosis. The co-expression of a single member of the *miR-17-92* family with c-Myc did not facilitate cancer growth, suggesting that the combined activity of all *miR-17* family members may be responsible for the cancer-promoting effects of the *miR-17* family. According to research, the *miR-17-92* gene cluster could represent a human oncogene^[11].

Another oncomiR, *miR-21*, is significantly upregulated in several solid tumor types, including aggressive glioma, breast, prostate, and lung cancers, indicating its oncogenic role in cancer development. The transfection of the gastric cancer cell line HEK-293 with a *miR-21* inhibitor resulted in the suppression of cancer growth and the induction of cancer cell apoptosis^[12].

3. The mechanisms of deregulated expression of miRNAs

Aberrant miRNA activity can lead to uncontrolled cell division or make tumor cells resistant to anti-tumor therapies. The mechanisms underlying the deregulation of miRNA expression in drug-resistant cells are crucial to understanding, as this knowledge can aid in developing strategies to counteract drug resistance. The primary causes of miRNA deregulation include amplification or deletion of miRNA genes, epigenetic regulation, degradation of transcription factors, and dysregulation of essential genes or proteins involved in miRNA biosynthesis and processing^[13].

3.1. Gene amplification and deletion

Nearly 60% of the human miRNA gene pool is located in regions vulnerable to cancer-related events, such as translocation boundaries and areas of deletion or amplification. These regions make the genes more susceptible to amplification, relocation, or deletion. For example, chromosomal deletions in the 13q14 region are common. It has been shown that *miRNA-15a* and *miRNA-16-1* are located on 13q14 and are often reduced or deleted in more than 50% of chronic lymphocytic leukemia (CLL) cases^[14]. In contrast, the *miRNA-17~92* family, found in the 13q31–q32 region, is frequently upregulated in various tumor types, which increases the number of mature miRNAs and promotes tumor growth. *miRNA-21* has also been found to be overexpressed in many cancers, contributing to drug resistance in tumor cells. One study demonstrated that the amplification of the 17q23–25 chromosomal region, which results in reduced PTEN expression, was responsible for the overexpression of *miRNA-21* in ovarian cancer^[15]. Another study showed that the amplification of 3q26.2 led to the overexpression of *miRNA-569* in some breast and ovarian cancers, which downregulated TP53INP1 and contributed to cell survival and proliferation^[16].

3.2. Methylation and histone modifications of miRNA genes

Although histone modification and DNA methylation do not change the DNA sequence, they can alter gene activity. DNA methylation, which leads to gene silencing, is an alternative mechanism of gene suppression.

Post-translational modifications, such as acetylation, methylation, and phosphorylation, occur on the amino-terminal histone tails and are closely associated with either gene activation or repression ^[17]. Acetylation of histones by histone acetyltransferases is often linked to gene activation, while hypoacetylation by histone deacetylases is linked to gene suppression. Histone methylation is associated with both gene activation and silencing. Epigenetic mechanisms, such as DNA methylation and histone modifications, contribute to the dysregulation of miRNA expression in cancers. These processes work together to silence tumor-suppressive miRNAs ^[18]. For instance, *miRNA-34a*, a known tumor-suppressor miRNA, is frequently downregulated in various tumor types, and its decreased expression is associated with increased tumor sensitivity to chemotherapy. A study found that 79.1% of primary prostate cancer samples had reduced *miRNA-34a* expression due to promoter methylation on the CpG island. Methylation of the *miRNA-34a* promoter was also observed in melanoma, bladder, lung, breast, kidney, pancreatic, and colon cancer cell lines ^[19].

3.3. Transcription elements mutation

Transcription factors are proteins that bind to cis-acting regions of a gene's promoter in eukaryotes, activating or suppressing gene transcription directly or indirectly, often in coordination with other proteins. In the context of miRNA regulation, p53 is the most well-known transcription factor. Following DNA damage, p53 expression increases and acts as a tumor suppressor, regulating hundreds of genes. In 2007, several studies simultaneously reported that p53 directly targets the *miRNA-34* family, including *miRNA-34a*, and significantly upregulates these genes. *miRNA-34a*, a tumor suppressor miRNA, enhances cancer cell sensitivity to chemotherapy. p53 initiates transcription of the *miRNA-34* family by directly recognizing their regulators. Two p53-related transcription factors, p63 and p73, have distinct roles, with p63 promoting cell division and survival, while p73 acts as a mediator of chemosensitivity. Research has shown that the expression of *miRNA-193a-5p* is regulated by p63 and p73 ^[19]. Pro-apoptotic isoforms of p73 *in vivo* stimulate *miRNA-193a-5p*, while p63 suppresses its expression in both normal and cancerous cells. Chemotherapy induces this miRNA in a p63/p73-dependent manner, which decreases chemosensitivity by inhibiting p73 through a miRNA-mediated feedback loop. Blocking *miRNA-193a* interrupts this feedback, reducing tumor cell survival and increasing their sensitivity to chemotherapy in both *in vitro* and *in vivo* experiments. Another study found that Δ Np63 α , an isoform of p63 and a member of the p53 family, regulates epithelial-mesenchymal transition (EMT) and stimulates the transcription of *miRNA-205* in human bladder cancer cells. Knockdown of Δ Np63 α reduced the expression of the *miRNA-205* host gene (*miRNA-205HG*), its primary and mature variants, and RNA Pol II interaction with the *miRNA-205HG* promoter ^[20].

Salmena *et al.* introduced the concept of competing endogenous RNAs (ceRNAs) in 2011. According to this theory, miRNA response elements (MREs) are present in pseudogenes, long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs). These sequences act as “miRNA sponges,” competing for limited pools of miRNAs, thereby reducing miRNA binding to their target genes ^[21]. The ceRNA hypothesis suggests that any RNA transcript containing MREs can sequester miRNAs from substrates with MREs, thus regulating the function of those targets ^[22]. Some studies indicate that ceRNAs may influence the regulation of chemoresistance-related miRNAs and their target transcripts. For instance, the tumor suppressor gene *PTEN* is frequently inactivated in cancer. Multiple miRNAs can bind to the 3'-UTR of *PTEN* mRNA, inhibiting its expression. *PTENP1*, a pseudogene of *PTEN*, shares significant homology with the same region in *PTEN* mRNA, particularly near the 3'-UTR. This similarity suggests that *PTENP1* can function as a ceRNA for *PTEN*. Both *PTENP1* and *PTEN* mRNA contain MREs capable of binding *miRNA-21* ^[23]. In clear-cell renal cell carcinoma (ccRCC), overexpression of *miRNA-21* promotes tumor growth and metastasis *in vivo*, as well

as cell proliferation, migration, and invasion *in vitro* ^[24]. Overexpression of *PTENP1* mimics the effects of *PTEN* activation by reducing cell proliferation, invasion, tumor formation, and metastasis in cells expressing *miRNA-21*. Additionally, the upregulation of *PTENP1* in ccRCC cells enhances their sensitivity to cisplatin (CDDP) and gemcitabine (GEM) treatments in both *in vitro* and *in vivo* settings. In patient samples, *PTENP1* and *PTEN* levels were inversely correlated with *miRNA-21* activity. Patients with ccRCC who did not express *PTENP1* had a lower chance of survival. These findings suggest that *PTENP1* acts as a ceRNA to inhibit cancer progression in ccRCC. Furthermore, ceRNAs are a relatively new concept, and many studies have shown a strong association between ceRNAs and cancer development and proliferation ^[25].

4. Role of miRNAs in cancer therapy

Restoring miRNA activity and inhibiting overexpressed miRNAs are two key strategies in developing miRNA-based cancer therapies, as imbalanced miRNA levels are linked to carcinogenesis. To restore endogenous miRNA function and reestablish the production of tumor-suppressor miRNAs, miRNA mimics and small molecules are typically employed ^[26]. On the other hand, small molecule inhibitors, which target specific oncomiRs elevated in cancer cells, are used to prevent overexpressed miRNAs. Since some miRNAs act as tumor suppressors, using miRNA mimics to restore reduced tumor-suppressor miRNA levels to healthy levels is a promising therapeutic approach ^[27].

Before the discovery of miRNAs, gene therapy techniques were used to restore gene activity in cancer cells. However, due to the limitations of viral carriers and DNA plasmids, these approaches had inconsistent efficacy. The recent advancements in miRNA research have provided alternative tools. Since miRNAs are much smaller than proteins, they can more easily enter cells through certain delivery methods.

Esquela-Kerscher *et al.* demonstrated that restoring *let-7* miRNA could significantly inhibit tumor growth in mouse models, marking the first validation of *let-7*'s role as a tumor suppressor and its potential as a cancer treatment ^[28]. Since then, interest in using miRNA mimics to restore miRNA activity has surged. The *let-7* family has been shown to suppress well-known oncogenes such as *NIRF*, *myc*, *HMGA*, *STAT3*, and *Ras*. Furthermore, reduced *let-7* production has been associated with poor prognosis in lung cancer, making the restoration of its function a potential treatment strategy ^[29]. Various administration methods were tested for *let-7* in mouse lung cancer models. The results demonstrated that *let-7* mimics could derepress direct *let-7* targets *CDK6* and *Ras*, induce necrosis, and slow tumor progression.

Similarly, the *miR-34* family, transcriptionally regulated by the tumor suppressor p53, is often deleted or downregulated in several cancers. *MiR-34a* has been shown to prevent metastasis and slow tumor progression by targeting CD44. When lipid-complexed *miR-34a* mimics were administered in mouse lung cancer models, tumor sizes were significantly reduced within a well-tolerated dose range, similar to the effects observed with *let-7* mimics ^[30].

In myc-driven liver cancer cells, *miR-26a* levels were lower than in normal cells. Reintroducing *miR-26a* mimics suppressed cyclin D2 and E2, halting the cell cycle. When *miR-26a* was administered to tumors *in vivo*, a significant response was observed, suggesting that restoring miRNA function using mimics could be an effective cancer treatment strategy ^[31].

In terms of overexpressed oncomiRs in cancer cells, considerable research has focused on suppressing these oncomiRs to develop new miRNA-based therapies. Small molecule inhibitors and complementary oligonucleotides—such as anti-miRNA oligonucleotides (AMOs), locked nucleic acid (LNA)-AMOs, antagomirs, and miRNA sponges—are the main types of miRNA inhibitors ^[32]. AMOs work by binding to the

target miRNA's complementary sequence, blocking its effects. AMOs are short DNA oligonucleotide strands that specifically bind to endogenous miRNA or its precursors, preventing them from binding to their target mRNA and thereby inhibiting miRNA activity. Subsequent research led to improved AMOs, such as 2'-O-methyl AMOs, 2'-O-methoxyethyl AMOs, and LNA-AMOS. LNA-AMOS, which features a modified structure, offers better selectivity, stability, and efficacy than earlier versions ^[33].

MiRNA sponges, like antagomirs, block miRNA from binding to its targets. Sponges are longer nucleic acids containing multiple miRNA-binding motifs and are typically DNA plasmids or transcribed RNA rather than short oligonucleotide strands. The study of pharmacological sites is crucial to developing new drugs ^[34]. Since miRNA plays a central role in gene regulation, it has emerged as a valuable tool for studying therapeutic targets. Traditional drugs are small chemical compounds that target individual cancer proteins, which has limitations in clinical settings. In contrast, miRNAs function at the core of multi-target regulatory networks and inherently regulate many receptor genes. Furthermore, miRNA production is tightly regulated by signaling pathways involving key enzymes, making the entire signaling cascade a potential therapeutic target.

Moreover, miRNA-based drugs can target molecules that conventional chemical or antibody-based drugs cannot reach, offering new avenues for treating diseases, particularly cancer, where traditional treatments have shown limited efficacy. As a result, miRNAs have gained increasing interest as therapeutic targets in drug development ^[35].

Certain small molecules, such as hypomethylating agents, can restore downregulated miRNAs. For example, the drugs decitabine and 5-azacytidine, used to treat myelodysplastic syndromes, increase the expression of several miRNAs. Additionally, enoxacin has been shown to stimulate miRNA production, leading to a general increase in miRNA levels in cell culture. In mouse xenograft models, enoxacin inhibited tumor growth by upregulating 24 mature miRNAs. These examples highlight the potential of small molecules to restore miRNA function in cancer treatment ^[36].

Another targeted strategy for miRNA restoration is the use of miRNA mimics—synthetic double-stranded RNA molecules that imitate endogenous miRNAs to regulate their activity. The primary challenge in applying miRNA mimics is developing an effective delivery system, such as nanoparticles, lipid emulsions, atelocollagen formulations, or adeno-associated viruses, due to the volatile nature of miRNA mimics in biological systems. In colon xenograft mouse models, the targeted delivery of *miR-34a* and *let-7* mimics via lipid formulations dramatically suppressed cancer proliferation. Similarly, *miR-26a* delivery using an adeno-associated virus vector inhibited cancer cell growth and reduced tumor size. Notably, a method for miRNA replenishment has been established in clinical trials for liver cancer using liposome-formulated *miR-34* mimic (MRX34) ^[37].

MiRNA sponges, which can absorb complementary miRNA molecules, are another effective miRNA regulator. By binding miRNAs, sponges prevent them from attaching to their target receptors, thus inhibiting miRNA function. Long non-coding RNA and circular RNA are known to function as natural miRNA sponges, binding miRNAs and interacting with miRNA target particles to negatively regulate their activity ^[38]. Shu *et al.* developed a sponge-based system that expresses circular miRNA inhibitors targeting *miR-223* and *miR-21*. This system was more effective at inhibiting cancer cell proliferation than its linear counterparts. Additionally, small molecule inhibitors of miRNA, as chemical entities, can be developed using conventional drug discovery methods. These inhibitors come in various forms, each with a distinct mechanism of action ^[39].

5. Altered microRNA expression in cancer cells

In the past decade, it has been discovered that cancerous cells and tissues exhibit aberrant miRNA expression

patterns. Certain miRNAs regulate key genes necessary for maintaining cellular homeostasis. When these genes are altered, abnormal biological processes, such as unchecked cell division, angiogenesis, metabolic dysregulation, and apoptosis, can occur, leading to cancer development ^[40]. Altered miRNA expression has been associated with numerous cancer hallmarks, including growth signaling (*let-7* family, *miR-21*), insensitivity to antigrowth signals, evasion of apoptosis, angiogenesis, invasion and metastasis (*miR-10b*, *miR-31*, *miR-200* family, *miR-21*), immune evasion (*miR-15b*), tumor-promoting inflammation (*miR-23b*, *miR-155*, *let-7d*), and genomic instability ^[41]. Cancer alters miRNA expression patterns that are specific to individual cells and cellular conditions, which differ from those seen in healthy tissues. Moreover, miRNAs can drive cancer progression by either upregulating oncogenes or suppressing tumor suppressor genes. This underscores the significance of miRNAs in cancer development and their potential as biomarkers for further subclassification of cancer types.

Tumor suppressor miRNAs (TS-miRNAs) and oncomiRs are the two main categories of tumor-related miRNAs. Lower expression of TS-miRNAs (e.g., *miR-133a*, *miR-145*, *miR-143*) allows for the translation of oncoproteins, while elevated expression of certain oncomiRs promotes cancer by inhibiting the translation of tumor-suppressing mRNAs (e.g., *miR-17-92*, *miR-125b*, *miR-125*) ^[42]. The dynamic nature of miRNA expression highlights their potential in cancer treatment strategies. As a result, miRNA mimics and antisense miRNA (anti-miRs) therapies have been developed and are commonly used to either replace TS-miRs or inhibit oncomiRs, respectively ^[43].

miRNAs are present in both the body's extrinsic and intrinsic environments. Recently, circulating miRNAs have emerged as a promising new class of biomarkers. For example, significantly elevated levels of *miR-31* have been found in the tissue and serum of individuals with oral cancer. *MiR-31* targets the enzyme SIRT3 (NAD-dependent deacetylase), which regulates metabolism and energy production ^[43]. Similarly, patients with gastric and germ cell cancers exhibit higher levels of *miR-371-3* in their tissue and serum, which directly targets the tumor suppressor gene *TOB1*, promoting cancer development and metastasis ^[44]. Furthermore, changes in miRNA expression have been associated with chemotherapy resistance. Overexpression of *miR-155* and *miR-221/miR-222* has been linked to poor outcomes in lung cancer and unfavorable responses to anti-estrogen therapies. The role of miRNAs in diagnostics and treatment is highly variable across different types of cancer.

6. MiRNA editing as cancer therapy

Some miRNAs hold potential as therapies because they are key regulators of carcinogenesis in certain types of cancer. For instance, in xenograft mice with stomach and breast tumors, augmenting the decreased levels of *miR-29b*, a driver of cell proliferation, growth, and angiogenesis, led to a reduction in tumor size. Another promising miRNA is *miR-34a*, which is typically underexpressed in various cancer types, particularly breast cancer. Some of *miR-34a*'s key targets include Fos-related antigen (Fra-1), which regulates apoptosis, development, and transformation; and NAD⁺-dependent histone deacetylase Sirtuin-1 (SIRT1), which removes the tumor suppressor p53 ^[45]. While miRNA regulates numerous biological processes, there are challenges in delivering miRNAs effectively. Crude miRNAs become unstable when exposed to different blood ribonucleases unless removed by phagocytosis through the reticuloendothelial system (RES). Additionally, the negative charge of crude miRNAs prevents them from crossing the vascular endothelium or cell membrane. To overcome these issues, various delivery methods for miRNAs are urgently needed ^[46].

Reducing oncomiRs, which are often overexpressed in human malignancies, restores tumor-suppressor activity, making it a potential therapeutic approach. Commonly used miRNA inhibitors either block miRNA production or inhibit miRNA-mRNA interaction. Examples include single-stranded antisense anti-miR

oligonucleotides (AMOs), locked nucleic acid (LNA) anti-miRs, antagomiRs, miRNA sponges, and small molecule inhibitors of miRNAs (SMIRs). For instance, antagomirs against *miR-16*, *miR-122*, *miR-192*, and *miR-194* were intravenously injected to significantly reduce their natural expression without inducing an immune response. Another example is the oncogenic *miR-21* antagonist, which inhibited EMT and angiogenesis in breast cancer by suppressing AKT and subsequently activating the Mitogen-Activated Protein Kinase (MAPK) pathway. MiRNA sponges, which are RNA compounds with repeated miRNA-binding sequences, can sequester specific miRNAs. This effectively suppresses the expression of *miR-23b* *in vitro* and *in vivo*, reducing angiogenesis, invasion, and migration of gliomas ^[47].

Another therapeutic approach is to introduce tumor-suppressive miRNAs into cancer cells. Tumor-promoting mRNAs can be targeted using pre-miR, plasmid-encoded miRNA genes, or synthetic double-stranded miRNA mimics, which compensate for lost tumor-suppressor miRNAs. The *miR-34* family, which is mutated in approximately 50% of human cancers, plays a key role in inhibiting tumor formation ^[48]. When *miR-34* mimics were introduced into cancer cells, they demonstrated a growth-inhibiting effect, confirming the potential of miRNA mimics as cancer therapies. However, challenges such as clearance by the RES and ribonucleases, as well as the negative charge of miRNAs, hinder their ability to cross the vascular endothelium and cell membrane. Even when they enter a cell, miRNAs are degraded by endolysosomes. Preserving normal tissue is essential for the successful delivery of cancer-specific medications. Tumor microenvironments, including compromised blood perfusion, act as barriers, limiting systemic delivery of miRNAs. Macrophages, neutrophils, and monocytes in the tumor microenvironment can nonspecifically absorb miRNAs carried within delivery mechanisms. Researchers have explored various strategies for delivering therapeutic miRNA mimics or blockers to address these issues ^[49].

7. Approaches for miRNA therapeutic delivery

Intrathecal injection of miRNA mimics or blockers is a more efficient delivery method than systemic approaches due to better absorption and lower toxicity. However, local administration is restricted to easily accessible and localized primary solid tumors, such as those in cervical, breast, and melanoma cancers. One advantage of local administration is minimal nonspecific uptake by healthy organs, which reduces unintended toxicity and immunogenicity ^[50]. miRNAs should be delivered to targeted cells, protected from early circulatory degradation, and allowed to enter cells easily without triggering an immune response. Systemic miRNA delivery systems and oligonucleotide modifications have been explored as enhanced therapeutic delivery methods for miRNA inhibitors and mimics. Chemically modified miRNA oligonucleotides increase stability and resist degradation by circulating nucleases. Both viral and non-viral vectors are frequently used for miRNA delivery. Viral vectors are effective but can elicit adverse immune responses, making non-viral vectors preferable for clinical research. Many labs are also investigating the use of nanoparticles as an alternative delivery mechanism for miRNAs ^[51].

Viral vectors deliver pre-miR or mature miRNA encoded in a plasmid into tumor cells, produce mature miRNA, use viral promoters to stimulate expression, and subsequently repress or degrade target mRNAs. Lentivirus, adenovirus, and adeno-associated virus (AAV) have all been effective in delivering miRNA mimics or antagonists to tumor cells, resulting in miRNA production and activation ^[52]. Kasar *et al.* demonstrated that *miR-15a/16* production, which was absent in chronic lymphocytic leukemia (CLL), was restored when these miRNAs were delivered via lentiviral vectors to a CLL model in New Zealand Black mice. In another study, intravenous delivery of lentiviruses encoding *miR-494* antagonists reduced the function of myeloid-derived suppressor cells (MDSCs), which promote angiogenesis and tumor formation ^[53,54]. Genetic modification

allows for the conjugation of targeting moieties to viral capsid proteins, improving the vectors' affinity for cancer-specific receptors and enabling targeted delivery into tumors ^[55]. However, it is important to note that lentiviruses integrate their viral DNA into the host genome, which may cause mutations and activate oncogenic pathways. In contrast, AAVs offer a safer alternative for therapeutic administration due to their episomal genome structure. Although viral vectors are an efficient way to deliver functional miRNA antagonists or mimics to tumor tissues, immunogenic responses remain a significant concern in practical applications. As a result, several non-viral delivery methods have been developed as safer alternatives for miRNA delivery ^[56].

Current research suggests that nanoparticles are a leading non-viral carrier for delivering exogenous nucleic acids, including DNA, mRNA, siRNA, and miRNA. However, concerns about safety and efficacy limit their practical use as therapeutic vectors, despite widespread application ^[57]. Nanoparticle-mediated miRNA delivery must overcome various barriers, including pharmacokinetics, endosomal escape, rapid renal clearance, and degradation. Notably, nanoparticles accumulate in tumors through the enhanced permeability and retention (EPR) effect, and particle size significantly impacts optimized targeting. Lipid-based vectors, the most common type of nanoparticles for in vivo gene therapy, consist of spherical structures made of phospholipid bilayers enclosing nucleic acids or drugs within an aqueous core. Liposome-based nanoparticles are favored for their low immunogenicity, flexibility, biocompatibility, and multiple delivery options. Advances in liposome design and modeling have enabled customization of their pharmacokinetics and absorption for specific clinical contexts ^[58].

Extracellular vesicles (EVs), such as exosomes or lipid-bilayer membrane-enclosed nanoparticles, are naturally produced by cells and facilitate intercellular communication by transferring nucleic acids (DNA, RNA) and proteins. EVs show potential for therapeutic and diagnostic applications, and much research is being conducted on their use in clinical settings. EVs can be broadly classified into apoptotic bodies, microvesicles, and exosomes. Microvesicles (50–1,000 nm) are released from the plasma membrane, while exosomes (30–150 nm) originate from the endosomal system. For convenience, “EV” is used as a general term for both exosomes and microvesicles of approximately 100 nm in size ^[59]. Although these subpopulations have different biosynthesis pathways, there is currently no reliable way to distinguish them. Compared to other delivery methods, EV-mediated miRNA transport offers several advantages as a biocompatible molecular carrier. EV surface receptors or proteins selectively deliver miRNAs to specific cell types. Unlike synthetic nanoparticles, EVs avoid endosomal entrapment within the cell, allowing miRNAs to act more quickly and effectively by suppressing gene targets ^[60]. EVs also protect miRNAs from RNase degradation and harsh conditions, enabling encapsulated miRNAs to travel through bodily fluids. EV-mediated miRNA delivery has been successfully demonstrated in the treatment of colon cancer (*miR-143*), gliomas (*miR-146b*), and lung cancer (*miR-145*). To improve cell selectivity, peptides can be conjugated to EV membranes. Ohno et al. developed EVs conjugated with the GE11 peptide, which targets epidermal growth factor receptor (EGFR) and was used to deliver *let-7a* to breast cancer cells expressing EGFR. These studies highlight EVs' potential to revolutionize therapeutic delivery strategies ^[61]. However, large-scale production and efficient encapsulation techniques are still under development.

8. Challenges and future perspectives of miRNA-based therapeutic approaches

While advancements have been made in miRNA-based therapies, several obstacles remain that hinder the transition of miRNA from research to clinical practice. The first challenge is delivery efficacy. Current chemically synthesized miRNA delivery methods exhibit limited cellular uptake capabilities. For therapeutic

benefits, miRNA must effectively cross cell membranes and navigate the complex vascular systems of various tissues ^[62,63]. The second challenge involves miRNA selectivity and off-target effects, which complicate the delivery of miRNAs to target cells. Unlike siRNA, the “multi-targeting” nature of miRNA can be both advantageous and problematic. While it may assist in treating diseases by influencing multiple factors involved in disease progression, it also leads to off-target effects. Despite sequence-specific targeting, miRNAs can inadvertently bind to unintended mRNAs with only partial complementarity, complicating precision targeting ^[64]. The third issue relates to miRNA-induced toxicity. Research has shown that some miRNAs can regulate the expression of enzymes involved in drug metabolism, such as bile acid synthase CYP7A1 and cytochrome P450s (CYPs). Dysregulation of CYP expression by certain miRNAs can disrupt drug metabolism, cause drug accumulation, and potentially lead to toxicity ^[65]. The fourth challenge is overcoming the rapid clearance of miRNA by the circulatory system.

Despite these limitations, miRNA holds great promise in cancer therapy. Certain miRNAs can directly influence cancer by regulating cell division, proliferation, and apoptosis, while others may have an indirect impact by targeting tumor suppressor genes and oncogenes. The study of miRNA’s role in tumor development and progression has garnered significant attention. Advances in molecular biology have improved techniques for detecting miRNAs, leading to the identification of an increasing number of cancer-associated miRNAs and a clearer understanding of the relationship between miRNA and target mRNA. Changes in miRNA expression patterns are key factors in tumor formation and carcinogenesis.

Since identifying miRNAs in cancer patients can aid in cancer prediction, treatment, and diagnosis, miRNAs may emerge as novel tumor biomarkers. As miRNAs regulate target genes that contribute to the biological characteristics of cancers, research-based therapeutics involving miRNAs hold promising applications in the coming decades. The use of miRNA in medicine depends on low-cost, high-efficiency, and sensitive detection techniques. However, limitations in current detection methods hinder the widespread clinical application of miRNAs. Once these challenges are addressed, miRNA detection and therapy are expected to enter clinical practice successfully and become novel targets for cancer treatment. The recently discovered natural delivery system involving exosomes/EVs, when combined with targeting ligands, offers great potential as a biocompatible delivery structure specific to cancer cell types. The expression of miRNAs is influenced by numerous factors, and dysregulated miRNA expression often leads to resistance to anticancer drugs. The entire regulatory network of miRNAs, which includes their interactions with mRNA, protein, and other non-coding RNAs, expands the possibilities for their use in future research and clinical applications.

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Effect of Jianpi Wenshen Granules on the Nutritional Control Status Score in Elderly Patients Undergoing Maintenance Hemodialysis

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Abstract: *Objective:* To explore the effect of Jianpi Wenshen Granules on the Controlling Nutritional Status (CONUT) score in elderly patients undergoing maintenance hemodialysis. *Methods:* Seventy elderly outpatients undergoing maintenance hemodialysis from January 2023 to January 2024 at the Blood Purification Centers of Taizhou Traditional Chinese Medicine Hospital and Taizhou Second People's Hospital were selected as the study subjects. The patients were randomly divided into two groups: the study group and the control group, with 35 patients in each group. Both groups received maintenance hemodialysis, while the control group received only conventional Western medicine treatment, and the study group was additionally treated with Jianpi Wenshen Granules. The changes in biochemical and inflammatory markers before and after treatment were compared between the two groups. The nutritional status of the patients was assessed using the Controlling Nutritional Status (CONUT) score, and detailed statistics were gathered on anemia and albumin levels in both groups. *Results:* After treatment, the CONUT score in the study group significantly decreased compared to the control group, showing a significant correlation ($P < 0.05$). Albumin and hemoglobin levels significantly increased, with a notable difference ($P < 0.05$). There were no significant differences in alanine aminotransferase and aspartate aminotransferase levels between the two groups before and after treatment ($P > 0.05$). *Conclusion:* In elderly patients undergoing maintenance hemodialysis, Jianpi Wenshen Granules improve the CONUT score and enhance nutritional status, demonstrating potential for clinical application and promotion.

Keywords: Jianpi Wenshen Granules; Maintenance hemodialysis; Controlling Nutritional Status score

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1. Introduction

Cardiovascular diseases and infections are the most common causes of death in elderly hemodialysis patients, while factors such as nutritional status and age have become important risk factors affecting their survival rates ^[1]. Maintenance hemodialysis is a common renal replacement therapy that can effectively prolong the life of patients with chronic kidney disease and improve their quality of life. Therefore, improving

dialysis techniques can help extend patient survival. As a large number of dialysis patients gradually enter old age, research has confirmed that age is an independent risk factor for mortality in dialysis patients. In addition, anemia, malnutrition, calcium-phosphorus metabolism disorders, and microinflammatory states are also associated with the mortality of dialysis patients ^[2]. The spleen and kidneys are important metabolic organs, crucial for elderly patients undergoing maintenance hemodialysis. However, long-term dialysis may damage the kidneys and spleen, affecting nutrient absorption and metabolic capabilities. Weakness of the spleen and kidneys is detrimental to elderly maintenance hemodialysis patient recovery and nutritional improvement. Based on this, and combined with years of clinical experience, this study suggests that improving the nutritional status of elderly maintenance hemodialysis patients must start with the spleen and kidneys, adopting the principle of “Jianpi Wenshen” (strengthening the spleen and warming the kidneys), applying a syndrome differentiation treatment approach to improve malnutrition, promote recovery, and thus improve the quality of life and survival status of patients ^[3].

2. Materials and methods

2.1. General information

From January 2023 to January 2024, 70 elderly outpatients undergoing maintenance hemodialysis at the Blood Purification Centers of Taizhou Traditional Chinese Medicine Hospital and Taizhou Second People’s Hospital were selected as study subjects.

Inclusion criteria: (1) Age 60–75 years; (2) Diagnosed with end-stage renal disease (ESRD); (3) Received maintenance hemodialysis (MHD) treatment for ≥ 6 months; (4) Clear consciousness and ability to communicate verbally; (5) Able to complete relevant scales and tests independently or with the help of family members; (6) Diagnosed with spleen and kidney yang deficiency syndrome: aversion to cold, cold limbs, fatigue, shortness of breath, lack of speech, poor appetite, weak waist and knees, cold pain in the waist, abdominal distension, loose stools, frequent nocturia. Pale tongue with teeth marks, weak pulse; (7) Voluntarily participated in the study and signed written informed consent.

Exclusion criteria: (1) Clinical inflammatory reactions such as pneumonia, fever, diarrhea, trauma, surgery, or malignant tumors within the past month; (2) Severe heart, lung, brain, or liver failure; (3) Received immunosuppressive or immunoenhancing therapy within the past month; (4) Incomplete data.

2.2. Methods

2.2.1. Control group (35 cases)

This group received purely Western medicine treatments, including a low-salt, low-phosphorus diet rich in high-quality protein, while correcting electrolyte and acid-base imbalances, anemia, and other complications. Basic treatments included blood pressure and blood sugar control.

2.2.2. Study group (35 cases)

In addition to the treatment received by the control group, the study group was also given Jianpi Wenshen Granules. The granules included Astragalus (30 g), Codonopsis (15 g), Atractylodes (15 g), Poria (12 g), Epimedium (12 g), Eucommia (15 g), Cornus (10 g), Cuscuta (10 g), Achyranthes (10 g), Curculigo (10 g), Jujube (12 g), and Ginger (10 g). These granules were prepared by the Chinese pharmacy at Taizhou Traditional Chinese Medicine Hospital, with the granule preparation provided by CR Sanjiu Chinese Herbal Formula Granules. The dosage was one dose per day, mixed with 150 mL of water, taken in two doses (75 mL each in the morning and evening), with a treatment course of 12 weeks.

2.3. Observation indicators

The therapeutic effects on the two groups were compared. The CONUT (Controlling Nutritional Status) score was used to assess the nutritional status of both groups, and the following data were collected.

2.3.1. CONUT score (before enrollment and 12 weeks after treatment)

- (1) Lymphocyte count: Reflects immune defense impairment due to malnutrition. Score range: $\geq 1,600$ cells/mm³ = 0 points, 1,200–1,599 cells/mm³ = 1 point, 800–1,199 cells/mm³ = 2 points, < 800 cells/mm³ = 3 points;
- (2) Total cholesterol (TC) level: Reflects energy reserve status. Score range: ≥ 4.65 mmol/L = 0 points, 3.60–4.64 mmol/L = 1 point, 2.6–3.6 mmol/L = 2 points, < 2.6 mmol/L = 3 points;
- (3) Albumin level: Reflects protein storage and utilization. Score range: ≥ 3.50 g/dL = 0 points, 3.00–3.49 g/dL = 2 points, 2.50–2.99 g/dL = 4 points, < 2.50 g/dL = 6 points. The total score is out of 12, and the nutritional status is categorized as 0–1 points = normal; 2–4 points = mild malnutrition; 5–8 points = moderate malnutrition; 9–12 points = severe malnutrition. Lymphocyte count, albumin, and total cholesterol were tested using a biochemical analyzer.

2.3.2. Inflammatory factors

C-reactive protein (CRP) is a non-specific inflammatory marker. CRP was tested using immunoassays (e.g., immunoturbidimetry or ELISA).

2.3.3. Anemia indicators

Hemoglobin is an important indicator of the improvement of renal anemia. When kidney function is impaired, the production of erythropoietin decreases, leading to a reduction in red blood cells and hemoglobin levels, resulting in anemia.

2.3.4. Traditional Chinese medicine syndrome score evaluation

Traditional Chinese Medicine (TCM) syndrome score was evaluated based on the severity of symptoms and categorized as severe, moderate, mild, or none, with scores of 3, 2, 1, and 0, respectively. Symptoms included shortness of breath, weak waist and knees, fatigue, and poor appetite. The score range was 0 to 12, with higher scores indicating more severe TCM syndrome symptoms.

2.3.5. Safety indicators

Safety indicators were assessed by monitoring liver function, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Elevated levels indicate increased side effects of the medication on elderly maintenance dialysis patients.

2.4. Statistical analysis

SPSS 26.0 was used for data processing. Measurement data were expressed as mean \pm standard deviation (SD) and analyzed using *t*-tests. Count data were expressed as *n* and percentages, and the chi-squared (χ^2) test was used. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Comparison of general data

As shown in **Table 1**, there were no statistically significant differences in gender and age between the two groups ($P > 0.05$).

Table 1. Comparison of demographic characteristics between the two groups

Groups	Gender (n)		Age (mean \pm SD, years)
	Male	Female	
Control group (n = 35)	22	13	68.94 \pm 4.59
Study group (n = 35)	28	7	67.23 \pm 5.48
χ^2 / t-value	2.520		1.415
P-value	0.114		0.162

3.2. Comparison of pre- and post-treatment CONUT scores and TCM syndrome scores between the two groups

After treatment, compared to the control group, the CONUT score of the study group decreased significantly, and the difference was statistically significant ($P = 0.048$). Additionally, the TCM syndrome score in the study group decreased significantly, with a highly significant difference ($P < 0.001$). See **Table 2**.

Table 2. Comparison of pre- and post-treatment CONUT scores and TCM syndrome scores between the two groups (mean \pm SD, points)

Groups	Before treatment		After treatment	
	CONUT scores	TCM syndrome scores	CONUT scores	TCM syndrome scores
Control group (n = 35)	4.71 \pm 1.18	6.09 \pm 1.28	4.15 \pm 1.08*	4.91 \pm 1.04*
Study group (n = 35)	4.77 \pm 1.23	6.14 \pm 1.35	3.66 \pm 0.95*	3.54 \pm 0.87*
t-value	0.208	0.159	2.015	5.978
P-value	0.836	0.874	0.048	< 0.001

Note: * indicates a comparison within the same group before and after treatment, $P < 0.05$

3.3. Comparison of pre- and post-treatment inflammatory factors and hemoglobin levels between the two groups

After treatment, compared to the control group, the CRP level in the study group decreased significantly, with a statistically significant difference ($P = 0.004$), and hemoglobin levels in the study group increased significantly, with a highly significant difference ($P < 0.001$). See **Table 3**.

Table 3. Comparison of pre- and post-treatment inflammatory factors and hemoglobin levels between the two groups (mean \pm SD)

Groups	C-reactive protein (mg/L)		Hemoglobin (g/L)	
	Before treatment	After treatment	Before treatment	After treatment
Control group (n = 35)	11.62 \pm 1.18	4.74 \pm 1.21*	114.14 \pm 15.76	118.48 \pm 10.37
Study group (n = 35)	11.93 \pm 1.16	1.90 \pm 0.32*	113.77 \pm 14.85	128.18 \pm 10.29
t-value	1.792	2.984	0.101	3.928
P-value	0.272	0.004	0.920	< 0.001

Note: * indicates a comparison within the same group before and after treatment, $P < 0.05$

3.4. Comparison of pre- and post-treatment serum albumin levels between the two groups

Table 4 shows that after treatment, compared to the control group, the serum albumin level in the study group increased significantly, with a statistically significant difference ($P = 0.045$).

Table 4. Comparison of pre- and post-treatment serum albumin levels between the two groups (mean \pm SD, g/L)

Groups	Albumin (g/L)	
	Before treatment	After treatment
Control group ($n = 35$)	35.85 ± 3.47	37.95 ± 3.83
Study group ($n = 35$)	36.27 ± 2.9	40.02 ± 4.60
<i>t</i> -value	0.543	2.046
<i>P</i> -value	0.589	0.045

3.5. Comparison of safety indicators between the two groups

Table 5 shows that there were no significant differences in ALT and AST levels between the control group and the study group before and after treatment ($P > 0.05$), indicating the safety of the medication.

Table 5. Comparison of safety indicators between the two groups (mean \pm SD, U/L)

Groups	ALT		AST	
	Before treatment	After treatment	Before treatment	After treatment
Control group ($n = 35$)	11.83 ± 4.04	10.23 ± 2.51	13.82 ± 4.01	16.13 ± 4.04
Study group ($n = 35$)	12.95 ± 4.21	10.12 ± 2.35	12.94 ± 4.16	14.47 ± 4.28
<i>t</i> -value	1.136	0.189	0.901	1.669
<i>P</i> -value	0.260	0.850	0.371	0.100

4. Discussion

According to TCM theory, the kidneys are considered the foundation of congenital essence in the human body, responsible for storing vital essence and playing a crucial role in growth, development, and reproductive functions. Prolonged kidney disease is primarily due to the dysfunction of internal organs, with spleen and kidney deficiency being the main causes. External factors, such as pathogenic invasion and excessive fatigue, lead to weakened vital energy and blood stasis, causing various symptoms^[4].

Malnutrition in dialysis patients is a common and serious problem, directly affecting their quality of life and survival rates. Insufficient dialysis is a major cause of malnutrition in MHD patients, leading to the accumulation of acidosis, toxins, and inflammatory mediators in the body. This results in gastrointestinal dysfunction, reduced intake and absorption of protein and calories, and decreased plasma albumin, further contributing to malnutrition, weakened immunity, and increased risk of infection. Malnutrition, in turn, affects the effectiveness of hemodialysis, creating a vicious cycle that reduces quality of life and increases the risk of death^[5]. Malnutrition is particularly pronounced in elderly MHD patients, resulting in higher infection and mortality rates. Adequate dialysis can remove toxins, improve intestinal and electrolyte balance, relieve indigestion, regulate acid-base balance, reduce protein catabolism, improve nutritional status and quality of life, and decrease mortality^[6,7].

Current methods for assessing malnutrition include the Modified Quantitative Subjective Global Assessment (MQSGA), the Malnutrition Inflammation Score (MIS), the Subjective Global Assessment (SGA), and the Mini Nutritional Assessment Short Form (MNA-SF). However, these methods are largely influenced by the patient's subjective factors ^[8]. The CONUT score is an objective parameter calculated based on laboratory data and assesses nutritional status by considering indicators such as lymphocyte count, albumin level, and total cholesterol level ^[9].

This study showed that the CONUT score and CRP levels in the study group significantly decreased, while albumin and hemoglobin levels significantly increased, indicating improvements in inflammation and nutrition. Moreover, the TCM syndrome scores decreased, highlighting the advantages of TCM intervention. In the formula used, Astragalus combined with Codonopsis invigorates qi and strengthens the spleen; Poria combined with Atractylodes invigorates the spleen and dispels dampness; Epimedium, Eucommia, and Cuscuta nourish the kidneys and strengthen yang; Achyranthes and Cornus nourish the liver and kidneys. Additionally, Achyranthes also tonifies the kidneys, strengthens yang, promotes blood circulation, and removes blood stasis, while Curculigo disperses cold and dampness. The entire formula strengthens qi, tonifies the spleen, warms the kidneys, and supports yang.

Modern pharmacology suggests that Astragalus regulates immunity, improves anemia, dilates blood vessels, and lowers blood pressure ^[10]; Codonopsis regulates the gastrointestinal system, suppresses inflammation, reduces lipid oxidation, and protects the kidneys ^[11]; Cornus has anti-inflammatory effects and protects against ischemia-reperfusion injury; Poria acts as a diuretic ^[12]; Achyranthes has analgesic and anti-inflammatory properties; Atractylodes promotes gastrointestinal secretion and absorption; and Epimedium enhances immunity ^[13].

The microinflammatory state is triggered by the stimulation of chemical substances, endotoxins, and immune compounds in the body, which activate monocytes and macrophages to release pro-inflammatory factors. This leads to chronic, sustained inflammation, increasing the risk of death ^[14]. The microinflammatory state in maintenance hemodialysis patients is closely related to malnutrition, and the two conditions influence each other. Malnutrition reduces immunity and promotes the occurrence of microinflammation, while microinflammation disrupts muscle protein metabolism, exacerbating malnutrition ^[15].

In summary, the CONUT score can be used as a tool to assess nutritional status. Jianpi Wenshen Granules help improve the nutritional control status score, nutritional status, and microinflammatory state in maintenance hemodialysis patients and are worth promoting in clinical practice.

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Advancing Cancer Stem Cell-Targeted Therapeutic Applications

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Abstract: Cancer stem cells (CSCs), first identified in blood cancers, are increasingly recognized as significant biomarkers and targets in tumor therapy due to their metastatic potential and role in cancer recurrence. Recent research has demonstrated the dedication of scientists in targeting CSCs to explore novel therapeutic strategies. Many types of cancer exhibit metastasis, heterogeneity, and resistance to treatment, all of which are influenced by CSCs. These cells utilize various transcription factors and signaling pathways to carry out these functions. By identifying and understanding these pathways, new therapeutic breakthroughs can be achieved. Thus, targeting cancer stem cells holds great potential and importance in cancer treatment. Moreover, CSCs offer promising avenues for treating otherwise incurable diseases. However, targeting CSCs presents challenges such as immunological rejection and disease recurrence. Advancing research into CSCs may reveal new insights in the fight against cancer and ultimately improve human health. This review explores the roles of CSCs in cancer development and treatment, aiming to uncover new therapeutic approaches.

Keywords: Cancer stem cells; Cancer; Therapy

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1. Introduction

Cancer stem cells (CSCs) are a specific subpopulation of tumor cells that possess unique characteristics and are known to initiate tumors. CSCs share traits with stem cells, such as the ability to self-renew and differentiate into various types of tumor cells, unlike most cancer cells. Even without CSCs, tumors can exhibit rapid growth, but CSCs are thought to be the primary drivers of tumor initiation, progression, and treatment resistance^[1]. Because of their ability to evade current cancer therapies, CSCs have become a major focus of research, raising questions about their potential for curative applications. A combination of stochastic and hierarchical factors is believed to play a critical role in the transformation of normal stem cells into CSCs. As stem cells continue to proliferate and differentiate into various cell types, they accumulate environmental influences and genetic changes over time^[2].

These cells are evolutionarily adapted to thrive in hostile conditions, allowing them to proliferate and become more aggressive regardless of their environment. An extensive network of transcription factors and signaling pathways regulates the maintenance, growth, and division of CSCs across different types of cancer^[3].

Transcription factors such as OCT4 and KLF4 serve as key regulators by controlling gene expression and maintaining the pluripotent state of CSCs. Additionally, signaling pathways like JAK-STAT, Hedgehog, and Wnt are crucial for the complex regulation of CSC behavior, with β -catenin playing a central role in promoting self-renewal and stemness^[4]. Notch and Hedgehog pathways help maintain stemness while sequentially limiting differentiation. The JAK-STAT pathway regulates the balance between CSC longevity and growth, while TGF/ β signaling, depending on the context, can either promote or inhibit CSC self-renewal. Furthermore, CSC differentiation can be driven by activating peroxisome proliferator-activated receptors (PPARs). These intricate biological components work together to form a dynamic regulatory system that governs CSC functions and growth in the context of cancer. Proper regulation of CSC survival and proliferation is critical for tumor progression and development^[5]. A comprehensive understanding of these molecular interactions is crucial for designing effective therapeutic strategies. Since CSCs are resistant to conventional chemotherapy, tumors driven by these cells are more dangerous, as even a single surviving CSC can lead to tumor recurrence. Moreover, CSCs may be protected by altered microenvironmental conditions, unlike normal stem cells. Targeting CSCs could revolutionize cancer therapies and significantly improve patient outcomes by eliminating the cells responsible for metastasis, relapse, and treatment resistance, increasing the chances of long-term recovery and improving survival rates^[6]. Developing drugs that specifically target CSCs offers new hope for individuals battling aggressive cancers.

Despite the progress made, researchers must exercise caution when advancing therapeutic strategies. The biological role of CSCs is still in its early stages, and new evidence is constantly emerging, either challenging or confirming current knowledge. It is crucial to address the cultural, ethical, and political implications as science moves from the lab to the clinic. While not every issue can be resolved, ongoing dialogue and collaboration are essential^[7]. Understanding the molecular mechanisms governing cell division is the key to achieving a controlled balance between self-renewal and differentiation, which could promote tissue repair. More specific guidelines for the optimal use of therapeutic cells are needed to improve patient's quality of life. Novel stem cell therapies have the potential to replace existing, often expensive and ineffective treatments. Additionally, stem cells are regarded as valuable research tools for studying cancer development, embryonic growth, and cell differentiation. In the near future, advancements in our understanding of basic biology may lead to improved therapies for both human and animal diseases. Therefore, this review highlights the crucial role that CSCs play in cancer treatment^[8].

2. Origination of cancer stem cells

In a physiological setting, stem cells seldom ever proliferate. Although stem cells are infinitely expanding, they divide rather infrequently—only 10% of bone marrow stem cells are in the reproduction, pre-division, or mitotic stages simultaneously. According to current theories, CSCs can arise from completely developed somatic cells, partially developed progenitor cells found in a particular niche, or normal stem cells. As a result, CSCs have characteristics that are comparable to those of normal stem cells. Nevertheless, some requirements must be satisfied for a regular stem cell to differentiate into a CSC, particularly the absence of cell cycle regulation and a buildup of epigenetic and genetic modifications^[9]. Because they enhance drug efflux pumps, produce detoxifying enzymes, and exhibit a strong potential to promote pro-survival and anti-apoptotic processes in addition to repairing DNA, CSCs are challenging to remove. Chemotherapy presently in practice is not effective enough to treat CSCs that have low repetition indices since it concentrates on quick cell division. The ability of CSC communities to smoothly transition between distinct stages of the cell cycle in reaction to

common cell cues caused by anticancer medications is one of the hypothesized mechanisms of CSC resistance to anticancer treatments. CSCs in the G0 phase can therefore withstand prolonged periods of dormancy because they are resistant to signals of cell cycle blockage accompanied by the apoptotic cascade's collapse ^[10].

3. Involvement of non-coding RNA in CSCs

There is little information on the control of CSC epigenetics, despite the extensive description of the roles played by miRNA, lncRNA, and circRNA in CSCs. These fragments demonstrate the role of non-coding RNA in CSC epigenetics. Research demonstrated that *miR-135a* can work on DNMT1 to decrease NANOG regulator DNA methylation, increasing the potential of CSC. LncRNA HotairM1 attracted many transcription factors (TFs) to the operator of its intended gene, *HOXA1*, which led to histone H3K27 trimethylation and epigenetic silencing of *HOXA1*. Afterward, H3K27 acetylation was triggered at the NANOG gene enhancer site, which increased the activity of *HOXA1* ^[11]. NANOG buildup subsequently suppresses *HOXA1*, creating a reciprocal regulatory circuit that keeps CSC growth and regeneration intact. Furthermore, prior work has documented that lncTCF7 attracts SWI/SNF clusters to the TCF7 promoter, inducing TCF7 transcription and consequently activating Wnt signaling, which in turn amplifies the tumorigenic potential of liver CSCs ^[12].

4. Cancer stem cell mechanisms and tissue-specific microenvironment

The formation and survival of stem cells are guaranteed by the particular milieu found in a specific area. The area where stem cells are stored in preparation for self-regeneration, proliferation, and specialization required to preserve tissue homeostasis is known as the stem cell niche. CSC-specific niches are characterized by an increase in cancerous cells and an immune system disturbance. In this regard, it is critical to remember that ongoing swelling is a normal promoter in niches known to promote tumors ^[13]. The buildup of cancer-associated fibroblasts, tumor-associated neutrophils, tumor-associated macrophages, and cell-mediated attachment, that govern contacts between cells as well as stromal, endothelial, and T cells, maintains particular features of CSC niches. Extracellular capsules, soluble variables, and the extracellular matrix are further constituents that aid the environment connected to cancer. Certain characteristics of CSCs, such as penetration, dissemination, and promotion of tumor-associated neovasculature, are favored in such an environment ^[14]. It is commonly recognized that reduced oxygen environments initiate neoangiogenesis; nevertheless, the neovasculature system exhibits abnormalities because of increased permeability and an undeveloped, twisted architecture. Furthermore, hypoxic niches sustain immature CSCs by restricting cell cycling and therefore reducing the rate of cell division, which triggers the G0 phase transition. It is interesting to note that hypoxia has an impact on cancer and creates a shield against damage to the DNA. Hypoxia-inducible factors (HIFs) influence CSCs' capacity for self-renewal, division of cells, and carcinogenic potential. Accordingly, some investigations have shown increased CD44⁺ and CD133⁺ expression in hypoxic circumstances ^[15]. In blood cancers, alteration of the bone marrow (BM) niche architecture is an ongoing condition. Leukaemia cell aggregation and invasion encourage the removal of healthy hematopoietic progenitor cells from bone marrow niches and create the perfect habitat for these cells to grow in. The altered BM habitat permits LSCs to engage in normal activities such as self-regeneration, inactivity, and evading apoptosis ^[16]. Furthermore, LSCs continue to occupy the changed BM niche, serving as an accumulation for leftover leukemia cells and encouraging relapse. It is important to note that the vascular niches and the osteoblastic niche, two distinct microenvironmental zones found in the BM niche, probably influence how LSCs cycle. These environments work well together to support the movement, self-renewal, proliferation, and organization of LSCs and BM-related stem cells ^[17].

Several alterations accumulate during the tumorigenesis process, and these changes, when subjected to selection by chance, lead to the growth of more vigorous groups of cells and ultimately accelerate the growth of the tumor. While this idea is hardly new, the process of experimentally identifying the cells that can cause cancers has only recently started. As seen in leukemia, breast cancer, and prostate cancer, a tiny percentage of cancer mass cells can proliferate when transplanted into susceptible animals and create new tumors. These cells are known as CSCs, and they resemble conventional stem cells in several ways ^[18]. Because both cell types can self-regenerate, the stem cell community can be maintained eternally, and new cells can be produced that can differentiate into at least one line. Spreading rates decline with differentiation, making ultimately differentiated cells less likely to proliferate and more likely to initiate the apoptotic pathway within a certain amount of time. As a result, the bulk of the tumor mass's cells are not carcinogenic ^[19]. Tumors are unique to each other because they are made up of distinct cells during various phases of development. Understanding the biology of cancers requires in-depth research on the impact of microenvironments on the survival of CSCs because stem cells depend on a particular microenvironment to sustain their ability for regeneration and because peritumoral organs affect the preservation of the tumor territory ^[20]. Two basic properties of stem cells that impact tumor progression are the elevated production of multidrug resistance proteins and the limited degree of proliferation. These characteristics suggest that conventional chemotherapy, which mostly targets cells that are reproducing, could prove useless in getting rid of these populations ^[21].

Chemotherapy causes cancer cells to go through senescence or dormancy. When ATR protein inhibitors are given before treatment, they successfully stop tumor cells from going into inactivity, according to research done on AML organoids and mice models. This strategy may enhance the efficacy of treatment for gastrointestinal, prostate, breast, and other cancers ^[22]. Moreover, research has demonstrated that some cancer cell subgroups that can hibernate may have a role in the return of disease. To prevent the illness from relapsing and enhance the effectiveness of therapy, it is crucial to address the dormant tumor cells. Subtypes of tumor cells that understand “hibernation” can cause cancer to return ^[23]. Even though more investigation is required to fully understand the complicated processes of diapause and its consequences for tumor development, understanding this biological phenomenon provides new opportunities for developing more effective cancer treatments. Researchers work to combat cancer by understanding the cellular processes governing diapause and creating methods to specifically target cancerous cells that are inactive. This helps to avoid tumor return, eliminate resistance to therapy, and eventually enhance the lives of patients ^[24]. **Table 1** shows comprehensive information about cancer stem cell therapy, possible challenges, and the main outcomes of the investigations.

Table 1. Cancer stem cell therapy

Challenge	Therapeutic approaches	Outcomes	References
Heterogeneous nature of CSCs	Notch, Hedgehog, and Wnt signaling pathways were studied	These pathways proved their key importance as anti-cancer target points because of their association with CSC self-renewal and survival	[25,26]
Effects of tumor microenvironment	CSC-TME association was disturbed	TME is extremely important for CSC development and expansion. So, these interactions are another significant therapeutic agent for cancer therapy.	[27]
Drug resistance	CSC metabolism was targeted	Treatments that target these metabolic cascades can enhance therapy effectiveness.	[28]
Combined approach	Comprehensive strategies to use CSC-targeted treatments with traditional therapies	The combined strategies can target both CSCs and cancer cells ultimately resulting in better clinical results.	[29,30]
Biomarker development	Identification of unique CSC biomarkers	These biomarkers are pivotal for better early-stage diagnosis, prognosis, and targeted therapies.	[31]

5. Models of CSCs working

Research platforms known as CSC models are designed to replicate the traits and actions of CSCs in a lab setting. These representations are crucial for researching the biology of CSCs, their function in the initiation, development, and failure of treatment of tumors, and the creation of new treatment approaches. CSC models can be created using known cell lines or cancer cell lines obtained from patient biopsies. In these models, stem-like cells that possess capabilities like self-renewal and differentiation potential are isolated and enriched. Through examining these cell populations, scientists can learn more about the biology of CSCs, pinpoint markers unique to CSCs, and find out how these cells react to different therapies. Models developed from cell lines provide many benefits for scientific study ^[32]. Many different types of study facilities can be them because of their affordability and ease of upkeep. Numerous cell lines develop quickly, which makes large-scale research possible. Some of these cell lines can also be genetically altered, which makes it possible to study the activities of particular genes.

Furthermore, using cell lines eliminates the moral dilemmas related to studying humans or animals, which makes them a useful option for a variety of clinical contexts. Cell line-derived models have drawbacks regardless of their benefits. There may be differences between the physiological reality and the results of experiments because they fall short of capturing the intricate *in vivo* settings and connections that exist throughout a whole body. A restricted variety of kinds of tissues constitute the source of many cell lines, which reduces their accuracy. Furthermore, cell lines may experience genetic and phenotypic movement, which could cause them to diverge from the initial features of the tumor tissue ^[33]. Living in laboratory settings, they do not interface with the extracellular matrix and have little biological significance. Ultimately, their reactions to medications might not precisely mirror *in vivo* reactions, which could result in false findings during the drug study and evaluation process. GEMMs entail modifying the biological composition of mice to induce particular cancer subtypes or to concentrate on particular genes connected to CSCs. Such models enable scientists to investigate the function of particular genetic mutations in the development of CSCs, the start of tumors, and their growth. The effectiveness of targeted treatments against CSCs in an additional intricate and changing framework can also be assessed using GEMMs. They are essential for numerous scientific purposes because they closely resemble illnesses in humans and allow exquisite genetic manipulation. They may not be appropriate for all conditions, require a lot of resources, and present ethical questions ^[34].

Immunodeficient mice are implanted with tumor tissues or CSC populations obtained from patients in PDX models. These models enable the investigation of tumor growth, spread, and response to treatment *in vivo*, and they provide a more accurate representation of the tumor surroundings. PDX models are useful resources for preclinical medication screening and customized treatment strategies because they preserve the genetic diversity and diversity of the underlying tumor. When it comes to simulating human cancers and evaluating medication responses, PDX models are quite advantageous. They do not have a human immune system, are resource-intensive, and might not be practical for all kinds of tumors. When selecting PDX models for their particular research objectives, researchers ought to critically analyze these advantages and disadvantages ^[35]. Organotypic models include a variety of cells and extracellular matrix elements in an attempt to replicate the intricacy of the cancer microenvironment. Cocultures of CSCs with additional cell kinds, such as immunological, stromal, or brain cells, can be included in these models to study how these cell types communicate and impact CSC activity. Organotypic models offer an environment for researching immune escape processes, CSC-mediated tumor-stroma connections, and possible treatments. With fewer ethical issues, organotypic models provide an extremely pertinent framework for researching medication reactions and illnesses unique to certain tissues. However, when employing patient-derived samples, they can be laborious, potentially not viable in the long

run, and show inconsistency^[36].

Tumor spheroids and organoids are examples of 3D culture methods that are designed to replicate the three-dimensional structure and molecular connections found in tumors. Wilson carried out a ground-breaking experiment in 1907 that revealed the extraordinary capacity for regeneration of sponge cells. Since then, stem cell scientists have accomplished great strides toward creating organoids from stem cells to study a variety of cancer kinds, such as breast, lung, colon, and pancreatic ductal adenocarcinoma^[37]. This marked the beginning of the technique for developing organoids. To research stomach cancer, there are currently stem cell clinical systems and inspired pluripotent stem cell organoids available. These models offer an additional biologically accurate setting for researching the behavior of CSCs, such as their differentiation, self-regeneration, and responsiveness to treatments. Tumor organoids have properties comparable to the original material and preserve the variability seen in individual tumors, however, organoids produced from benign conditions are useful models for comprehending tumor formation and examining tumor-related alterations^[38]. Because of this characteristic, they are potentially useful instruments for bridging investigation and targeted therapy, providing a strong foundation for investigating the biology of cancer and individualized treatment approaches. Either known cancer cell lines or patient specimens can be used to create 3D culture systems. For example, homologous recombination deficit, chromothripsis, tandem-duplicator phenotype, and whole genome duplication are some of the mutational pathways generating chromosomal instabilities that have been effectively studied using patient-derived organoids in high-grade epithelial ovarian cancer^[39].

They can direct the emergence of targeted therapies and be utilized for evaluating chemical sensitivity. Heterogeneous reactions to distinct treatments have been examined in colorectal cancer organoids. Colorectal carcinoma organoids can forecast treatment reactions and assist in the design of tailored cancer therapies when combined with additional methods such as mass spectrometry and RNA-seq^[40].

Organoid research in the context of cancer is however subject to many restrictions, nevertheless its enormous potential. Organoid model generation outcomes vary widely, and to improve reliability, the circumstances for organoid growth need to be optimized. Organoids' inability to contain particular kinds of cells, including stromal cells, makes it more difficult to assess the effectiveness of immunomodulatory therapies and make precise predictions about the clinical effects^[41]. Furthermore, the incapacity of current organoid models to accurately replicate the intricacy of *in vivo* settings stems from their lack of vascularization and the inability to simulate connections between various tissues and organs. The wider integration of organoid equipment into medical facilities also depends on the standardization of protocols and the development of affordable production techniques. Consequently, organoids have great promise for the study of cancer; but, to fully realize their value as trustworthy preclinical and clinical models for medication assessment and customized therapy, it will be imperative to overcome these issues and advance organoid development^[42].

6. Role of CSCs in clinical findings

CSCs can serve as biomarkers for the early detection, diagnosis, and prognosis of various types of cancer. CSCs can also aid in drug radiation resistance and tumor initiation. Their presence and characteristics can provide valuable insights into tumor aggressiveness, treatment response, and the likelihood of recurrence. Identifying and targeting CSC-specific biomarkers can aid in personalized treatment strategies and monitoring disease progression^[43]. SCs can be utilized for drug screening and testing novel therapeutic agents. By culturing CSCs *in vitro* or developing animal models with CSC populations, researchers can assess the efficacy of different drugs and identify potential candidates for further clinical development. This approach enables the identification of drugs that specifically target CSCs, ultimately leading to more effective treatment options.

Researchers have discovered new biomarkers in CSCs that govern the survival and spread of cancer, and hope is rising that drug discovery to kill CSCs can follow suit ^[44]. Biomarkers can help clinicians detect that an abnormal process may be underway and can appear as an array of aberrant proteins, such as hormones, enzymes, or signaling molecules, and may vary from patient to patient.

Tumor cell dissemination, from primary origin to secondary sites, is strongly related to cancer-associated mortality in two out of every three solid tumors. The CSC paradigm assumes that solid tumors and leukemias are hierarchically defined, with CSCs at the top of this pyramid, leading to tumor development, spread, relapse, and drug resistance. Interestingly, higher CSC counts have been detected in leukemias and lymphomas, while solid tumors presented lower numbers ^[45]. However, it is considered that higher-grade tumors show higher percentages of CSCs. Nevertheless, according to the CSC model, not all of those cells are able to trigger cancer progression. Tumor spreading depends on a more anomalous and particular subpopulation of CSCs ^[46]. Thus, there is a need to identify at least two CSC/LSC subpopulations: early-stage (pre-neoplastic) and late-stage (pro-metastatic) CSCs/LSCs. To fully understand the relationship between CSCs and cancer progression, it is important to note that dysregulation of vascular homeostasis facilitates tumor progression ^[47]. Transcription factors specific for mesenchymal cells (Twist1, Slug, and Snail) and antigens (Vimentin and N-cadherin) are expressed on the surface of CSCs, helping them to undergo epithelial-mesenchymal transition (EMT) and trigger the formation of secondary malignant phenotypes, cell migration, and apoptosis-resistant CSCs. Furthermore, the upregulation of stemness-related components, including Oct4, Notch, ALDH1, and SOX1, confirms the ability to effectively switch between CSC and non-CSC states ^[48].

The EMT is stimulated by mediators released from the niche, i.e., transforming growth factor β (TGF- β), hepatocyte growth factor (HGF), HIF, Hedgehog, Wnt, and Notch. The Wnt/ β -catenin, Hedgehog, Notch, and PI3K/Akt/mTOR signaling pathways are upregulated in all solid and non-solid tumors, leading to the enhancement of CSC/LSC-specific properties. The Wnt pathway enhances cancer cell division, motility, and drug resistance, while the self-renewal of CSCs/LSCs is mediated by the Hedgehog and Notch pathways ^[49]. However, the research so far has not allowed us to fully understand and control the mechanism by which CSCs/LSCs contribute to cancer invasion. Nevertheless, the above-indicated signaling pathways provide a mechanism for explaining the differences in behavior between early-stage (pre-tumorigenic stem cells) and late-stage CSCs/LSCs ^[50]. The Wnt/ β -catenin pathway is fundamental to preserving the self-renewal ability of early-stage stem cells in leukemias; breast, lung, and liver cancers; and melanomas, whereas the Notch signaling pathway has been implicated in stemness of late-stage cancer stem cells in AML, breast cancer, colon cancer, and glioblastoma. Stemness of late-stage CSCs in glioblastoma, colon cancer, and pancreatic cancer involves the Hedgehog signaling pathway ^[51].

Hematological malignancies are highly heterogeneous concerning the diversity of clinical presentation, cytogenetics, and molecular profiles, as well as a future outcome that is associated with patient- and leukaemia-related factors. Hematological malignancies arise not only from the genetic alterations in malignant cells but also due to their communication/symbiotic relationship with the microenvironment. The evolution of the disease is strongly associated with reciprocal communication between stroma and malignant cells, which promotes anti-apoptotic signals in LSCs during their migration to the secondary space ^[52]. Many studies demonstrated that CSCs are quiescent or slowly dividing, whereas leukemia progenitors are able to divide rapidly by escaping the dormant state. Indisputably, LSCs hold great importance in the pathogenesis and relapse of leukemia; thus, hematological malignancies should be treated based on stemness patterns. Furthermore, the heterogeneous LSC population shows diversity at the level of functionality, since there exist sub-colonies that display the unfavorable phenotypes of dormancy, long-term neoplasm propagation, and drug insensitivity.

This has modified the understanding of therapeutic needs in hematological malignancies because unfavorable phenotypes of dormancy are reversible and give space to use LSC-targeted treatments that prolong remission periods ^[53].

7. Treatment and side effects associated with stem cell therapy

Addressing CSCs in particular is a potential malignancy therapeutic strategy. Although fast-dividing cells are the main focus of traditional cancer procedures like radiation and chemotherapy, CSCs may not be eliminated since they are frequently more susceptible to these remedies. CSCs may endure the first course of treatment and aid in the spread and return of tumors. To create effective customized therapies, the separation and recognition of malignant tissues must be precisely programmed and constructed ^[54]. This involves comprehending the processes that control the growth of cancer stem cell populations and the emergence of drug tolerance. Immunomodulation, immunological evasion, and impact resistance induced by CSCs profoundly disrupt the homeostasis of the native defense system. Tumor advancement can be controlled by utilizing the signaling pathways of notch receptor, mammalian target of rapamycin, Wnt/ β -catenin, and sonic hedgehog. The creation of medications that specifically target CSCs or interfere with their surroundings that support their existence is another aspect of CSC-based therapy. These strategies seek to prevent CSCs from proliferating, stimulate them to differentiate into noncancerous cell types, or make them more susceptible to standard therapeutics ^[55]. The use of immunotherapy for fighting CSCs is another new field of study. Immunotherapies use the immune response of the body to identify and eradicate CSCs. To boost the immune system towards CSCs and enhance therapeutic results, techniques such as immune checkpoint inhibitors, chimeric antigen receptor (CAR) T-cell therapy, and tumor vaccinations are being investigated ^[56].

The use of CSCs in therapeutics has the potential to enhance cancer medication results. However, this method carries some dangers and possible negative consequences, just like various medical methods. While the likelihood of these adverse reactions and consequences is usually low, some of the documented adverse consequences of CSC therapy involve nausea, vomiting, and sore throats and mouths. Blood-related issues have also required transfusions. Inflammation and bleeding are other risks, which are typical worries with any invasive medical operation ^[57]. Additional possible hazards associated with CSC therapy encompass hepatic hyperplasia and hepatic veno-occlusive disease. Nonetheless, care should be taken to reduce any possible dangers related to the therapy. One way to increase the likelihood of effective malignant tissue growth in the given surroundings is to minimize using immune-suppressive drugs during the course of therapy. Strict control of any adverse reactions and ongoing surveillance are required to guarantee the efficacy and efficacy of CSC intervention ^[58]. Researchers and medical experts are always trying to increase our knowledge of these risks and create plans to reduce them. The objective is to give patients more secure and efficient therapies for treating their cancer by decreasing the hazards and negative consequences related to CSC therapies.

CSCs have been thoroughly investigated for their possibility as immunizations since they are essential in enhancing the body's immune response to combat cancer. According to new investigations, putting CSCs into the body can boost the body's defenses in attacking different kinds of cancer and assist fight growth-promoting proteins. T-cancer cells are specific tumor cells found in humans that continuously scan the outside of malignant tissues for anomalies or possible hazards to the body. Viral or bacterial infection can be suggested by alterations in the patterns of cancer cells, such as mutations or changed transcription [59]. But as they have grown, cancers have evolved defense processes, making it difficult to find and cure throughout their initial phases. The body's immune reaction is weakened as a result of this escape, which lets cancer spread. Although surgery is frequently

regarded as the most successful way to remove tumors, it cannot ensure that all cancer cells are completely eradicated. Regenerating residual cells has the potential to cause illness relapse. This highlights the necessity of complementing strategies that might fortify the immune system and improve its capacity to identify and eradicate cancerous cells. Approaches for vaccination based on CSCs have a lot of potential to do this ^[60]. Through the utilization of CSCs' distinct qualities, scientists hope to create novel treatments that focus on and stimulate the immune response to combat cancer. These initiatives aim to strengthen the immune system's ability to combat malignant cells to conquer the obstacles presented by tumor avoidance strategies and increase therapeutic efficacy ^[61]. In the continuing struggle over malignancy, the adoption of CSC-based vaccination techniques offers new prospects for more potent and specialized therapy.

8. Future of CSCs

Future breakthroughs in CSC study will open the door to important new insights into the genetics of cancer and the creation of creative treatment strategies. Characterization of CSC indicators, which entails discovering particular DNA fingerprints and genetic characteristics linked with CSCs in diverse kinds of cancer, is one noteworthy area of development. By isolating and examining CSC populations, researchers can gain a better knowledge of their biology and behavior as well as identify prospective targets for treatment therapies. Investigating CSC diversity among tumors is a crucial component of CSC research ^[62]. It is now acknowledged that CSC populations show notable inclusion with several subpopulations exhibiting unique traits and activities. The processes causing this variation and their consequences for therapeutic opposition, metastasis, and tumor development are the subject of intense scientific investigation. Gaining an understanding of CSC heterogeneity is essential to creating tailored therapy plans that target particular CSC groups, improving therapeutic outcomes, and lowering the risk of cancer resurgence. Additionally, a lot of work is being done to target signaling systems unique to CSCs. Important functions such as CSC self-renewal, distinction, and preservation are regulated by these pathways. Researchers hope to impede tumor development and disturb CSC stability by focusing on these mechanisms. It has been determined that pathways like notch, Wnt, and hedgehog could be candidates for a prevention approach ^[63]. To improve therapeutic success, strategies that incorporate CSC-targeted medicines with traditional procedures including radiation and chemotherapy are being investigated. Targeting both the bulk tumor cells and the CSC subpopulations at the same time is intended to improve tumor shrinkage, avoid a return, and overcome drug resilience.

Research on CSCs is beginning to show potential with immunotherapies as well. When it comes to identifying and getting rid of tumor cells, particularly CSCs, the immune response is essential. To strengthen the body's reaction against CSCs and boost results for patients, researchers are creating immunotherapeutic strategies such as immune checkpoint inhibitors and adoptive cell treatments. To interfere with CSC niches and the relationships that sustain them, researchers are also concentrating on altering the tumor habitat ^[64]. The functioning of CSCs and the growth of tumors are significantly influenced by the tumor microenvironment, which includes immune cells, stromal cells, and extracellular matrix elements. Inhibiting CSC proliferation and metastasis through therapeutic targeting of the microenvironment has promise. Additionally, efforts are being made to create medications that particularly target CSCs, such as CSC destruction mediated by nanoparticles ^[65]. Drugs that precisely attack CSCs are designed to eradicate them while preserving normal stem cells, minimizing negative consequences, and enhancing the effectiveness of medication. Different strategies, outside of nanomedicine, are being investigated for their ability to target CSC-specific pathways or indicators, including small molecule inhibitors and antibodies. Individualized healthcare techniques are becoming possible due to

the genetic identification of CSCs made possible by technological advancements in single-cell sequencing and genomics ^[66]. Customized medicines can be created to address the distinctive traits of each patient's CSC community by comprehending the genetic and epigenetic changes particular to CSCs within their tumor, hence enhancing therapy results and patient mortality percentages. Research on cancer stem cells is quickly progressing due to continuing advancements in CSC biology and treatment approaches ^[67]. These developments have enormous potential to further our knowledge of CSCs, enhance the results of cancer treatments, and eventually provide cancer victims with greater flexibility and potent treatments. For CSC-based cancer therapy methods to reach their maximum effectiveness, more study and cooperation in this field are essential.

9. Conclusion

Strong populations of CSCs and LSCs possess the capacity to permanently transition between distinct stages of the cell cycle, enabling them to halt pro-apoptotic cues and extend their longevity in an inactive form. Therefore, to recognize individuals who are at high risk for repeated illnesses, more advanced but user-friendly approaches for identifying CSCs/LSCs are desperately needed. Decades of research have led to constant advances in the field of cancerous cell research, with stem cell therapies demonstrating great success. Notwithstanding the difficulties that still exist, every new study deepens our knowledge of the potential applications of stem cells in regenerative therapy. Cancer stem cell treatment has amazing possibilities for managing illnesses that were thought to be irreversible. Owing to the invention of pluripotency, it is now possible to use sufferers' malignant tissues. This has resulted in the creation of tissue banks, becoming an invaluable tool for restorative medicine in the treatment of a variety of illnesses. Cancer stem cell therapy has had an unparalleled effect on prolonging human life, presenting bright future possibilities. One of the most fascinating and potential branches of cancer study nowadays is the development of therapies that utilize cancer stem cells. Researchers hope that maximum approaches will be discovered in which such cancer stem cells will be used for therapeutic purposes and will emerge as a new treatment option for cancer with comprehensive treatment options.

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The Effect of Immunotherapy on the Gut Microbiota, Intestinal Barrier, and Immune Function in Patients with Gastric Cancer

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Abstract: *Objective:* To explore the effect of immunotherapy on the gut microbiota, intestinal barrier, and immune function in patients with gastric cancer. *Methods:* From July 2023 to July 2024, 60 patients with gastric cancer from our hospital were randomly divided into two groups, the control group and the study group, with 30 patients in each group. The control group received conventional treatment, while the study group received immunotherapy. A comparative analysis was conducted between the two groups on gut microbiota content (*Bifidobacterium*, *Fusobacterium nucleatum*, *Streptococcus*, *Lactobacillus acidophilus*), intestinal barrier indicators [D-lactate (D-LA), diamine oxidase (DAO), lipopolysaccharide (LPS)], immune function indicators [Immunoglobulin A (IgA), Immunoglobulin G (IgG), Immunoglobulin M (IgM)], adverse reactions, and treatment effects. *Results:* After treatment, the content of *Bifidobacterium* and *Fusobacterium nucleatum* in the study group was higher than in the control group, while the content of *Streptococcus* and *Lactobacillus acidophilus* was lower than in the control group ($P < 0.05$). The levels of D-lactate and DAO in the study group were lower than in the control group, while the LPS level in the study group was higher ($P < 0.05$). The levels of IgA and IgG in the study group were lower than in the control group, and the IgM level was also lower than in the control group ($P < 0.05$). After treatment, the total incidence of adverse reactions in the study group was lower than in the control group ($P < 0.05$). The overall treatment efficacy rate in the study group was higher than in the control group ($P < 0.05$). *Conclusion:* Immunotherapy in patients with gastric cancer can improve gut microbiota, intestinal barrier, and immune function, reduce the occurrence of adverse reactions, and promote better clinical treatment outcomes, making it worthy of clinical recommendation.

Keywords: Immunotherapy; Gastric cancer; Gut microbiota; Intestinal barrier; Immune function

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1. Introduction

As one of the most common malignant tumors worldwide, gastric cancer shows a high incidence and mortality rate. With the continuous evolution of social changes, living environments, and lifestyle transformations in China, the incidence and mortality rates of gastric cancer have been gradually rising^[1]. Surgery is a common

treatment for early-stage gastric cancer and has certain therapeutic effects, but postoperative recurrence rates are high. When gastric cancer progresses to the late stage, the significance of surgical treatment declines substantially, making the search for an effective and safe treatment method all the more crucial. Clinical investigations have revealed that immunotherapy has shown favorable effects in antitumor treatment in recent years. The mechanism of immunotherapy lies in its ability to stimulate the patient’s immune system, thereby effectively enhancing the body’s ability to recognize and eliminate tumor cells. However, its clinical mechanism remains unclear. Some scholars have noted in their research that immunotherapy, as an innovative approach to cancer treatment, has demonstrated remarkable efficacy in the treatment of gastric cancer. However, due to the complexity of the immune system, further in-depth research is required on the interaction between drugs and the immune system to enhance the antitumor effects of immunotherapy in the future ^[2,3]. Based on this, this study selected gastric cancer patients treated in our hospital, provided immunotherapy, and analyzed its effect on the gut microbiota, intestinal barrier, and immune function of gastric cancer patients, thus providing some reference data for the subsequent clinical treatment of gastric cancer patients.

2. Materials and methods

2.1. General information

From July 2023 to July 2024, 120 gastric cancer patients from The First Affiliated Hospital of Yangtze University were selected and randomly divided into a control group and a study group. The baseline data between the two groups showed no significant difference ($P < 0.05$), as shown in **Table 1**.

Inclusion criteria: Patients diagnosed with gastric cancer through gastroscopic biopsy, pathology, and medical imaging; complete clinical follow-up data; patients and their families agreed to treatment and signed informed consent; and approved by the hospital’s ethics committee.

Exclusion criteria: Participants with liver dysfunction; those with cognitive impairments accompanied by mental health issues that hindered their ability to cooperate effectively in the study; patients with unstable vital signs.

Table 1. Comparison of general information (mean ± SD)

General information		Control group (n = 30)	Study group (n = 30)	t / χ^2	P
Gender	Male	16	17	0.162	0.614
	Female	14	13		
Average age		58.50 ± 2.80	60.00 ± 4.00	0.795	0.831
Average BMI		20.00 ± 2.40	20.50 ± 2.80	0.058	0.931

2.2. Methods

Both groups of patients received routine treatment. The control group was given chemotherapy: oral capecitabine (approval number: National Medicine Standard H20143365) at 2,500 mg/m² daily for two weeks, followed by a one-week break; intravenous drip of oxaliplatin (batch number: 2009312707) at 130 mg/m² on day 1.

Treatment method for the study group: Sintilimab (approval number: National Medicine Standard S20180016) at 200 mg every three weeks, or Tislelizumab (batch number: 2018112618) at 200 mg every three weeks, for a total of 6 cycles.

2.3. Observation indicators

2.3.1. Comparison of intestinal flora content between the two groups

Before treatment, 3–5 g of fecal samples were collected, processed under sterile conditions, and cultured on appropriate media plates for bacterial growth. The bacterial content in fecal dilutions (expressed as \log_{10}) was calculated to assess levels of *Bifidobacterium*, *Fusobacterium nucleatum*, *Streptococcus*, and *Lactobacillus acidophilus*.

2.3.2. Comparison of intestinal barrier function between the two groups

Four milliliters of fasting venous blood were collected from the patient's elbows, and centrifuged to remove the supernatant, and the double-antibody sandwich method was used to detect diamine oxidase (DAO). The enzyme colorimetric method was used to detect D-lactate and lipopolysaccharides (LPS), assessing intestinal barrier function.

2.3.3. Comparison of immune function between the two groups

The levels of immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) in the supernatant were detected using the immunoturbidimetric assay.

2.3.4. Comparison of adverse reactions between the two groups

The incidence of bone marrow suppression, gastrointestinal reactions, and other adverse events in the two groups was compared.

2.3.5. Comparison of clinical efficacy between the two groups

CT scans were used to evaluate tumor size in the tumor area before and after treatment. The overall response rate was calculated as (complete response + partial response) / total cases \times 100%.

2.4. Statistical analysis

Statistical analysis was performed using SPSS 26.0 software. Measurement data were expressed as mean \pm standard deviation (SD), and the *t*-test was used for analysis. Count data were expressed as percentages (%) and analyzed using the χ^2 test. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Comparison of intestinal flora content between the two groups

Before treatment, there was no statistically significant difference in the levels of *Bifidobacterium*, *Fusobacterium nucleatum*, *Streptococcus*, and *Lactobacillus acidophilus* between the two groups ($P > 0.05$). After treatment, the levels of *Bifidobacterium* and *Fusobacterium nucleatum* increased, while *Streptococcus* and *Lactobacillus acidophilus* decreased in both groups. The levels of *Bifidobacterium* and *Fusobacterium nucleatum* were significantly higher in the study group than in the control group, while the levels of *Streptococcus* and *Lactobacillus acidophilus* were significantly lower in the study group compared to the control group, with a statistically significant difference ($P < 0.05$). See **Table 2**.

Table 2. Comparison of intestinal flora content before and after treatment (mean \pm SD)

Groups	Bifidobacterium		Fusobacterium nucleatum		Streptococcus		Lactobacillus acidophilus	
	Before	After	Before	After	Before	After	Before	After
Control group ($n = 30$)	7.89 \pm 0.88	8.12 \pm 0.62	7.66 \pm 0.66	8.05 \pm 0.44	9.33 \pm 0.85	8.94 \pm 0.65	8.14 \pm 0.66	7.94 \pm 0.54
Study group ($n = 30$)	8.01 \pm 0.72	9.52 \pm 0.77	7.85 \pm 0.74	8.76 \pm 0.85	9.21 \pm 0.75	8.44 \pm 0.51	8.26 \pm 0.64	7.55 \pm 0.62
<i>t</i>	0.667	8.957	1.212	4.692	0.669	3.186	0.825	3.000
<i>P</i>	0.506	0.001	0.229	0.001	0.505	0.002	0.411	0.003

3.2. Comparison of intestinal barrier function indicators between the two groups

Before treatment, there was no statistically significant difference in the intestinal barrier function indicators between the two groups ($P > 0.05$). After treatment, the levels of D-lactate and DAO in both groups significantly decreased, while the levels of LPS significantly increased. The study group had lower levels of D-lactate and DAO compared to the control group, and higher LPS levels compared to the control group, with a statistically significant difference ($P < 0.05$). See **Table 3**.

Table 3. Comparison of intestinal barrier function indicators before and after treatment (mean \pm SD)

Groups	D-lactate (mg/L)		DAO (U/L)		LPS (EU/L)	
	Before	After	Before	After	Before	After
Control group ($n = 30$)	5.92 \pm 0.54	3.45 \pm 0.66	5.59 \pm 0.62	2.55 \pm 0.96	0.52 \pm 0.11	1.45 \pm 0.36
Study group ($n = 30$)	5.96 \pm 0.63	2.55 \pm 0.74	5.65 \pm 0.61	1.65 \pm 0.45	0.56 \pm 0.18	0.86 \pm 0.21
<i>t</i>	0.304	5.741	0.436	5.369	1.199	8.953
<i>P</i>	0.761	0.001	0.663	0.001	0.234	0.001

3.3. Comparison of immune function between the two groups

Before treatment, there was no statistically significant difference in immune function between the two groups ($P > 0.05$). After treatment, the levels of IgA and IgG decreased, and IgM levels increased in both groups. The study group had lower levels of IgA and IgG compared to the control group, and higher levels of IgM compared to the control group, with a statistically significant difference ($P < 0.05$). See **Table 4**.

Table 4. Comparison of immune function before and after treatment (mean \pm SD)

Groups	IgA (ng/L)		IgG (ng/L)		IgM (ng/L)	
	Before	After	Before	After	Before	After
Control group ($n = 30$)	4.45 \pm 1.02	3.02 \pm 0.96	20.45 \pm 2.56	14.77 \pm 2.23	3.36 \pm 0.68	3.76 \pm 0.66
Study group ($n = 30$)	4.33 \pm 1.05	3.84 \pm 0.88	19.85 \pm 2.46	17.48 \pm 2.84	3.23 \pm 0.42	2.74 \pm 0.54
<i>t</i>	0.518	3.982	1.402	4.666	1.029	7.565
<i>P</i>	0.605	0.001	0.164	0.001	0.306	0.001

3.4. Comparison of adverse reactions between the two groups

Table 5 shows that after treatment, the overall incidence of adverse reactions in the study group was lower than that in the control group, with a statistically significant difference ($P < 0.05$).

Table 5. Comparison of adverse reactions [*n* (%)]

Groups	Chest tightness	Dyspnea	Gastrointestinal reactions	Total incidence
Control group (<i>n</i> = 30)	4 (13.33)	3 (10.00)	2 (6.67)	9 (30.00)
Study group (<i>n</i> = 30)	1 (3.33)	0 (0.00)	1 (3.33)	2 (6.67)
χ^2				5.164
<i>P</i>				0.023

3.5. Comparison of treatment efficacy between the two groups

Table 6 shows that after treatment, the overall efficacy rate in the study group was higher than that in the control group, with a statistically significant difference ($P < 0.05$).

Table 6. Comparison of treatment efficacy [*n* (%)]

Groups	Significantly effective	Effective	Ineffective	Overall effective rate
Control group (<i>n</i> = 30)	12 (40.00)	10 (33.33)	8 (26.67)	22 (73.33)
Study group (<i>n</i> = 30)	15 (50.00)	13 (43.33)	2 (6.67)	28 (93.33)
χ^2				4.904
<i>P</i>				0.027

4. Discussion

As a global disease, gastric cancer ranks among the most serious malignant tumors of the digestive tract. The main clinical treatments for gastric cancer include surgery and chemotherapy. However, due to the non-specific symptoms of early-stage gastric cancer, such as abdominal discomfort and morning acid reflux, diagnosis is often difficult. As a result, most patients are diagnosed in the middle or late stages, leading to limitations in clinical treatment^[4,5].

In recent years, immunotherapy has emerged as a research hotspot with promising prospects in the field of cancer treatment, showing great potential^[6,7]. Clinical investigations have found that the immune system in the body has the ability to identify and eliminate antigenic foreign substances. Since malignant tumors are masses formed by the mutation of normal cells, the immune system can recognize and eliminate them. Therefore, targeting the immune system for treatment holds tremendous research potential^[8,9].

The results of this study showed that in the study group, *Bifidobacterium* and *Fusobacterium nucleatum* increased, while *Streptococcus* and *Lactobacillus acidophilus* decreased. After treatment, the intestinal barrier indicators D-lactate and DAO levels decreased, with the study group showing lower levels than the control group, and LPS levels increased, with the study group showing higher levels than the control group. Immunotherapy effectively altered the structure and metabolic function of the intestinal flora, improved the diversity of gut microbiota, and enhanced the thickness of the intestinal mucosal layer, significantly improving the mucosal defense capability. Furthermore, this study found that patients' immune functions improved, and adverse reactions were lower, indicating good clinical value.

In conclusion, immunotherapy for gastric cancer patients results in significant improvements in intestinal flora and immune function, demonstrating effective treatment outcomes and warranting further clinical promotion.

Disclosure statement

The authors declare no conflict of interest.

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Exploring the Pivotal Association of AI in Cancer Stem Cells Detection and Treatment

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Abstract: Cancer stem cells (CSCs), or tumor-initiating cells (TICs), are cancerous cell subpopulations that remain while tumor cells propagate as a unique subset and exhibit multiple applications in several diseases. They are responsible for cancer cell initiation, development, metastasis, proliferation, and recurrence due to their self-renewal and differentiation abilities in many kinds of cells. Artificial intelligence (AI) has gained significant attention because of its vast applications in various fields including agriculture, healthcare, transportation, and robotics, particularly in detecting human diseases such as cancer. The division and metastasis of cancerous cells are not easy to identify at early stages due to their uncontrolled situations. It has provided some real-time pictures of cancer progression and relapse. The purpose of this review paper is to explore new investigations into the role of AI in cancer stem cell progression and metastasis and in regenerative medicines. It describes the association of machine learning and AI with CSCs along with its numerous applications from cancer diagnosis to therapy. This review has also provided key challenges and future directions of AI in cancer stem cell research diagnosis and therapeutic approach.

Keywords: Artificial intelligence; Cancer; Cancer stem cells; Health

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1. Introduction

Artificial intelligence (AI) powerful statistical abilities have an opportunity to completely transform cancer research, diagnosis, and therapy. Strong computing skills are therefore required to merge information with complex results from studies, a potential made possible by big malignant study information that has never been seen before ^[1]. To address the high complexity faced by healthcare facilities, including that which is presented by biological system anomalies like cancer, AI uses algorithmic mathematical principles that replicate the functioning of the human brain. Modern medical diagnostics and treatment will likely be dominated by AI and machine learning (ML), which are already expected to have an important effect on everyday life ^[2]. Recent advances in AI and ML have made it possible for tools for diagnosing autonomic neuropathy disease to use massive amounts of data to address the difficulty of human disease identification at an incredibly early stage, especially in cancer ^[3]. Cancer is among the two leading reasons for mortality globally and is a major public

health concern. Uncontrolled cell division that results in aberrant cell creation and quick body expansion is a hallmark of cancer. Complex in nature, cancer arises from healthy cells primarily due to aberrant cell division and an elevated burden of mutations ^[4]. It is a complex condition that makes early diagnosis challenging. For this reason, the scientific community and researchers are primarily concerned with multifactorial disorders and cancer therapy. Cancer is one of the toughest conditions, and even with international initiatives, the prevalence of cancer patients is rising ^[5]. Initial cancer screening and diagnosis are less precise, and the majority of patients do not receive a diagnosis using the methods and procedures that are currently in use. Current breakthroughs in combination chemistry, genomics, and proteomics have made a multitude of databases containing living and chemical information available. These advancements have the potential to greatly advance our knowledge of the molecular biology of cancer ^[6]. A deeper comprehension of cancer biology could have a big impact on the way we diagnose and treat patients with cancer in hospitals.

Similar to other kinds of cells, tumors also sense sensory signals from their environment, which affect DNA transcription, cell conduct, and efficiency. Cancer cells are only one component of the procedure of cancer development ^[7]. The extracellular matrix (ECM), non-cellular constituents, invasive cell elements, and signaling chemicals form a complex structure that is known as the tumor microenvironment (TME) ^[8]. Software developers and healthcare scientists can now collaborate directly to use multi-factor assessment to enhance prediction because of scientific improvements. It has been reported that such analyses yield much better results than actual estimations. Scientists are currently focusing on creating models that use AI algorithms to recognize and anticipate cancer as part of the AI application. These methods are now being applied to increase the precision of their existence, return, and cancer prognosis ^[9].

The TME's many genomic and noncellular elements exchange information back and forth. Tumor development may be influenced by biochemical signals as well as topography, rigidity, shear stresses, interstitial flow, and fluid pressures. In a tumor wound, an abnormal circulation that produces a differential of nutrients, oxygen, and metabolic waste products leads to the development of a necrotic core ^[10]. The cells in the core region become dormant and change making their eradication difficult. They also produce hypoxia-inducible proteins and other cytokines that may affect the bodily functions of surrounding cells ^[11]. Furthermore, the way malignant cells spread is regulated by relationships between cells and the ECM in the manner of extracellular matrix transformation, perivascular tissues, fibroblast application, and immune systems. The TME is essential to the tumor's evolution because it actively communicates with the adjacent environment in both directions, encouraging and stimulating the development of the tumor and its spread ^[12]. Moreover, a substantial body of research has demonstrated the TME's protective function in halting tumor growth and spread in the initial levels of tumor formation, and how "re-programming" the TME in these stages afterward could greatly advance the growth of cancer treatments ^[13].

Hypoxia is necessary for cancer cells to thrive, so to support cancerous cell life, hypoxia-inducible factor-1 (HIF-1) binds to react to components of hypoxia, activating numerous genes in the process. HIF-1-specific genes majorly regulate tumor metastasis, angiogenesis, pH, attachment, and metabolic processes. The TME, composed of stromal cells, secreted chemicals, and ECM proteins, is a complex mechanism including tumor cells ^[14]. A malignant composed of changed, constant cell division is not the sole formation caused by cancerous cells. The growth, advancement, and aggressiveness of tumors depend critically on their association with these microenvironmental components ^[15].

The metabolic regulation of metabolic processes constitutes one of the features of cancer that contributes to its growth. Oncogenic processes lead to abnormal metabolic processes and confer unfair benefits on tumor cells that facilitate increased proliferation and survival in unfavorable surroundings ^[16]. Other ways that a repaired

physiology effectively encourages the growth of tumors include the synthesis of oncometabolites connection to signals, and metabolite-dependent regulatory control. The alterations in the metabolic cycle of tumor cells have an impact on nearby TME cells and aid in regulating significant events involved in the growth of malignancy, such as angiogenesis, inflammation, and cancer resistance^[17]. Therefore, analyzing the biochemical interactions between cancer and healthy cells in the TME could identify vulnerabilities associated with cancer and provide fresh possibilities for cancer detection and therapy^[18].

The clinical oncology-based investigation is primarily concerned with determining how tumor cells proliferate to shed light on the disease's biological origin. Additionally, it sought to address the growing global cancer mortality toll by using machine learning to handle massive amounts of data from millions of appropriate situations^[19]. Furthermore, it is anticipated that applying AI to medical decision-making could facilitate the use of high-resolution imaging and NGS to forecast and detect disorders early. AI can be used to synthesize fresh biomarkers for tumor diagnosis. This requires the creation of novel, individualized medications as well as the provision of prospective therapy regimens through the creation of sizable databases and the application of bioinformatics techniques^[20]. The goal of AI working is to create an environment that is appropriately educated to effectively advise on whether or not an individual will need immunotherapy. AI can determine which individuals require additional evaluations, such as whole genome spectroscopy, and which immunotherapy medication will have the greatest effect on a patient's rehabilitation^[21].

The increased use of AI in healthcare is combined with real-world case research that has been published up to this point to fully address the gaps and restrictions in the diagnosis of different kinds of cancer. Also, a thorough explanation of the structure needed for AI to function is provided. The primary goal of the present review was to examine how biological and artificial intelligence might be used to tackle cancer-related health problems in future decades to keep scientists informed about how AI will be used in computerized forensics and detection and how it will impact cancer therapy over time.

2. AI and common therapeutic approaches

AI in cancer treatment concentrates heavily on the interaction between patients and medications. The control of chemotherapy medication intake, the estimation of chemotherapy drug dependence, and the improvement of chemotherapy programs are among the primary applied accomplishments of AI. AI has the power to streamline and expedite the procedure of optimizing combination chemotherapy^[22]. Choudhary *et al.* have created a deep learning-based diagnostic method that can identify tumor cells with HR defects with 74% reliability and identify patients who could profit from PARP medicines. A machine learning algorithm created by Khan *et al.* can forecast if breast cancer would tolerate chemotherapy. The research managed to discern the connection between the adverse reactions of two chemotherapy medications, taxol and gemcitabine, by examining the connection between the patient's genes and the treatments^[24]. Furthermore, research has demonstrated that the deep learning technique outperforms the Epstein-Barr Virus-DNA-based framework in terms of risk division and initial chemotherapy direction for nasopharyngeal cancer. This suggests that the deep learning approach's directing function could be a useful predictor of single-induction chemotherapy for developed nasopharyngeal cancer^[25]. **Table 1** summarizes the key roles of artificial intelligence in cancer stem cell detection and therapy from previous studies.

Table 1. Artificial intelligence in cancer stem cell study

Application	Used AI strategies	Explanation	Advantages
Identification	ML image analysis	ML algorithms used to recognize CSCs using images	Better and more precise recognition of CSCs than manual analysis ^[65,66]
Treatment	ML for suitable drug discovery	AI models help to set and identify underlying drugs for CSCs	The significant discovery of novel therapeutic strategies to target CSCs and mitigate sources of cancers ^[67,68]
Treatment	Predictive modeling	Patient reactions to the used treatment strategy were assessed against CSCs	Increases precise therapeutic approaches and diminishes side effects targets ^[69,70]

The use of AI innovation has a greater emphasis during cancer radiation treatment. AI can be used by radiologists to autonomously plan radiotherapy schedules or map out regions of interest. Considering a precision of 79%, Chen *et al.* worked on automated nasopharyngeal cancer delineation using the three-dimensional convolutional neural network (3D CNN) corresponds to that of radiation professionals ^[26]. Using machine learning and radiomics—a technique for obtaining picture attributes from radiographic images developed a prediction model that assesses how well bladder cancer treatments will work. By using deep learning gadgets, one of the studies created computer programs that cut the time needed to schedule radiation treatment down to a brief period ^[27].

AI is mostly used in cancer immunotherapy applications to assess therapeutic efficacy and assist doctors in modifying the medication regimen. A study created a machine learning-based artificial intelligence system to precisely forecast the curative effects of programmed cell death protein 1 (PD-1) antagonists ^[28]. This system is useful for assessing the impact of immunotherapy in patients with PD-1 inhibitor-sensitive progressive solid malignancies. A machine learning technique derived from the human leukocyte antigen (HLA) mass spectrometry database was developed and has the potential to enhance the effectiveness of tumor immunotherapy by improving the detection of cancer neoantigens ^[29]. To prevent patients from receiving unnecessary treatments, an algorithm can efficiently recognize precancerous abnormalities from computerized pictures of women's cervixes that require therapy. A machine learning method can lessen the overtreatment of tumors thought to be breast cancerous. With the use of this technology, physicians can prevent needless surgery and establish informed choices regarding treatment by identifying high-risk breast lesions that have the potential to develop into cancer ^[30].

Deep learning technologies improve the intelligence of cancer therapeutic decisions. Artificial Intelligence can identify the best course of therapy for physicians based on its understanding of medical large amounts of information from cancer victims. A Clinical Decision Support System (CDSS) built on deep learning algorithms was formed ^[31]. It can produce alternative therapies for cancer by extracting and analyzing an enormous quantity of diagnostic information from health records. The study highlights how crucial AI innovation is to doctors' efforts to enhance patients' cancer therapy regimens. AI's capacity for deep learning and innovative solving issues has brought its capabilities to the attention of scientists and researchers ^[32].

3. AI in anticancer drug formation

Models that anticipate how tumor cell lines, as well as individuals, will react to novel medications or treatment mixtures can be created by training machine learning techniques on high-throughput testing information. By employing machine learning to design and construct reverse synthesis processes for chemicals, researchers are speeding up the process of finding new drugs. A great deal of information is generated throughout the

entire procedure of developing a new medication ^[33]. Our ability to analyze chemical information and produce outcomes that will aid in creating drugs is greatly enhanced by machine learning. We may analyze information gathered over years or even centuries in a relatively brief period with the assistance of machine learning. Furthermore, it will enable us to arrive at better-informed choices than we would have to if we had to rely just on experience and predictions ^[34].

Drug development is one of the numerous fields in which deep learning, a novel machine-learning method, has demonstrated outstanding results. Although the use of deep learning in pharmaceutical effect projection has only just been investigated, these algorithms have special qualities that could render them better suited for difficult challenges involving the simulation of drug responses employing chemical and biological information ^[35]. Current developments in deep learning have allowed computers to gather data from photographs like never before. Exciting fresh possibilities for drug recycling have arisen from the development of deep learning models utilizing large-scale collection of data ^[36].

4. Using AI in regenerative medicine

AI has emerged as a critical component in the execution of computational models and in silico investigations for healthcare purposes. It has several benefits over conventional therapeutic inquiry strategies, including quicker outcomes and reduced expenses. Numerous major tasks are now being undertaken to integrate AI into a variety of industries, including but not limited to healthcare, drugs, and medical care ^[37]. These initiatives seek to improve and expedite some procedures, including medication creation, illness diagnosis, and medical care, by utilizing AI. Researchers and practitioners believe that incorporating AI will lead to more precise and effective results, which will ultimately improve people's standard of living individually and in groups ^[38]. In particular, by automating processes like sifting through massive molecular and biological records and finding relationships and trends that human scientists would overlook, deep learning can hasten the discovery of regenerative medicines. By doing so, scientists may be able to create more potent treatments to target the underlying illness processes. It discusses a few of many significant areas in regenerative medicine where artificial intelligence may prove beneficial ^[39].

The chemical universe contains an enormous quantity of compounds, which presents advantages and difficulties for the search and creation of new drugs. Drug development in the setting of regenerative therapy is the procedure of locating compounds, biological products, or similar medicinal substances that can support tissue regeneration and operational restoration. The absence of modern technology hinders the creation of new medications. Since conventional drug research procedures need the synthesis and screening of several molecules to find possible therapeutic candidates, they can be costly and time-consuming ^[40]. Making sure that possible treatment options are both healthy and efficient is a key consideration in the medication development process. AI has become a potent instrument in the fight against these obstacles, able to anticipate which medicines are most effective for particular ailments by analyzing enormous collections of chemical components. Through the analysis of chemical compositions and characteristics, trends and correlations have been found, which can aid in the identification of viable medication ideas. Chemicals could be ranked in order of priority for more research and production using this data ^[41]. The drug goal, or the particular biological substance or route that a medicine wants to communicate with, could potentially be validated with the use of AI. Scientists can save valuable along with by employing AI to learn more about the purpose and prospective efficacy of the medicinal subject. By examining the chemical patterns and characteristics of possible medication prospects, it can also forecast their level of hazard. This may mitigate the chance of serious side effects by assisting in the earlier

identification of any potential risks during the medication discovery phase ^[42]. AI can also help in the creation of novel compounds that are tailored for particular treatment uses. Additionally, it can make it easier to identify novel compounds that have a higher chance of being successful therapies for specific illnesses. Even though AI has the potential to greatly improve drug development management, scientists and physicians still need to deal with concerns with data quality, openness, and regulations. They can develop AI solutions and raise the efficacy and speed of drug research by tackling these obstacles ^[43].

5. Cellular therapy and AI

Cell treatment, a potentially exciting area of regenerative medicine, uses living cells to substitute or heal sick or wounded organs and tissues. It is predicated on the assumption that since cells can both divide and recover, they are perfectly suited to heal wrecked tissues and organs. Alternative therapies for several chronic diseases and injuries that are presently restricted or nonexistent could be revolutionized by cell therapy ^[44]. The application of stem cells to medical treatment represents one of its most exciting frontiers. Undifferentiated progenitors with the capacity to develop into several cell kinds are known as stem cells. They are available from several sources, such as adult and embryonic tissues as well as umbilical cord blood. Even though cell therapy has yielded encouraging leads to clinical studies, there are still many obstacles to be overcome to find the right cells, guarantee their security, and maximize their beneficial effects ^[45]. AI has a chance to completely transform cell therapy by allowing scientists to examine enormous volumes of data and gain fresh perspectives regarding the way cells function. AI's capacity to assist in determining which cells are optimal for a certain patient constitutes one of the main advantages of utilizing it in cell therapy. AI algorithms are capable of predicting which cells could probably be beneficial in curing a patient's illness by examining their genetic data and medical records. AI can also assist scientists in determining the best environments for cell growth ^[46].

One crucial stage in cell therapy that can have an enormous effect on the treatment's outcome is getting the cells to the target place. By streamlining the management process and guaranteeing that the cells arrive at the intended location, AI can enhance the transport of cells. To optimize therapeutic advantages, AI can also assist in determining the ideal dose and timing of cell distribution. It can also help in following the cells after they are delivered, analyzing their movement and existence, and identifying any negative consequences. This may help modify the therapeutic strategy and enhance patient results ^[47]. The application of AI in cell treatment has certain restrictions in addition to its possible advantages. A primary constraint is the caliber and volume of accessible data. For AI algorithms to forecast results with accuracy, vast volumes of high-quality information are needed. Unfortunately, patient information in the discipline of cell treatment is frequently sparse and varied, which makes it difficult to successfully train AI programs. Because AI models could be just as accurate as the information they have been taught, the precision of AI estimates may be impacted by flaws or irregularities in the information being analyzed. The intricacy of biological processes is a further drawback. The interactions between cells and tissues in cell therapy are extremely complex, which makes it challenging for many machine learning and deep learning algorithms to effectively simulate them ^[48].

6. Detection of skin cancer

The most common cancer among humans is skin cancer. Most cases of skin cancer are diagnosed visually, starting with a clinical test and potentially advancing to a biopsy, histological testing, and dermoscopic study ^[49]. The natural manifestation of skin concerns that cannot be detected sooner makes the identification of wounds and their scanning difficult. Several deep-learning neural networks and machine learning are available currently.

One of the most deadly malignancies for a person's life and well-being is lung cancer. Despite being the most frequently detected cancer overall, most lung cancer sufferers receive a more advanced detection ^[50]. The association between AI and the diagnosis of lung cancer is seen in several freshly published instances. The division technique for the neural network-based lung cancer models demonstrated good recognition efficacy in a case test ^[51]. After lung cancer, prostate cancer is the second-leading cause of cancer in men to be diagnosed. To determine whether a patient has prostate cancer, physicians have to examine the findings of central needle samples. AI models were developed to address issues arising from the growing responsibilities of physicians, labor shortages, and variations in the assessment of histopathology ^[52]. Breast cancer is a particularly widespread kind of malignancy in women and the primary source of cancer-related deaths among women worldwide. A statistical analysis indicates that the fatality rate from breast cancer has risen dramatically in all countries, although it has increased most in the less wealthy areas. The most effective approaches for avoiding and treating this type of cancer are through breast testing and enhanced diagnostic tools ^[53].

One of the most common malignancies worldwide is colorectal cancer. It is critical to identify and recognize patients as soon as possible, assess the efficacy of their therapies, and make precise prognostic predictions to reduce cancer incidence by increasing the lifespan of patients. AI has demonstrated significant potential in the clinical domain of colorectal cancer (CRC) in the past decade, thanks to growing omics and clinical data and ongoing machine learning development ^[54]. These tools have given scientists fresh techniques to identify high-risk patients and select individualized treatment plans. Gastric cancer is sixth in the world's cancer rates per year, making it a significant condition on an international scale. Due to its advanced phase of evaluation, gastric cancer has a significant fatality pace, making it the third-leading source of fatalities due to cancer globally in 2018 (784,000 deaths). Gastric cancer is double as common in men as in women ^[55].

AI has surfaced in the pharmaceutical sector as a prospective remedy for problems discovered by classical chemistry, which hinders the creation of new drugs. Computer-aided drug design (CADD) has profited from the rising usage of AI methods such as ML and DL due to advances in technology and the creation of powerful computers ^[56]. The in-depth understanding of clinicians could potentially improved by the automated AI capacities. The ability to monitor multiple lesions simultaneously, precisely define the volumetric tumor size as time passes, understand individual tumor outcomes by cross-referencing them to records of possibly endlessly numerous similar instances, and translate phenotypic to genotypic implications are among the many instances of the way these automated capacities might strengthen medical knowledge ^[57].

7. Limitations of AI

AI is capable of handling multitasking, nonlinear complicated interactions, and development. Considering that it is adaptable and capable of handling parallel processing of both quantitative and qualitative data. Its practical application has been demonstrated by numerous research findings from several disciplines ^[58]. AI is now used in medical and clinical settings in a variety of ways. It not only capitalizes on various aspects of clinical variety, but it additionally assists in correction. AI has enormous promise to transform and expedite the creation of regenerative medicines. By evaluating enormous molecular and genomic information that is incomprehensible to humans, AI can offer perspectives on a variety of topics, from improving drug development to improving tissue engineering and cellular therapy ^[59]. Even though AI has the potential to further exploration and progress in regenerative medicine, there are still many important technical problems that need to be resolved until these advances are extensively used. The absence of serious, accurate information required to train complex machine-learning algorithms is one of the main drawbacks. Complicated biological relationships involved in regenerative

therapies are hard to adequately represent in statistics ^[60].

Furthermore, because of our incomplete knowledge of cellular and chemical processes, creating precise computer models that can mimic and anticipate cell behavior across time has enormous technical difficulties. Comprehensive research is necessary to validate AI algorithms and obtain legislative clearance; this process is time- and resource-consuming ^[61]. It is similarly crucial to tackle issues with data privacy, security, and bias as well as to provide fair and equal accessibility to fresh technologies. Furthermore, overcoming uptake barriers will be necessary to get physician buy-in for innovations that promise more efficient individualized treatment. To get over these challenges and turn artificial intelligence's theoretical promise in regenerative medicine into practical solutions that enhance patient results, a significant amount of continuing research is still required ^[62]. The development of suitable protections, supervision procedures, and rules for the application of AI in regenerative medicine requires collaboration between researchers, legislators, healthcare providers, and AI developers. The potential for improving and tailoring AI algorithms, particularly for regenerative medicine applications will rise as AI technology advances and further excellent information becomes accessible ^[63].

Future advancements in fields like robotics, computer goals, natural language interpreting, and machine learning may yield fresh perspectives that completely alter the method of creating and implementing regenerative medicine. When AI is combined with other cutting-edge technologies like genome editing, nanotechnology, and 3D bioprinting, it could result in previously unheard-of breakthroughs in the development of customized regenerative treatments. The future of AI-driven regenerative medicine is bright, provided the necessary legal and ethical guidelines are in place ^[64].

8. Conclusion

Because AI and ML can be applied in healthcare settings, the prospects of medical disciplines, particularly in the detection and therapy of cancer, look bright in this era of rapidly advancing technology. Faster guiding visualizations for particular therapies are made possible by AI, which will be essential in the future as the population grows. Researchers and technicians dare to enhance conventional ways of advancement because of AI's accuracy and precision. AI has brought something special to the research and therapy of anticancer drugs. Since human knowledge is limited, it might be challenging to develop the best course of action. According to this perspective, patients might overlook important therapy possibilities and their health may even worsen if doctors prescribe improper care. It can tailor each cancer patient's medication and offer significant data and knowledge that cannot be found through human recognition. AI has the potential to significantly accelerate the development of anticancer treatments by expediting the discovery of novel materials. Finally, AI-enabled disease modeling can help discover new treatment strategies for malignant cells and offer an understanding of the mechanisms underlying illness. To find patients who might benefit from regenerative therapies and to better tailor treatment regimens, AI can enhance predictive modeling. The creation of tailored medicine strategies based on a patient's genetic and medical data can be made possible by AI. AI can help with cell delivery and monitoring optimization as well as the identification of the best cell types for cell treatments. AI may be utilized to continually track patients to identify alterations and potential hazards. AI is able to offer patient training resources that are customized based on each patient's requirements and preferences. AI can increase traceability, and transparency, and improve evaluation of information to boost regulatory compliance. AI is also used in adjacent domains like immunotherapy, which can improve cancer therapies from the start. AI is anticipated to play a significant role in advancing human cancer research and therapy in the future. We anticipate that at some point in the future, AI will significantly alter medical technology.

Disclosure statement

The author declares no conflict of interest.

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Advancements and Challenges in Biomarkers for Colorectal Cancer Detection: A Comprehensive Review

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Abstract: This study provides an overview of the current landscape of biomarkers for colorectal cancer (CRC) detection, focusing on genetic, proteomic, circulating microRNA (miRNA), and metabolomic biomarkers. CRC remains a significant global health challenge, ranking among the most prevalent cancers worldwide and being a leading cause of cancer-related deaths. Despite advancements in screening methods such as colonoscopy, sigmoidoscopy, and fecal occult blood tests (FOBT), the asymptomatic nature of early-stage CRC often results in late diagnoses, negatively impacting patient outcomes. Genetic biomarkers like *APC*, *KRAS*, *TP53*, and microsatellite instability (MSI) play critical roles in CRC pathogenesis and progression. These biomarkers, detectable through polymerase chain reaction, next-generation sequencing, and other advanced techniques, guide early detection and personalized treatment decisions. Proteomic biomarkers such as CEA, CA 19-9, and novel signatures offer insights into CRC's physiological changes and disease status, aiding prognosis and treatment response assessments through enzyme-linked immunosorbent assay and mass spectrometry. Circulating miRNAs, including *miR-21* and *miR-92a*, present promising non-invasive biomarkers that can be detected in blood and stool samples, reflecting CRC presence, progression, and therapeutic response. Metabolomic biomarkers, encompassing amino acids, lipids, and TCA cycle intermediates, provide further insights into CRC-associated metabolic alterations, which are crucial for early detection and treatment monitoring using mass spectrometry and nuclear magnetic resonance. Despite these advancements, challenges such as biomarker validation, standardization, and clinical utility remain. Future research directions include integrating multi-omics approaches and leveraging technologies like liquid biopsies and AI for enhanced biomarker discovery and clinical application. By addressing these challenges and advancing research in biomarker development, CRC screening and management could potentially be revolutionized, improving patient outcomes and reducing the global burden of this disease.

Keywords: Colorectal cancer; Biomarker; Diagnosis; Detection

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1. Introduction

Colorectal cancer (CRC) is one of the most significant global health challenges, ranking as the third most common cancer and the second leading cause of cancer-related deaths. The burden of CRC is substantial, with over 1.9 million new cases and approximately 935,000 deaths estimated worldwide in 2020 ^[1]. The incidence of CRC varies by geographic region, reflecting differences in risk factors such as diet, lifestyle, and access to healthcare services ^[2].

Early detection of CRC is crucial for improving patient outcomes, as the prognosis is significantly better when the disease is diagnosed at an early stage. The five-year survival rate for localized CRC exceeds 90% but drops to less than 15% for metastatic cases ^[3]. Despite the clear benefits of early detection, many CRC cases are diagnosed at advanced stages due to the asymptomatic nature of early-stage disease and limitations in current screening methods ^[4]. Traditional screening techniques, including colonoscopy, sigmoidoscopy, and fecal occult blood tests (FOBT), such as fecal immunochemical tests (FIT), are commonly used. While colonoscopy is considered the gold standard for CRC detection and prevention, it is invasive, expensive, and often associated with patient discomfort and low compliance rates ^[5]. FOBT and FIT, though non-invasive and more cost-effective, have variable sensitivity and specificity, leading to false positives and negatives ^[6].

In recent years, there has been growing interest in developing biomarkers for CRC detection. Biomarkers are biological molecules found in blood, body fluids, or tissues that indicate a normal or abnormal process, or the presence of a disease ^[7]. They offer the potential for non-invasive, accurate, and early detection of CRC, thereby improving patient compliance and screening efficacy ^[8].

The term “biomarker” encompasses a wide range of biological entities, including genetic mutations, epigenetic alterations, protein expression patterns, metabolites, and circulating tumor components. These biomarkers can be detected in various biological samples such as blood, stool, urine, and tissue biopsies ^[9]. The ideal biomarker for CRC detection should have high sensitivity and specificity, be easily accessible, cost-effective, and applicable across diverse populations ^[10].

This review aims to provide a comprehensive update on the current state of biomarkers for CRC detection. It will explore various types of biomarkers, including genetic, epigenetic, proteomic, metabolomic, and circulating biomarkers, and discuss their roles in early detection and diagnosis. Furthermore, the review will examine the clinical utility of these biomarkers, the challenges associated with their implementation, and future perspectives in the field.

By offering an overview of the latest advancements and ongoing research in CRC biomarkers, this review seeks to highlight the potential of these novel approaches to transform CRC screening and improve patient outcomes. Understanding the current landscape of CRC biomarkers is essential for researchers, clinicians, and policymakers aiming to reduce the global burden of CRC through innovative and effective screening strategies ^[11].

2. Genetic biomarkers

Genetic biomarkers are pivotal for understanding the molecular underpinnings of CRC and play a significant role in early detection and personalized treatment strategies. CRC arises from the accumulation of genetic mutations that drive the transformation of normal colonic epithelium into adenocarcinoma ^[12]. Several key genetic biomarkers have been identified, each contributing to different stages of CRC development and progression. This section delves into the primary genetic biomarkers associated with CRC, including *APC*, *KRAS*, *TP53*, and microsatellite instability (MSI), discussing their roles, detection methods, and clinical implications ^[13-16].

2.1. APC mutations

The adenomatous polyposis coli (*APC*) gene is a tumor suppressor that plays a crucial role in the Wnt signaling pathway. Mutations in *APC* are considered one of the earliest events in colorectal tumorigenesis and are present in approximately 80% of sporadic CRC cases^[17]. These mutations lead to the loss of APC protein function, resulting in the accumulation of β -catenin in the nucleus, where it activates transcription of genes involved in cell proliferation and survival^[18,19].

2.2. KRAS mutations

The *KRAS* gene, encoding a GTPase involved in the EGFR signaling pathway, is another critical genetic biomarker in CRC. Mutations in *KRAS* occur in approximately 35%–45% of CRC cases, typically in codons 12 and 13. These mutations lead to the constitutive activation of the KRAS protein, promoting cell proliferation, survival, and metastasis^[20,21].

2.3. TP53 mutations

The *TP53* gene, which encodes the tumor suppressor protein p53, is often referred to as the “guardian of the genome” due to its role in maintaining genomic stability. *TP53* mutations are present in approximately 50% of CRC cases and often occur at later stages of tumorigenesis, marking the transition from adenoma to carcinoma^[22,23].

2.4. Microsatellite instability

MSI is a form of genomic instability resulting from defects in the DNA mismatch repair (MMR) system. MSI is characterized by the accumulation of insertion or deletion errors in microsatellite regions of the genome. Approximately 15% of CRC cases exhibit high-level MSI (MSI-H), which is commonly associated with Lynch syndrome (hereditary nonpolyposis CRC, HNPCC) and some sporadic CRC cases^[24,25].

2.5. Emerging genetic biomarkers

In addition to well-established genetic biomarkers, ongoing research continues to identify novel genetic alterations with potential clinical utility in CRC detection and management. Emerging biomarkers include:

- (1) *BRAF* mutations: *BRAF* V600E mutations occur in approximately 10% of CRC cases and are associated with poor prognosis and resistance to certain targeted therapies. Detection of *BRAF* mutations can guide treatment decisions and identify patients who may benefit from BRAF inhibitors combined with other therapeutic agents^[26,27].
- (2) *PIK3CA* mutations: Mutations in the *PIK3CA* gene, encoding a subunit of the PI3K enzyme, are found in about 15%–20% of CRC cases. These mutations drive tumor growth and resistance to therapies, making them valuable targets for novel therapeutic strategies^[28,29].
- (3) *NRAS* mutations: *NRAS* mutations, though less common than *KRAS* mutations, are present in a subset of CRC cases. Like *KRAS*, *NRAS* mutations can impact response to anti-EGFR therapies, underscoring the importance of comprehensive RAS testing in CRC^[30,31].

2.6. Detection methods and clinical implications

The detection of genetic biomarkers in CRC utilizes a variety of advanced techniques to ensure accurate identification and subsequent clinical action. *APC* mutations, early events in CRC tumorigenesis, can be detected through polymerase chain reaction (PCR), next-generation sequencing (NGS), and digital droplet PCR (ddPCR), with non-invasive stool DNA testing, such as the Cologuard test, offering a practical option^[32,33].

KRAS mutations, present in about 35%–45% of CRC cases, are identified using PCR-based methods, NGS, and allele-specific oligonucleotide PCR, with liquid biopsy offering a minimally invasive alternative ^[34,35]. *TP53* mutations, found in roughly 50% of CRC cases, are detected through Sanger sequencing, NGS, immunohistochemistry (IHC), and circulating tumor DNA (ctDNA) analysis ^[22,36]. MSI, associated with defects in the MMR system, is assessed using PCR to analyze microsatellite markers and IHC for MMR protein expression, with NGS panels providing comprehensive profiling ^[25,37,38].

Clinically, these biomarkers are crucial for guiding CRC management and treatment. *APC* mutation identification aids in early detection, particularly in individuals with familial adenomatous polyposis (FAP), enabling preventive measures ^[32,33]. *KRAS* mutation status is critical for determining eligibility for anti-EGFR therapies, such as cetuximab and panitumumab, as patients with these mutations do not benefit from such treatments ^[34,35]. The presence of *TP53* mutations, often associated with aggressive disease and poorer prognosis, informs the need for therapies targeting DNA repair pathways or reactivating mutant p53 ^[22,36]. MSI testing, essential for all CRC patients, identifies those likely to respond to immune checkpoint inhibitors and helps in screening for Lynch syndrome ^[25,37]. Emerging biomarkers like *BRAF* and *PIK3CA* mutations also provide insights into therapy resistance and potential targets for novel treatments, while *NRAS* mutations underscore the importance of comprehensive RAS testing to optimize anti-EGFR therapy decisions ^[25,30].

3. Proteomic biomarkers

Proteomic biomarkers, which involve studying the complete set of proteins expressed by a genome, cell, tissue, or organism, hold great promise for the detection and management of CRC. Unlike genetic biomarkers, which provide information about the potential for cancer development, proteomic biomarkers reflect real-time physiological changes and disease states. The dynamic nature of the proteome makes it a rich source for identifying disease-specific alterations that can aid in early detection, prognostication, and therapeutic targeting ^[39,40].

This section explores key proteomic biomarkers in CRC, including carcinoembryonic antigen (CEA), cancer antigen 19-9 (CA 19-9), and novel proteomic signatures, highlighting their detection methods and clinical implications ^[41-43].

3.1. Carcinoembryonic antigen

CEA is one of the most extensively studied and widely used proteomic biomarkers in CRC. CEA is a glycoprotein involved in cell adhesion, and its expression is significantly elevated in colorectal tumors compared to normal tissues ^[41,44].

3.2. Cancer antigen 19-9

CA 19-9 is another glycoprotein that serves as a tumor marker in various gastrointestinal cancers, including CRC. Although CA 19-9 is more commonly associated with pancreatic cancer, it can also be elevated in a subset of CRC patients ^[41,45].

3.3. Novel proteomic signatures

Advances in proteomics technologies, such as mass spectrometry and protein microarrays, have facilitated the discovery of novel proteomic signatures that can improve CRC detection and management. These signatures often comprise multiple protein biomarkers that together enhance diagnostic accuracy and provide insights into the molecular mechanisms of CRC ^[42,43].

3.4. Circulating tumor proteins

Circulating tumor proteins, released by tumor cells into the bloodstream, represent another important category of proteomic biomarkers. These proteins can be detected in blood samples, providing a minimally invasive approach to CRC detection and monitoring ^[42,43].

3.5. Detection methods and clinical implications

Proteomic biomarkers for CRC, such as CEA, CA 19-9, novel proteomic signatures, and circulating tumor proteins, are detected using advanced techniques and have significant clinical implications. CEA and CA 19-9 levels are typically measured using enzyme-linked immunosorbent assays (ELISAs) or chemiluminescent immunoassays (CLIAs) in serum samples, providing a non-invasive method for monitoring disease progression and treatment response ^[41,44]. While CEA is valuable for postoperative monitoring and detecting recurrence, CA 19-9, though less specific to CRC, can complement CEA in assessing disease status ^[45].

Novel proteomic signatures, identified through high-throughput platforms like mass spectrometry (MS) and protein microarrays, offer comprehensive profiling that enhances diagnostic accuracy and provides insights into CRC's molecular mechanisms ^[42,43]. These signatures are crucial for early detection, patient stratification, and identifying therapeutic targets. Circulating tumor proteins, detected through techniques like ELISA, bead-based multiplex assays, and MS, provide a minimally invasive approach for early detection and monitoring. Elevated levels of these proteins in blood samples can indicate tumor presence, progression, and treatment response, aiding in timely CRC management ^[42,43].

Integrating these proteomic biomarkers into clinical practice enhances early detection, informs prognosis, guides personalized therapy, and ultimately improves patient outcomes.

4. Circulating microRNAs

Circulating microRNAs (miRNAs) have emerged as promising non-invasive biomarkers for CRC detection, prognosis, and monitoring. These small, non-coding RNA molecules, typically 19–25 nucleotides in length, regulate gene expression post-transcriptionally and can be found in various body fluids, including blood, serum, plasma, and stool ^[46,47]. The stability of miRNAs in circulation, owing to their protection within exosomes, microvesicles, or protein complexes, makes them attractive candidates for clinical applications. This section explores key circulating miRNAs, their detection methods, and their clinical implications in CRC.

4.1. Key circulating miRNAs

- (1) *miR-21*: One of the most extensively studied miRNAs in CRC, *miR-21* is frequently overexpressed in CRC tissues and detectable at elevated levels in the blood of CRC patients ^[48,49].
- (2) *miR-92a*: Part of the *miR-17-92* cluster, *miR-92a* is upregulated in CRC and is associated with tumor growth and metastasis ^[50,51].
- (3) *miR-29a*: This miRNA is involved in the regulation of apoptosis and cell proliferation, with increased levels observed in the blood of CRC patients ^[52,53].
- (4) *miR-17-3p* and *miR-20a*: Both are part of the *miR-17-92* cluster and are implicated in CRC progression and metastasis ^[54,55].
- (5) *miR-145*: Generally downregulated in CRC, *miR-145* acts as a tumor suppressor, and its reduced expression correlates with more advanced disease stages ^[56,57].

4.2. Detection methods and clinical implications

Circulating miRNAs are detected using techniques such as quantitative PCR (qPCR), ddPCR, NGS, microarrays, and bead-based multiplex assays. These methods enable the sensitive and specific quantification of miRNAs in body fluids ^[58,59]. Clinically, circulating miRNAs hold significant potential for early CRC detection, prognosis, and monitoring of treatment response. Elevated levels of miRNAs, such as *miR-21*, *miR-92a*, and *miR-29a*, in blood samples can indicate the presence of CRC, even at early stages, facilitating timely intervention ^[53,60]. Furthermore, the expression profiles of specific miRNAs provide prognostic information, correlating with disease progression and patient outcomes ^[49,61]. Changes in miRNA levels during and after treatment can reflect therapeutic efficacy and help detect recurrence, aiding in the development of personalized treatment strategies.

The non-invasive nature of circulating miRNA testing offers a patient-friendly alternative to traditional biopsies, making routine monitoring more accessible and less burdensome. Integrating miRNA profiles into clinical practice enhances the precision of CRC diagnosis, prognosis, and treatment, ultimately improving patient outcomes through personalized medicine.

5. Metabolomic biomarkers

Metabolomics, the comprehensive study of metabolites in biological systems, has become an invaluable approach for identifying biomarkers in CRC ^[62]. Metabolites are small molecules involved in various metabolic pathways, reflecting the physiological state of cells, tissues, and organisms ^[63]. Changes in metabolite levels can indicate alterations in metabolic processes associated with cancer development and progression. This section explores key metabolomic biomarkers in CRC, their detection methods, and their clinical implications.

5.1. Key metabolomic biomarkers

- (1) Amino acids: Altered levels of amino acids, such as tryptophan, glutamine, and arginine, have been observed in CRC patients. These changes reflect disruptions in amino acid metabolism, which is crucial for tumor growth and survival ^[64,65].
- (2) Lipid metabolites: Abnormal lipid metabolism is a hallmark of cancer. Elevated levels of certain phospholipids, sphingolipids, and free fatty acids are commonly found in CRC patients ^[66,67].
- (3) Carbohydrate metabolites: Changes in carbohydrate metabolism, including elevated levels of glucose and lactate, are indicative of the Warburg effect—a phenomenon where cancer cells preferentially utilize glycolysis for energy production even in the presence of oxygen ^[68,69].
- (4) Bile acids: Altered bile acid profiles have been linked to CRC. Elevated levels of primary and secondary bile acids can reflect changes in gut microbiota and hepatic function ^[70,71].
- (5) Tricarboxylic acid cycle intermediates: Disruptions in the tricarboxylic acid (TCA) cycle, such as altered levels of citrate, succinate, and fumarate, indicate metabolic reprogramming in cancer cells ^[72,73].

5.2. Detection methods and clinical implications

Metabolomic biomarkers in CRC are detected using advanced analytical techniques, such as MS, nuclear magnetic resonance (NMR) spectroscopy, capillary electrophoresis-mass spectrometry (CE-MS), Fourier transform infrared (FTIR) spectroscopy, and high-performance liquid chromatography (HPLC) ^[63,65]. These methods enable the sensitive and specific identification and quantification of metabolites in biological samples, providing a detailed metabolic profile of both the tumor and the host. Clinically, metabolomic biomarkers

have significant implications for CRC management. They facilitate early detection through the identification of specific metabolic changes associated with cancer onset ^[64,69]. Certain metabolites, such as altered amino acids, lipids, carbohydrates, bile acids, and TCA cycle intermediates, offer prognostic value by correlating with disease stage, progression, and patient outcomes ^[70,72]. Metabolomic biomarkers are also valuable for monitoring treatment response and detecting recurrence, as changes in metabolite levels can reflect therapeutic efficacy and disease status ^[66,73]. Furthermore, metabolomic profiling can guide personalized therapy by identifying metabolic vulnerabilities that can be targeted with specific treatments ^[65,68]. The non-invasive nature of metabolomic testing, using fluids such as blood, urine, and stool, enhances patient comfort and facilitates routine monitoring. Integrating metabolomic data with other omics data provides a comprehensive understanding of CRC, leading to improved disease management and patient outcomes through personalized medicine.

6. Challenges and future directions

Biomarker research for CRC faces several significant challenges that must be addressed to realize its full potential in clinical practice. One critical challenge is the standardization and validation of biomarkers. Biomarker discovery often involves diverse methodologies and sample types across different studies, leading to variability in results. Standardizing protocols for biomarker identification, validation, and clinical implementation is essential to ensure reproducibility and reliability across various research settings and populations.

Another key hurdle is demonstrating the clinical utility of biomarkers and integrating them into routine clinical practice. While biomarkers show promise in research, their adoption in clinical settings requires robust evidence of their effectiveness in improving patient outcomes. Biomarkers must demonstrate clear benefits in terms of sensitivity, specificity, cost-effectiveness, and impact on clinical decision-making to justify their incorporation into screening and diagnostic algorithms.

The heterogeneity of CRC presents another challenge. CRC includes various molecular subtypes and clinical manifestations, requiring biomarkers that can accurately reflect this diversity. Biomarkers must be validated across different patient populations to ensure their efficacy in stratifying patients for personalized treatment strategies.

Ethnic and geographic variations also influence biomarker performance. Genetic, lifestyle, and environmental factors can affect biomarker expression and efficacy across different ethnic groups and geographic regions. Developing biomarkers that are effective and reliable across diverse populations is crucial for their global applicability and adoption in clinical practice.

Additionally, the transition from single biomarkers to multimodal biomarker panels represents a promising future direction in CRC research. Single biomarkers may lack sufficient sensitivity or specificity for accurate CRC detection and prognosis. Combining multiple types of biomarkers, such as genetic, proteomic, and metabolomic markers, could enhance diagnostic accuracy and reliability, paving the way for more effective screening and personalized treatment approaches.

Looking ahead, advancements in multi-omics approaches—including genomics, epigenomics, proteomics, and metabolomics—hold great potential for improving biomarker discovery and validation. Integrating data from multiple omics layers can provide a more comprehensive understanding of CRC biology and facilitate the development of robust biomarker panels. Moreover, leveraging technologies such as liquid biopsies and artificial intelligence (AI) for biomarker detection and analysis could further enhance the clinical utility and predictive power of biomarkers in CRC management.

Addressing these challenges and pursuing these future directions will be instrumental in advancing biomarker research for CRC. By overcoming these obstacles, biomarkers have the potential to revolutionize CRC screening, diagnosis, and treatment, ultimately improving patient outcomes and reducing the global burden of this disease.

7. Conclusion

Biomarkers present a promising pathway for the early detection and management of CRC. Genetic, proteomic, circulating miRNA, and metabolomic biomarkers have demonstrated potential in enhancing screening accuracy and improve patient outcomes. However, significant challenges remain in the standardization, validation, and clinical integration of these biomarkers. Future research should focus on developing robust, multimodal biomarker panels and utilizing technological advancements to strengthen CRC detection and screening programs. With continued efforts, biomarker-based screening could become a cornerstone in the fight against CRC, ultimately reducing its global impact.

Disclosure statement

The authors declare no conflict of interest.

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Clinical Analysis of Acupuncture Combined with Acupoint Injection for the Treatment of Chronic Nonbacterial Prostatitis

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Abstract: *Objective:* To evaluate the therapeutic effect of acupuncture combined with acupoint injection on chronic nonbacterial prostatitis (CNP). *Methods:* A total of 72 CNP patients admitted between March 2022 and October 2023 were selected. The patients were randomly divided into two groups using a random number table. The combined treatment group (36 cases) received acupuncture combined with acupoint injection therapy, while the control group (36 cases) received conventional Western medicine treatment. The overall efficacy rate, symptom severity, prostatic fluid indicators, incidence of adverse reactions, and recurrence rates were compared. *Results:* The overall efficacy rate of the combined treatment group was higher than that of the control group ($P < 0.05$). After 10 days of treatment, the symptom severity score of the combined treatment group was higher than that of the control group, and the prostatic fluid indicators were lower than those of the control group ($P < 0.05$). The incidence of adverse reactions in the combined treatment group was lower than in the control group ($P < 0.05$). During the follow-up period of 1–6 months, the recurrence rate in the combined treatment group was lower than that in the control group ($P < 0.05$). *Conclusion:* Acupuncture combined with acupoint injection can alleviate CNP disease symptoms, improve prostate function, and prevent post-treatment adverse reactions. It also has a lower recurrence risk and demonstrates excellent efficacy.

Keywords: Acupuncture; Acupoint injection; Chronic nonbacterial prostatitis; Symptom severity; Adverse reactions

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1. Introduction

Chronic nonbacterial prostatitis (CNP) is a highly prevalent prostate disease, mainly affecting men over 50 years old. Symptoms include frequent urination, lower abdominal pain, and incomplete urination. The pathogenesis of this disease is complex, involving factors such as microbial infection, endocrine disorders, and immune dysfunction, which significantly reduce the health of the reproductive system. Oral Western medications, particularly antibiotics, are commonly used to treat this disease. These drugs can alleviate symptoms and reduce inflammation in the body, thereby controlling the disease ^[1]. However, the treatment cycle of Western medicine

is long and may cause side effects such as abdominal pain or nausea, which can affect long-term efficacy. In contrast, traditional Chinese acupuncture and acupoint injection therapies have the advantages of fewer side effects and significant efficacy. As external, non-invasive treatments, they result in higher patient adherence. Therefore, this study selected 72 CNP patients to evaluate the efficacy of acupuncture combined with acupoint injection therapy.

2. Materials and methods

2.1. General information

A total of 72 CNP patients admitted between March 2022 and October 2023 were selected for the study. They were randomly divided into two groups using a random number table: 36 patients in the combined treatment group and 36 patients in the control group. In the combined group, the patients' ages ranged from 22 to 67 years, with an average age of 40.29 ± 4.37 years, and the disease duration ranged from 0.3 to 6 years, with an average duration of 2.19 ± 0.59 years. In the control group, the patients' ages ranged from 23 to 65 years, with an average age of 40.32 ± 4.41 years, and the disease duration ranged from 0.4 to 7 years, with an average duration of 2.24 ± 0.62 years. There was no significant difference in the general data between the two groups ($P > 0.05$).

Inclusion criteria: Patients exhibiting typical symptoms such as frequent urination, a burning sensation in the urethra, and urinary pain; prostatic palpation revealed inflammatory nodules or an uneven texture in the prostate; prostatic fluid microscopy showed a white blood cell count of no fewer than 10/high-power field (HP); clinical data were relatively complete; the patients met the indications for acupuncture and acupoint injection.

Exclusion criteria: Urethral stricture; bacterial prostatitis; bladder neck hypertrophy; comorbid heart, liver, or kidney disease; hematologic disorders; allergies to the study medication; withdrawal from the study midway.

2.2. Methods

The control group received conventional Western medicine treatment: oral administration of Azithromycin capsules, taken 30 minutes after meals, with a dose of 0.5 g once daily for 10 days.

The combined treatment group received acupuncture combined with acupoint injection therapy:

- (1) Acupuncture: Patients were placed in a supine position, and the acupoints were disinfected with 75% alcohol cotton balls. A 1.5-inch acupuncture needle (Huatuo brand) was used to quickly insert into Baihui, Sanyinjiao, and Xuehai acupoints at a depth of 1.0–1.5 inches. Another 1.5-inch acupuncture needle was used to insert into Guanyuan and Zhongji acupoints at a depth of 1.0–1.5 inches, applying the lifting, thrusting, and twisting method until a sensation of soreness, distension, or pain was achieved. The needles were retained for 30 minutes and then quickly removed, with the needle holes disinfected with alcohol. For those with bleeding, sterile cotton balls were applied for pressure to stop the bleeding. Next, patients were placed in a prone position, and the following acupoints were treated: Shenshu, Zhishi, Zhibian, Mingmen, Pishu, and Ciliao, using the same needle retention time as before.
- (2) Acupoint injection: After acupuncture treatment, acupoint injection was performed at the Zhibian and Ciliao acupoints. The needle was inserted to a depth of 3 cm, and if no blood was drawn upon aspiration, Houttuynia injection was administered, with a total dose of 4 ml. After withdrawing the needle, the needle holes were disinfected with sterile cotton balls to prevent infection. Acupuncture and acupoint injections were performed once daily for 10 days.

2.3. Observation indicators

- (1) Symptom severity: The National Institutes of Health Chronic Prostatitis Symptom Index (NIH-CPSI) was used, which includes pain or discomfort (40 points), quality of life (6 points), impact of symptoms (6 points), and urinary symptoms (10 points), for a total of 62 points. Symptom severity was scored positively.
- (2) Prostatic fluid indicators: Before and after treatment, 1 mL of prostatic fluid was collected, and secretory immunoglobulin A (SIgA) and vascular cell adhesion molecule-1 (VCAM-1) levels were measured using enzyme-linked immunosorbent assay (ELISA).
- (3) Incidence of adverse reactions: Observing adverse reactions such as abdominal pain, hematoma, nausea, and needle fainting.
- (4) Recurrence rate: Patients were followed up for 1 to 6 months, and the recurrence rate was recorded.

2.4. Efficacy evaluation criteria

- (1) Cure: No disease symptoms, normal prostate texture, normal prostatic fluid test results, and no recurrence during 6 months of follow-up.
- (2) Significant efficacy: Mild symptoms, nearly normal prostate texture, a reduction in white blood cell count by $\geq 60\%$.
- (3) Preliminary efficacy: Moderately obvious symptoms, uneven prostate texture with tenderness, a reduction in white blood cell count by 30%–59%.
- (4) No efficacy: Severe symptoms, hardened prostate texture, a reduction in white blood cell count by $< 30\%$.

2.5. Statistical analysis

Data were processed using SPSS 21.0 software. Measurement data were expressed as mean \pm standard deviation (SD) and compared using *t*-tests. Count data were expressed as [*n* (%)] and compared using χ^2 -tests. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Comparison of overall efficacy between the two groups

Table 1 shows that the overall efficacy of the combined treatment group was higher than that of the control group ($P < 0.05$).

Table 1. Comparison of overall efficacy between the two groups [*n* (%)]

Groups	<i>n</i>	Cure	Significant efficacy	Preliminary efficacy	No efficacy	Overall efficacy
Combined treatment group	36	16 (44.44)	10 (27.78)	9 (25.00)	1 (2.78)	35 (97.22)
Control group	36	6 (16.67)	13 (36.11)	10 (27.78)	7 (19.44)	29 (80.56)
χ^2	-	-	-	-	-	5.063
<i>P</i>	-	-	-	-	-	0.024

3.2. Comparison of symptom severity scores between the two groups

Before treatment, there was no significant difference in symptom severity scores between the two groups ($P > 0.05$). After treatment, the symptom severity scores in the combined group were lower than those in the control group ($P < 0.05$). See **Table 2**.

Table 2. Comparison of symptom severity scores between the two groups before and after treatment (mean \pm SD, points)

Groups	<i>n</i>	Pain or other discomfort		Quality of life		Symptom impact		Urinary symptoms	
		Before	After	Before	After	Before	After	Before	After
Combined treatment group	36	20.15 \pm 3.16	34.16 \pm 3.87	2.59 \pm 0.74	4.68 \pm 0.97	2.71 \pm 0.33	4.70 \pm 0.41	4.26 \pm 0.88	7.18 \pm 1.29
Control group	36	20.19 \pm 3.21	30.11 \pm 3.79	2.62 \pm 0.78	4.15 \pm 0.92	2.75 \pm 0.36	4.23 \pm 0.37	4.29 \pm 0.87	6.57 \pm 1.22
<i>t</i>		0.053	4.486	0.167	2.379	0.491	5.106	0.145	2.061
<i>P</i>		0.958	0.000	0.868	0.020	0.625	0.000	0.885	0.043

3.3. Comparison of prostatic fluid indicators between the two groups

Before treatment, there was no significant difference in prostatic fluid indicators between the two groups ($P > 0.05$). After treatment, the prostatic fluid indicators in the combined group were significantly better than those in the control group ($P < 0.05$), as shown in **Table 3**.

Table 3. Comparison of prostatic fluid indicators between the two groups before and after treatment (mean \pm SD, ng/mL)

Groups	<i>n</i>	SIgA		VCAM-1	
		Before	After	Before	After
Combined treatment group	36	117.95 \pm 12.36	59.28 \pm 5.91	93.75 \pm 10.27	51.23 \pm 4.38
Control group	36	117.82 \pm 12.15	85.41 \pm 6.13	93.71 \pm 10.22	71.92 \pm 6.47
<i>t</i>	-	0.045	18.412	0.017	15.889
<i>P</i>	-	0.964	0.000	0.987	0.000

3.4. Comparison of adverse reaction rates between the two groups

Table 4 shows that the adverse reaction rate in the combined group was lower than that in the control group ($P < 0.05$).

Table 4. Comparison of adverse reaction rates between the two groups [*n* (%)]

Groups	<i>n</i>	Abdominal pain	Hematoma	Nausea	Needle fainting	Adverse reaction rate
Combined treatment group	36	0	0	1 (2.78)	1 (2.78)	2 (5.56)
Control group	36	3 (8.33)	3 (8.33)	2 (5.56)	0	8 (22.22)
χ^2	-	-	-	-	-	4.181
<i>P</i>	-	-	-	-	-	0.041

3.5. Comparison of recurrence rates between the two groups

At different follow-up times, the recurrence rate in the combined group was lower than that in the control group ($P < 0.05$), as shown in **Table 5**.

Table 5. Comparison of recurrence rates between the two groups [*n* (%)]

Groups	<i>n</i>	Follow-up 1 month	Follow-up 3 month	Follow-up 6 month
Combined treatment group	36	0	1 (2.78)	1 (2.78)
Control group	36	4 (11.11)	6 (16.67)	7 (19.44)
χ^2	-	4.235	3.956	5.063
<i>P</i>	-	0.040	0.047	0.024

4. Discussion

Chronic prostatitis is a high-risk urinary system disease, which can be subdivided into bacterial and non-bacterial inflammation, with CNP being the most common form ^[2]. The symptoms of this disease include difficulty urinating, lumbosacral pain, frequent urination, etc. The causes involve poor lifestyle habits, endocrine disorders, and other factors, which increase the physical and mental burden on patients and may even lead to serious complications. Western medicine, primarily azithromycin, is the basic treatment for this disease, as it effectively reduces inflammation and clears prostate infections, thereby improving treatment outcomes ^[3,4]. However, the curative effect of Western medicine is often limited, necessitating the combination with other therapies.

According to traditional Chinese medicine, CNP falls under the category of “Jing Zhuo” (essence turbidity), and its etiology involves factors such as long-term fatigue, excessive alcohol consumption leading to weakened immunity, excessive sexual activity, endocrine dysfunction ^[5], and chronic congestion of the prostate. The pathological basis includes inflammatory infiltration of glandular epithelium and destruction of glandular epithelium. The disease mechanism involves kidney Qi deficiency, damp-heat stagnation, and blood stasis interacting with each other. Based on these mechanisms, this study selected acupuncture combined with acupoint injection as the treatment. Acupuncture can adjust the balance between organs, enhance the metabolic ability of prostate tissue, improve its repair capacity, and regulate local microcirculation to promote the absorption of inflammatory reactions ^[6].

In terms of acupoints, Baihui is selected to boost central Qi and improve microcirculation, while Guanyuan enhances the phagocytic function of leukocytes and regulates immune function. Sanyinjiao facilitates the lower jiao, Xuehai harmonizes and nourishes blood, and Guanyuan and Zhongji improve prostate microcirculation and stimulate the nerves in the perineal area. The combination of Guanyuan, Zhongji, and Sanyinjiao has the effect of consolidating the foundation, eliminating dampness, and strengthening the spleen. Acupuncture at Shenshu, Pishu, Zhishi, and Mingmen also supports both prenatal and postnatal aspects ^[7]. Additionally, the Shenshu point helps to tonify the kidneys and consolidate essence.

Acupoint injection is a widely used local drug delivery method, allowing the drug to directly reach the treatment site while minimizing drug loss. Houத்துynia injection was selected for acupoint injection due to its anti-inflammatory, stasis-resolving, analgesic, diuretic, and dampness-eliminating effects. Its main components, flavonoids, and volatile oils, possess strong anti-inflammatory and antibacterial properties, as well as the ability to enhance immunity ^[8].

The results showed that the overall efficacy of the combined treatment group was higher than that of the control group. After 10 days of treatment, the symptom severity score of the combined group was higher than that of the control group, and the prostatic fluid indicators were lower than those of the control group. The adverse reaction rate in the combined group was lower than in the control group. During follow-up from 1 to 6 months, the recurrence rate in the combined group was lower than in the control group ($P < 0.05$). These findings are consistent with those of Guo and Wang ^[9]. The reason is that acupuncture can improve endocrine levels, enhance the glandular secretion function, and produce nerve stimulation at the related acupoints, thereby increasing catecholamine secretion,

regulating vascular permeability, and reducing prostatic inflammatory exudation and glandular edema ^[10]. The combined effect of acupoint injection provides quick results with lower drug doses, allowing it to resolve stasis, expel pus, clear heat, detoxify, and eliminate dampness, thereby alleviating CNP symptoms ^[11].

In conclusion, acupuncture combined with acupoint injection can improve CNP symptoms, regulate prostate function, reduce treatment side effects, and lower the long-term recurrence rate, demonstrating significant clinical efficacy.

Disclosure statement

The authors declare no conflict of interest.

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Immunoregulatory Effect and Mechanism of Epigallocatechin-3-Gallate in A Mouse Oral Cancer Model

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Abstract: *Objective:* This investigation delineates the anti-cancer potency of epigallocatechin-3-gallate (EGCG) in an oral cancer mouse model, with a focus on its effect on T-cell activation. *Methods:* An oral cancer model was established in male Balb/c mice using 4-nitroquinoline 1-oxide (4-NQO). The mice were systematically grouped and administered graded concentrations of EGCG. Key parameters such as body weight, hydration levels, tumor volume, and mass were meticulously tracked. T-cell activity and cytokine expression profiles, focusing on interleukin-2 (IL-2), interferon-gamma (IFN- γ), and tumor necrosis factor-alpha (TNF- α), were quantified using ELISA. A comprehensive statistical evaluation included one-way ANOVA, Tukey's HSD multiple comparison test, and the Kruskal-Wallis non-parametric assessment. *Results:* EGCG-administered cohorts exhibited a pronounced reduction in tumor size and mass, with the high-dose group showing the greatest efficacy. ELISA findings corroborated a significant increase in T-cell activity and concomitant upregulation of key cytokines, including IL-2, IFN- γ , and TNF- α ($P < 0.05$). *Conclusion:* This investigation confirms the tumor-suppressive efficacy of EGCG in a murine oral squamous cell carcinoma model. The therapeutic effects of EGCG are mediated through T-cell activation and the upregulation of pivotal cytokine expression, highlighting its potential immunomodulatory role in oral cancer treatment.

Keywords: Epigallocatechin-3-gallate (EGCG); Oral squamous cell carcinoma (OSCC); 4-nitroquinoline 1-oxide (4-NQO); Peripheral blood mononuclear cell (PBMC); Enzyme-linked immunosorbent assay (ELISA)

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1. Introduction

Head and neck malignancies, particularly oral cancer, have emerged as a significant public health challenge on the global health agenda. According to the Global Cancer Observatory (GLOBOCAN) 2020 data on global cancer statistics ^[1], over 377,713 new cases of oral cancer were diagnosed worldwide, with more than 177,757 deaths, underscoring its substantial impact on public health. The incidence and mortality rates of oral cancer are steadily rising globally, a trend particularly pronounced in developing countries such as China. For instance, in Hunan Province, the number of new cases annually has reached approximately 51,600, with around 23,200

deaths ^[2]. This increasing trend is closely associated with factors such as lifestyle changes, increased tobacco and alcohol consumption, and population aging.

Green tea, a distinctive type of tea in China, contains epigallocatechin-3-gallate (EGCG) as its primary active component, which is responsible for its anti-inflammatory, antioxidant, and anti-tumor properties. In recent years, research on EGCG has expanded, and clinical studies have found that EGCG, when combined with standard radiotherapy and chemotherapy, can enhance the treatment efficacy for oral cancer. However, the specific roles of EGCG in treatment and the mechanisms through which it exerts its effects remain unclear, and its broader application potential is still being explored.

In terms of treatment, traditional methods such as surgery, radiotherapy, and chemotherapy have achieved some success, but they remain limited in improving patients' long-term survival rates and quality of life. Therefore, there is an urgent need to explore and develop new therapeutic strategies ^[3]. In this context, EGCG has garnered widespread attention in the scientific community due to its significant antioxidant, anti-inflammatory, and anti-tumor effects ^[4].

2. Materials and methods

2.1. Experimental materials

Male Balb/c mice, aged 6–8 weeks and weighing 20–22 grams, were provided by Changsha Tianqin Biotechnology Co., Ltd. 4-Nitroquinoline-1-oxide (4-NQO, Sigma-Aldrich) was used to establish the oral cancer model, while epigallocatechin-3-gallate (EGCG, Sigma-Aldrich) was employed to test its inhibitory effects on the model. Ficoll solution (GE Healthcare) was utilized to isolate peripheral blood mononuclear cells (PBMCs) from whole blood samples collected from the mice. Enzyme-linked immunosorbent assay (ELISA) kits for interleukin-2 (IL-2), interferon-gamma (IFN- γ), and tumor necrosis factor-alpha (TNF- α) [BD Biosciences] were used to measure cytokine levels. Anti-CD3 and anti-CD28 antibodies (Abcam) were applied to activate T cells *in vitro*, and an ELISA microplate reader (Bio-Rad) was used to read the results.

2.2. Methods

2.2.1. Establishment of mouse oral cancer models

A total of 60 male Balb/c mice were acclimatized for one week in the animal facility of the Life Sciences Building at Changsha Medical University. The mice were housed with free access to food and water under a 12-hour light/dark cycle, with the temperature maintained between 23°C and 26°C, and humidity controlled at 50 \pm 5%. A 1% aqueous solution of 4-NQO was prepared with sterile distilled water and stored at 4°C in a light-protected refrigerator. For administration, it was diluted to 200 mg/L in tap water and provided in light-protected bottles for ad libitum drinking. The mice were randomly divided into five groups, with 12 mice per group. Throughout the experiment, the activity of each group was observed daily. Every two days, the remaining water in the bottles was measured using a graduated cylinder, and weekly records of water consumption, drug intake, and body weight were maintained. Starting from the 12th week, the oral mucosa of the mice was examined weekly under mild anesthesia for any changes. The appearance of grayish-white exophytic masses was considered indicative of a successful oral cancer model establishment.

2.2.2. Administration of EGCG in the mouse oral cancer model

Mice with successfully induced oral cancer were divided into four groups: a model group, a low-dose group, a medium-dose group, and a high-dose group. EGCG was prepared in aqueous solutions of 30 mg/100 mL, 50

mg/100 mL, and 100 mg/100 mL using sterile distilled water. The mice were administered these solutions via gavage at a dosage of 30 mL per day, and their conditions were monitored throughout the experiment. At the end of the study, the mice were euthanized via cervical dislocation, and the tumor tissues were collected and weighed. The tumor inhibition rate (IR) was calculated using the formula: $IR = (1 - \text{average tumor weight of the experimental group} / \text{average tumor weight of the control group}) \times 100\%$.

2.2.3. Isolation of peripheral blood mononuclear cells

A total of 10 mL of whole blood was drawn from the mice and heparinized to prevent coagulation. Two 15 mL centrifuge tubes containing 5 mL of Ficoll solution were prepared. The blood was diluted with an equal volume of phosphate-buffered saline (PBS) and gently layered over the Ficoll solution to prevent mixing. The tubes were centrifuged at 2,000 rpm for 20 minutes with the brake disengaged to ensure a gradual deceleration. After centrifugation, a white layer containing PBMCs was visible and carefully transferred into a clean 15 mL centrifuge tube using a pipette. Five volumes of PBS were added to achieve a final volume of 10–15 mL, followed by centrifugation at 1,500 rpm for 10 minutes. This washing step was repeated twice. After discarding the supernatant, 10 mL of 1640 medium was added, and the solution was thoroughly mixed before counting and preparing the cells for further use.

2.2.4. Detection of T cell activity and cytokine expression of IL-2, IFN- γ , and TNF- α by ELISA

The isolated PBMCs were cultured in a medium containing anti-CD3 antibodies (1 $\mu\text{g/mL}$) and anti-CD28 antibodies (2 $\mu\text{g/mL}$). The PBMCs were incubated at 37°C in a 5% CO₂ incubator for 2–3 days to activate T cells. After incubation, the culture medium was gently mixed and centrifuged at 1,500 rpm for 10 minutes to pellet the cells. The supernatant was collected, and ELISA kits were used to measure IL-2, IFN- γ , and TNF- α levels. The absorbance was measured at 450 nm using a microplate reader.

2.3. Statistical methods

Statistical analyses were performed using SPSS version 26. Quantitative data are presented as mean \pm standard deviation (SD), while categorical data are expressed as rates or proportions. Analysis of variance (ANOVA) was conducted, followed by Tukey's Honestly Significant Difference (HSD) multiple comparison test and the Kruskal-Wallis non-parametric test. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Establishment of the oral cancer model and effects of EGCG treatment

After 17 weeks of 4-NQO induction, 44 mice across the five groups successfully developed oral cancer, yielding a success rate of approximately 73.3% (**Figure 1**). Following 12 weeks of EGCG treatment, a dose-dependent anti-tumor effect was observed in the EGCG-treated groups. The average tumor volume in the control (untreated) group was 500 mm³, while the average tumor volumes in the low, medium, and high-dose EGCG groups decreased to 375 mm³, 297 mm³, and 196 mm³, respectively. Correspondingly, the average tumor weights were reduced from 1.514 g in the control group to 0.775 g, 0.523 g, and 0.315 g in the low, medium, and high-dose EGCG groups. The calculated tumor inhibition rates were 13.3%, 26.7%, and 46.4%, respectively.

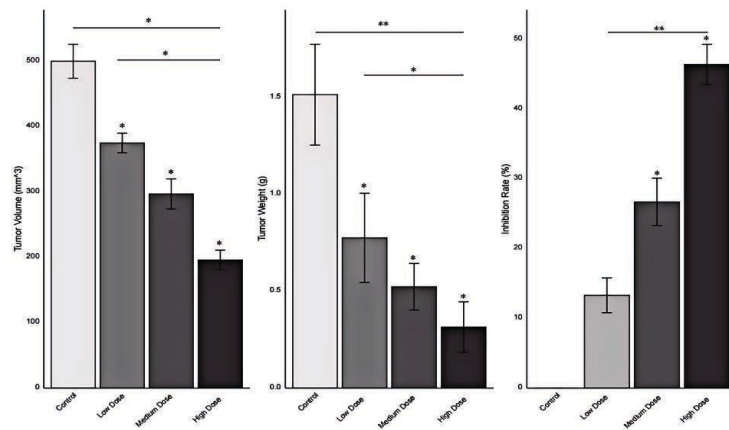


Figure 1. (A) Tumor volume; (B) Tumor weight; (C) Tumor inhibition rate (* $P < 0.05$, ** $P < 0.01$)

3.2. Changes in T cell activity and cytokine expression

ELISA results demonstrated a significant increase in T-cell activity in the EGCG-treated groups compared to the control group (**Figure 2**). The PBMC counts in the low, medium, and high-dose EGCG groups were $(1.10 \times 10^6 \pm 0.03 \times 10^6)$, $(1.20 \times 10^6 \pm 0.03 \times 10^6)$, and $(1.30 \times 10^6 \pm 0.02 \times 10^6)$, respectively, which were significantly higher than the control group's $(1.01 \times 10^6 \pm 0.05 \times 10^6)$. Additionally, the levels of IL-2, IFN- γ , and TNF- α in the control group were 6.18 ± 0.23 , 5.07 ± 0.34 , and 4.03 ± 0.28 , respectively. These levels increased to 76.97 ± 3.27 , 91.25 ± 2.92 , and 69.25 ± 2.92 in the high-dose EGCG group.

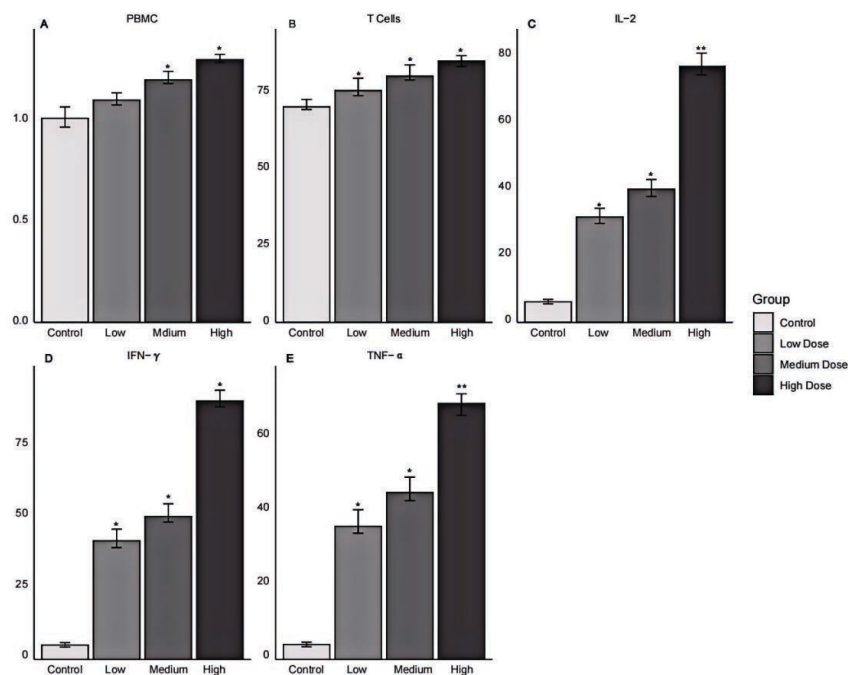


Figure 2. (A) PBMC; (B) T cell ratio; (C) IL-2; (D) IFN- γ ; (E) TNF- α (* $P < 0.05$, ** $P < 0.01$)

3.3. Statistical analysis

One-way ANOVA revealed that different EGCG doses significantly affected gene expression levels, with a P -value < 0.01 . ANOVA results also showed significant differences between groups for PBMC count, T cell proportion, and levels of IL-2, IFN- γ , and TNF- α ($P < 0.05$). Tukey's HSD multiple comparisons test further highlighted significant differences in IL-2, IFN- γ , and TNF- α levels between the high-dose EGCG group and the control, low-dose, and medium-dose groups. The Kruskal-Wallis test also indicated significant differences between groups for all variables.

4. Discussion

This study conducted an in-depth investigation into the effects of EGCG, a major active component of green tea, in a mouse oral cancer model, with a focus on its anti-tumor mechanisms. EGCG, a naturally occurring polyphenol, demonstrates significant biological activity, particularly in antioxidation, anti-inflammation, and modulation of cellular signaling pathways^[5]. In terms of EGCG's tumor-suppressive effects, a dose-dependent reduction in tumor volume and weight was observed in mice as the EGCG dosage increased. This effect may be associated with EGCG's regulatory influence on the cell cycle. EGCG can inhibit critical checkpoints in the tumor cell cycle, such as the G1/S or G2/M transitions, thereby blocking tumor cell proliferation^[6].

Regarding T-cell activity and cytokine production, ELISA analysis revealed that EGCG significantly increased both the proportion and activity of T cells, as well as the levels of key cytokines such as IL-2, IFN- γ , and TNF- α . These cytokines are essential for activating and regulating T-cell immune responses^[7]. IL-2 promotes T cell growth and differentiation, IFN- γ plays a role in antiviral and anti-tumor immunity, and TNF- α contributes to inflammation and cell death^[8]. By elevating the levels of these cytokines, EGCG may enhance the cytotoxicity of T cells, enabling them to more effectively target and eliminate tumor cells^[9]. This finding underscores EGCG's potential to strengthen the immune system, particularly through T cell-mediated immune responses.

In conclusion, EGCG exerts significant anti-tumor activity via multiple mechanisms affecting both tumor cells and the immune system. First, EGCG directly inhibits tumor cell proliferation and induces apoptosis^[10]. Second, through its antioxidant and anti-inflammatory effects, EGCG may improve the tumor microenvironment, reducing the survival and metastatic potential of tumor cells^[11].

As the focus on healthy lifestyles grows, natural medicines and dietary therapies have become increasingly popular topics of public interest. Green tea, a traditional beverage, has become deeply integrated into daily life, and its health benefits are progressively being validated by scientific research^[12]. Against this backdrop, the current study not only highlights the anti-cancer potential of EGCG, a component of green tea, but also offers the public a simple and feasible method for promoting health. This approach, which combines traditional dietary culture with modern scientific research, is gradually becoming a part of global health management and disease prevention strategies^[13].

Despite the positive results of this study, it is important to acknowledge the limitations of natural medicine therapies. Issues such as the bioavailability of natural compounds, potential side effects, and interactions with conventional treatments require further exploration. Additionally, while this experimental model provides robust evidence, there are inherent differences between human and animal models. Therefore, the clinical translation of EGCG still necessitates additional research to support and validate its use. Future studies should focus on preclinical research on EGCG, including pharmacokinetic and toxicological assessments, and clinical trial design, to provide a stronger scientific basis for its clinical application.

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Disclosure statement

The author declares no conflict of interest.

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Writing – review & editing: all authors

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Ultrasonic-Assisted Extraction of Fucoxanthin from Marine Macroalga *Padina australis*: Optimization, Bioactivity, and Structural Characterization

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Abstract: Macroalgae serve as a potential feedstock for fucoxanthin extraction. Fucoxanthin, a bioactive pigment found in the chloroplasts of marine algae, exhibits significant pharmacological properties. As a member of the carotenoid family, fucoxanthin plays a crucial role in both the food and pharmaceutical industries. This research explores the effects of ultrasonics on the extraction of fucoxanthin from the marine macroalga *Padina australis*. In addition, various extraction techniques and the influence of solvents on the efficient separation of fucoxanthin from algae have been studied and compared. Using methanol, chloroform, and a combination of methanol and chloroform (1:1, v/v), conventional fucoxanthin extraction from *Padina australis* yielded 8.12 mg of fucoxanthin per gram of biomass. However, the ultrasonic-assisted extraction resulted in a significantly higher yield of 16.9 mg of fucoxanthin per gram of biomass, demonstrating that the use of ultrasonics enhances the extraction rate compared to conventional methods. Therefore, the efficient separation of fucoxanthin from *Padina australis* is highly dependent on ultrasonic-assisted extraction. The process conditions for the extraction were optimized to maximize the yield of fucoxanthin from seaweeds. The following parameters were selected for optimization studies: moisture content, particle size, mixing speed, extraction temperature, extraction duration, and solid-to-solvent ratio. The extracted fucoxanthin exhibited various biological activities, including antimicrobial and antioxidant properties, and its structure was elucidated through FTIR and NMR spectroscopy. Additionally, thin-layer chromatography of the crude algae extracts confirmed the presence of fucoxanthin in the marine algae. Given these findings, the optimized extraction process holds the potential for scaling up to large-scale fucoxanthin production. Fucoxanthin, as a potent pharmacological agent, offers promising applications in the treatment of various ailments.

Keywords: Marine algae; Fucoxanthin; Ultrasonics; Extraction; Optimization; TLC; DPPH; Antimicrobial antioxidant activities

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1. Introduction

The marine environment is a rich source of bioactive compounds, and marine algae, also known as macroalgae, hold great potential as a renewable resource for these valuable substances. Algae, which are photosynthetic organisms, form the foundational biomass of the intertidal zone ^[1]. Approximately 6,000 species of algae have been identified and classified into three major groups: green algae (chlorophytes), brown algae (phaeophytes), and red algae (rhodophytes). These classifications are based on the algae's nutrient content, pigments, and chemical composition ^[2,3]. Like terrestrial plants, algae contain numerous inorganic and organic compounds with the potential to benefit human health. Due to their ability to produce a wide range of secondary metabolites with diverse biological activities, algae are an excellent reservoir of bioactive compounds ^[4,5]. Bioactive compounds with antioxidant, antiviral, antifungal, and antibacterial properties can be found in brown, red, and green algae ^[6,7].

Although algae generally have lower lipid content compared to microalgae, lipids from brown algae are particularly noteworthy as they contain a variety of bioactive substances, including omega-3 polyunsaturated fats (PUFAs), fucoxanthin, fucosterols, and polyphenols ^[3,8,9]. Fucoxanthin, a key nutraceutical derived from brown algae, stands out due to its unique physiological effects stemming from a distinctive molecular mechanism ^[10]. Found in the chloroplasts of brown algae, fucoxanthin is a major carotenoid, accounting for over 10% of the estimated global carotenoid production, making it the most abundant carotenoid in these organisms ^[11]. Its complex formation with chlorophyll protein plays a crucial role in capturing and protecting light, ensuring efficient energy utilization, and regulating photosynthesis.

The unique structure of fucoxanthin, characterized by an allenic bond and a notable 5,6-monoepoxide linkage, is rare among natural carotenoids, with only about 40 out of 700 naturally occurring carotenoids possessing this feature. Fucoxanthin from brown algae, peridinin from microalgae, neoxanthin from higher plants, and fucoxanthin metabolites such as fucoxanthinol and amarouciaxanthin are the primary allenic carotenoids. The unique allenic bond of fucoxanthin enables it to function as an antioxidant even in hypoxic conditions, where other carotenoids may fail to counteract oxidative stress—an uncommon feature in natural food products. Fucoxanthin offers a wide range of health benefits, including anti-cancer, anti-inflammatory, antioxidant, and anti-obesity properties. It has demonstrated a potent inhibitory effect on prostate cancer, likely due to its antioxidant activity and its impact on apoptosis-related biomolecules. Furthermore, fucoxanthin has shown greater efficacy against human colon cancer cells than β -carotene and astaxanthin, highlighting its enhanced biological activity.

Given these promising effects, fucoxanthin has significant potential for commercial applications. In addition to its anti-obesity properties, it may reduce the risk of disorders such as type 2 diabetes by facilitating the removal of misfolded proteins. Fucoxanthin also has the potential to protect the liver and blood vessels, and it offers benefits for the brain, bones, skin, and eyes ^[12]. Although brown seaweed contains 3–10 g/kg of fucoxanthin, the chemical synthesis of the compound is challenging due to its high costs and low recovery rates.

Additionally, the occurrence of algal blooms can disrupt ecosystems by altering food chains and wildlife communities. Utilizing this biomass for fucoxanthin extraction not only provides a source of the compound but also helps mitigate environmental damage ^[13–15].

This preliminary study aimed to investigate and evaluate the fucoxanthin content of *Padina australis*. This research focused on optimizing the extraction process to maximize the yield of this bioactive compound from macroalga.

2. Materials and methods

2.1. Preparation of the sample

To remove contaminants such as sand, gravel, and mud, samples were first washed with fresh water and then

drained. All samples were transported to the laboratory for further examination after being wrapped in black plastic bags and stored in a cold box. The extraction of bioactive molecules from seaweeds followed previously reported protocols, which were also adopted for this optimization study. The methods used are outlined below.

2.1.1. Method 1

This methodology was based on a proposed extraction procedure for *Padina australis* algae specimens. The specimens were washed, dried, ground into powder, and subjected to extraction using methanol, chloroform, and a methanol-chloroform mixture. These extractions produced extracts representing the polar, non-polar, and low-polarity components of the algae. The extracts were then combined, concentrated under reduced pressure, and the fractions evaporated to dryness. The weight of the crude extract from each sample was recorded.

2.1.2. Method 2

In this approach, a modified version of a previous study was used to extract and purify fucoxanthin from *Padina australis* algae. The algae were collected, cleaned, shade-dried, ground into powder, and then used for further experiments. A mixture of acetone and methanol was added to the dried and powdered algae, homogenized, and filtered. This process was repeated three times. The resulting extracts were combined and evaporated, and the residue was dissolved in methanol.

2.1.3. Method 3

In this extraction procedure for marine algae *Padina australis*, the samples were washed, freeze-dried, and blended. The samples were then ground into powder and treated with 80% methanol for 24 hours at room temperature. The methanolic extracts were filtered and dried under a vacuum. Proper washing and handling of the samples were critical steps in this extraction process.

2.1.4. Ultrasonics-based method

Cell disruption is a crucial step in recovering intracellular components from biomass, improving the bioavailability and absorption of pigments within cells. In a study focusing on the marine macroalga *Padina australis*, ultrasonication of dried and powdered samples was employed to enhance fucoxanthin extraction efficiency. The application of ultrasound was found to induce acoustic cavitation in the solvent, facilitating deeper penetration of the solvent into the tissue. Additionally, ultrasound increased the interface area between the solid and liquid phases, promoting rapid diffusion of solutes from the solid phase into the solvent. Three different extraction techniques utilizing ultrasonication were employed for fucoxanthin extraction. In this method, 1 g of dried powdered macroalgae was placed in a glass flask with a solid-to-solvent ratio of 1:10. The mixture was stirred with a magnetic stirrer and subjected to ultrasound-assisted extraction in a sonication water bath. The ultrasound parameters were set to a fixed frequency of 40 kHz and a power of 250 W. The extract was then filtered and analyzed to determine the fucoxanthin content ^[16].

2.2. Optimization of extraction parameters

Several factors can affect fucoxanthin extraction; however, six parameters were chosen and optimized to increase the extraction yield. The parameters selected for optimization were the solid-to-solvent ratio, temperature, time, mixing speed, moisture content, and particle size.

Table 1. Ranges of extraction parameters for fucoxanthin optimization

Extraction parameters	Range of the parameters
Moisture content (%)	2.08–9.82
Particle size (mm)	0.246–0.053
Mixing speed (rpm)	200–800
Extraction temperature (°C)	35–65
Extraction time (min)	10–70
Solid-to-solvent ratio	1:2–1:14

2.2.1. Moisture content

Padina australis was thoroughly washed with fresh water, transported to the laboratory in iced conditions, and shade-dried ($38 \pm 2^\circ\text{C}$) for about 20 hours. The moisture content of the samples varied between 2.08% and 9.82%. The moisture content was determined using the following equation:

$$\text{Moisture content (\%)} = \frac{M_i - M_f}{M_i} \times 100$$

where M_i is the initial weight of the sample (g), and M_f is the final weight after drying (g). The dried samples, with specific moisture contents, were extracted using ultrasonication and a methanol-chloroform (1:2) solvent system. The sample was stirred for 30 minutes at 400 rpm, and ultrasound-assisted extraction was performed in a sonication bath. The extraction temperature was maintained at 40°C . The extracts were then evaporated under reduced pressure using a rotary evaporator and analyzed.

2.2.2. Particle size

The dried sample with the optimal moisture content, determined from the above procedure, was ground into powders with particle sizes ranging from 0.246 mm to 0.053 mm. By holding the other parameters constant, the same procedure was used to extract fucoxanthin from these samples.

2.2.3. Mixing speed

The dried sample with optimal moisture content and particle size was used for fucoxanthin extraction. The same protocol was followed, but mixing was performed at varying speeds (200–800 rpm). After extraction, the fucoxanthin content was analyzed.

2.2.4. Extraction temperature

Following the previous procedure, the dried sample with an optimal moisture content of 8.17% and particle size of 0.063 mm was used for fucoxanthin extraction. Ultrasonication was performed using a 1:1 methanol-chloroform solvent mixture and a mixing speed of 700 rpm. The extraction temperature was varied from 35°C to 65°C while maintaining the extraction time at 30 minutes with a solid-to-solvent ratio of 1:10.

2.2.5. Extraction time

Extraction time is a key parameter for fucoxanthin extraction from *Padina australis*. It helps determine the optimal duration for the extraction process. The dried sample with optimal moisture content, particle size, mixing intensity, and temperature was used for fucoxanthin extraction. Extraction times varied from 10 to 70 minutes, after which the extracts were analyzed.

2.2.6. Solid-to-solvent ratio

The solid-to-solvent ratio for fucoxanthin extraction was varied between 1:12 and 1:14 while maintaining the other parameters at their optimal levels: moisture content at 8.17%, particle size at 0.063 mm, mixing speed at 700 rpm, extraction temperature at 60°C, and extraction time at 60 minutes.

2.3. Analysis of fucoxanthin extract

Fucoxanthin extracted from *Padina australis* was analyzed using Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) spectroscopy, and high-performance liquid chromatography (HPLC) techniques. A modified version of the Wright *et al.* [17] HPLC method was employed, with solvent gradients consisting of methanol (A), acetonitrile (B), and pure ethyl acetate (C). A linear solvent gradient program was used (Table 2). The solvents were degassed using a sonicator and filtered through a 0.25 µm filter before use. The injection volume was 20 µL, and the flow rate was 1 mL/min. All samples were dissolved in a 1:1:2 methanol solution. Separation was carried out at room temperature using a C18 column, and fucoxanthin was detected using a UV-VIS detector at 450 nm.

Table 2. Gradient program for the separation of brown seaweed pigments

Time (min)	Flow rate (mL/min)	A (%)	B (%)	C (%)
0–10	1	80	10	10
10–25	1	80	16	4

Abbreviation: A, methanol; B, acetonitrile; C, pure ethyl acetate.

2.4. Bioactivity studies on fucoxanthin applications

2.4.1. Antimicrobial activity of fucoxanthin

A well-diffusion assay was conducted. After autoclaving, 1% of 24-hour cultures of indicator organisms (*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Salmonella typhi*) were added to nutrient agar medium, cooled to 40°C, and aseptically poured into sterile Petri dishes (100 × 15 mm). Each plate was left to solidify for three hours, after which wells were bored into the agar. Different concentrations (250 µg, 500 µg, 750 µg) of crude fucoxanthin extract were added to each well. The plates were incubated at 37°C for 24 hours, and the diameters of inhibition zones around the wells were measured horizontally and vertically and averaged.

2.5. Qualitative analysis of antioxidant activity

2.5.1. Thin layer chromatography (TLC)

Plant extracts were loaded onto pre-coated silica plates and developed using a methanol-chloroform solvent system (0.75:9.25 ratio). Spots were visualized under iodine vapor, far light, and UV light. The R_f value was calculated as the ratio of the solute's travel distance to the solvent's travel distance.

2.5.2. TLC bioautography for antioxidant screening

Plant extracts were applied to thin-layer chromatography sheets, developed, and allowed to dry. The plates were sprayed with a 0.2% DPPH solution in methanol or ethanol, left to sit at room temperature for 30 minutes, and observed under white light. Antioxidant activity was indicated by a color change from purple (DPPH) to yellow.

2.6. Antioxidant assay for DPPH radical scavenging activity

The effect of fucoxanthin extract from *Padina australis* on DPPH radicals was assessed. Fucoxanthin extracts (10, 20, 50 µg) were added to 4 mL of distilled water and combined with 1 mL of methanolic DPPH solution.

After one minute of vortexing, the mixture was left to stand at room temperature in the dark for 30 minutes. Absorbance was measured at 517 nm. The scavenging effect was calculated using the formula:

$$\text{Scavenging effect (\%)} = \left[1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}} \right] \times 100$$

where A_{control} is the absorbance of the control (DPPH solution without sample), A_{sample} is the absorbance of the test sample (DPPH solution plus test sample), and A_{blank} is the absorbance of the sample alone (without DPPH solution) ^[18].

3. Results

Various extraction methodologies and the influence of solvents on the effective isolation of fucoxanthin from algae have been reported and compared. Methanol, chloroform, and a methanol-chloroform combination (1:1 v/v) followed by chloroform resulted in a fucoxanthin yield of 8.12 mg per gram of biomass using Method 1 for fucoxanthin extraction from *Padina australis*. The application of an ultrasonic-assisted approach led to a fucoxanthin yield of 16.9 mg per gram of biomass, demonstrating that ultrasonic assistance enhances extraction efficiency compared to the non-ultrasonic method. Hence, the effective separation of fucoxanthin from the marine macroalga *Padina australis* is contingent upon ultrasonic-assisted extraction ^[16]. When compared to other techniques, ultrasound-assisted extraction of fucoxanthin from *Padina australis* produced a greater yield. The optimal technique demonstrates the potential for process development and scaling up for fucoxanthin extraction from seaweeds. This bioactive compound can be utilized to treat cancer by inducing apoptosis in various cancer cell types, exhibiting anti-tumor properties, inhibiting cancer cell invasion, and blocking enzyme activity in cancer cells.

3.1. Optimization of parameters influencing extraction

3.1.1. Effect of moisture content

Moisture content is a crucial factor in fucoxanthin extraction. **Figure 1** illustrates how moisture levels affect the extraction of fucoxanthin, with a range of 2.08% to 9.82% moisture content tested. By holding other parameters constant—such as particle size (0.147 mm), stirrer speed (400 rpm), extraction temperature (40°C), extraction time (30 minutes), and solid-to-solvent ratio (1:10)—the impact of moisture content on fucoxanthin extraction from *Padina australis* was investigated. Based on **Figure 1**, it can be deduced that fucoxanthin extraction increases from 2.08% to 8.17% moisture content, then gradually decreases as moisture content continues to rise.

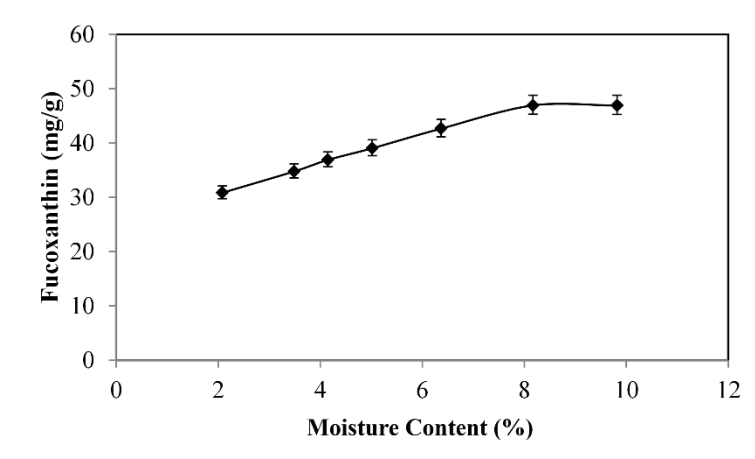


Figure 1. Effect of moisture content on fucoxanthin extraction

3.1.2. Effect of particle size

Particle size significantly affects fucoxanthin extraction from *Padina australis*. Smaller biomass particles provide a greater interfacial area between the solid and liquid phases, leading to higher fucoxanthin yield. Maximum extraction was achieved with a particle size of 0.063 mm and optimal moisture content of 8.17% at 40°C, 400 rpm, and a solid-to-solvent ratio of 1:10 for 30 minutes. **Figure 2** shows that the fucoxanthin yield gradually increased as particle size decreased from 0.246 mm to 0.063 mm. However, further reduction in particle size did not significantly improve fucoxanthin extraction.

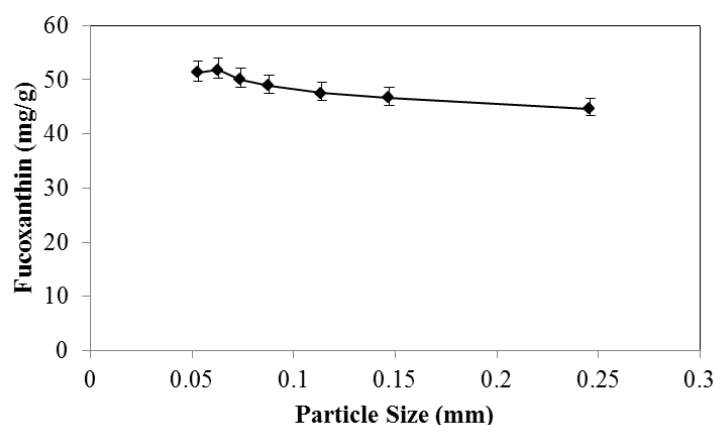


Figure 2. Effect of particle size on fucoxanthin extraction

3.1.3. Effect of mixing speed

Mixing intensity is a key factor in fucoxanthin extraction. **Figure 3** shows how stirrer speed affects extraction, with speeds ranging from 200 to 800 rpm. It was discovered that increasing the stirrer speed from 200 to 700 rpm enhanced fucoxanthin extraction, with the highest yield achieved at 700 rpm. However, speeds above 700 rpm did not significantly increase extraction efficiency.

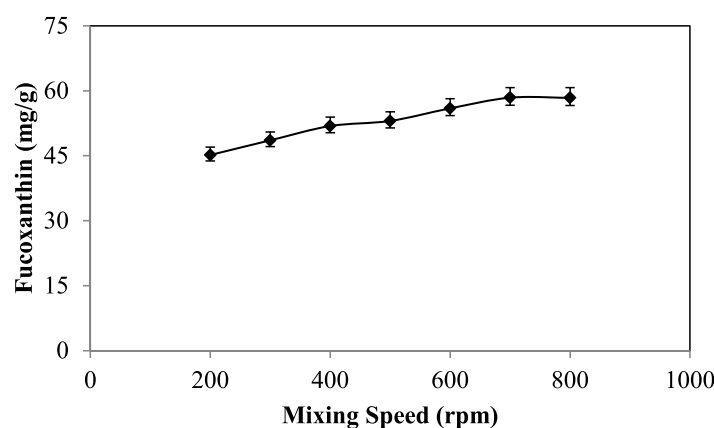


Figure 3. Effect of mixing speed on fucoxanthin extraction

3.1.4. Effect of extraction temperature

Temperature is one of the most crucial factors in fucoxanthin extraction. **Figure 4** illustrates the effect of temperature on extraction from *Padina australis*, tested within a range of 35°C to 65°C. By keeping other parameters constant at optimal levels (moisture content: 8.17%, particle size: 0.063 mm, stirrer speed: 700 rpm) and using a solid-to-solvent ratio of 1:10 for 30 minutes, the impact of temperature was investigated. It was

found that fucoxanthin extraction increased as the temperature rose, due to the improved dissolution capacity of the solvent system. According to **Figure 4**, extraction efficiency increased as temperature rose from 35°C to 60°C, then gradually decreased with further temperature increases.

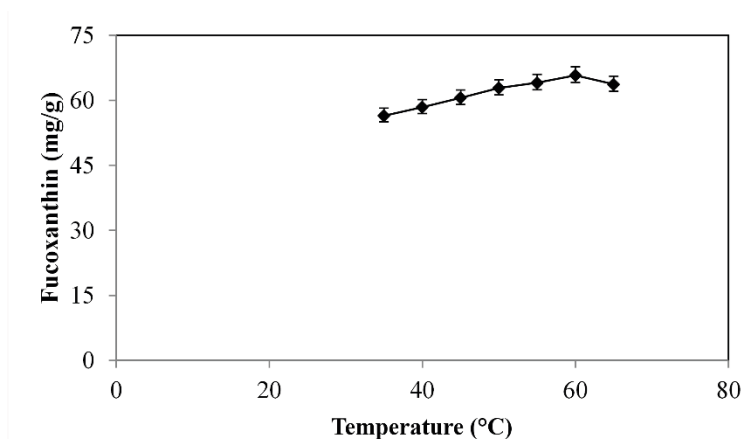


Figure 4. Effect of extraction temperature on fucoxanthin extraction

3.1.5. Effect of extraction time

The effect of extraction time was examined using intervals ranging from 10 to 70 minutes (**Figure 5**). The findings showed that fucoxanthin extraction increased over time, reaching its peak at 60 minutes under optimal conditions (8.17% moisture content, 0.063 mm particle size, 700 rpm stirrer speed, and 60°C temperature). Extending the extraction time beyond 60 minutes to 70 minutes did not significantly improve the yield. Thus, the optimal extraction time was determined to be 60 minutes.

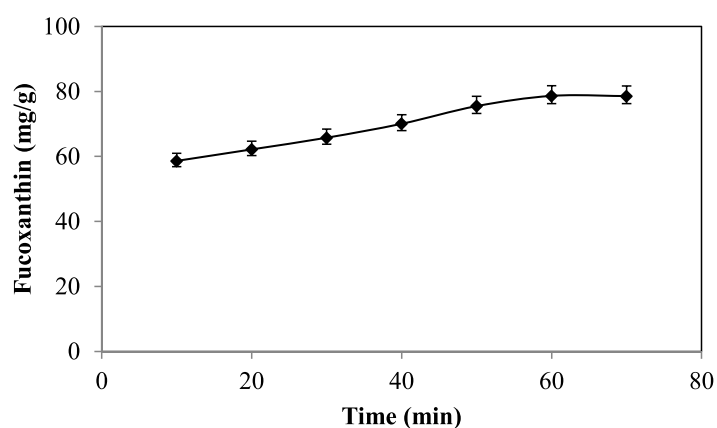


Figure 5. Effect of extraction time on fucoxanthin extraction

3.1.6. Effect of solid-to-solvent ratio

The solid-to-solvent ratio is another crucial element in the extraction process. A larger solvent volume can improve extraction yield by more efficiently dissolving the target components, but excessive solvent use leads to waste. Conversely, a lower solvent volume results in lower yields. **Figure 6** illustrates the effect of the solid-to-solvent ratio on fucoxanthin extraction. By keeping all other parameters at optimal levels, the impact of ratios ranging from 1:2 to 1:14 was investigated. The fucoxanthin yield increased as the solid-to-solvent ratio rose from 1:2 to 1:14, but further increases beyond a ratio of 1:12 did not significantly improve extraction. Thus, the optimal solid-to-solvent ratio of 1:12 was selected for process scale-up.

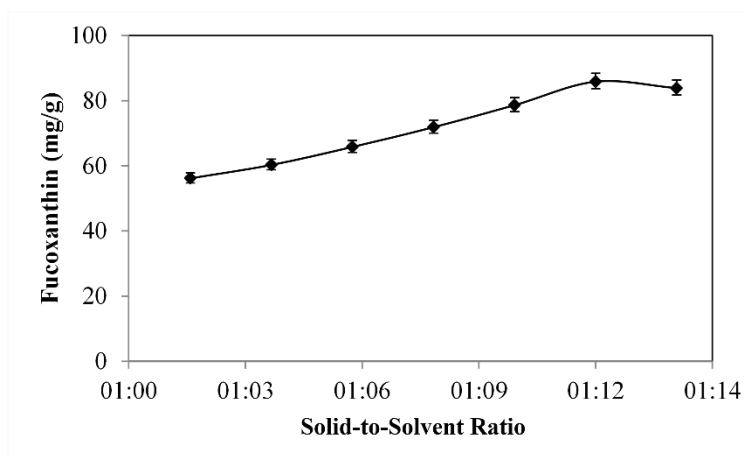


Figure 6. Effect of solid-to-solvent ratio on fucoxanthin extraction

3.2. Characterization of fucoxanthin

3.2.1. Fourier transform infrared (FTIR) analysis

FTIR analysis was conducted on the fucoxanthin extract from *Padina australis* to identify the associated functional groups (**Figure 7**). The FTIR spectrum of the sample was compared with that of standard gallic acid. The FTIR spectrum of the fucoxanthin extract showed the same number of peaks, ranging between 3904.5 cm^{-1} and 1108.2 cm^{-1} .

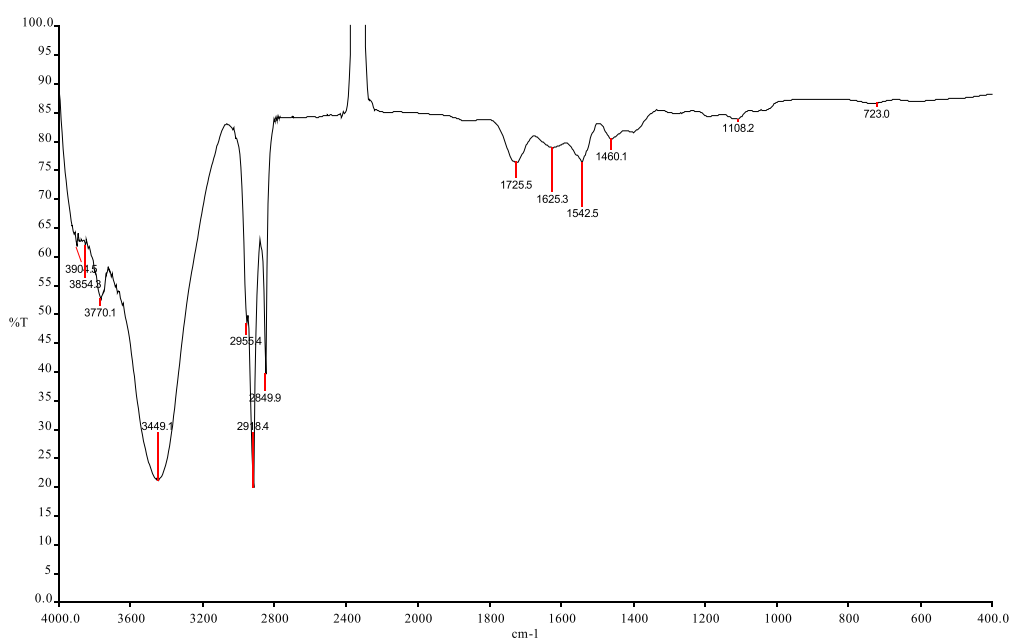


Figure 7. FTIR analysis of fucoxanthin

Table 3. FTIR spectral analysis of fucoxanthin extract

Frequency range (cm^{-1})	Bond	Compound type
3600–3200	O-H	Hydrogen bonded – Alcohols, Phenols (stretch)
2950–2850	C-H	Alkanes (stretch)
1725–1640	C-H	Phenyl ring substitution
1470–1350	C-H	Alkanes (bending)
1110–1000	C-O	Alcohols, Ethers, Esters (stretch)

3.2.2. Nuclear magnetic resonance spectroscopy

Fucoxanthin was characterized using NMR spectroscopy (**Figure 8**). Based on the NMR spectral analysis, the fucoxanthin extract was identified as all-trans fucoxanthin because its NMR spectral data matched those of an authentic all-trans fucoxanthin compound, as reported by Englert *et al.* ^[19].

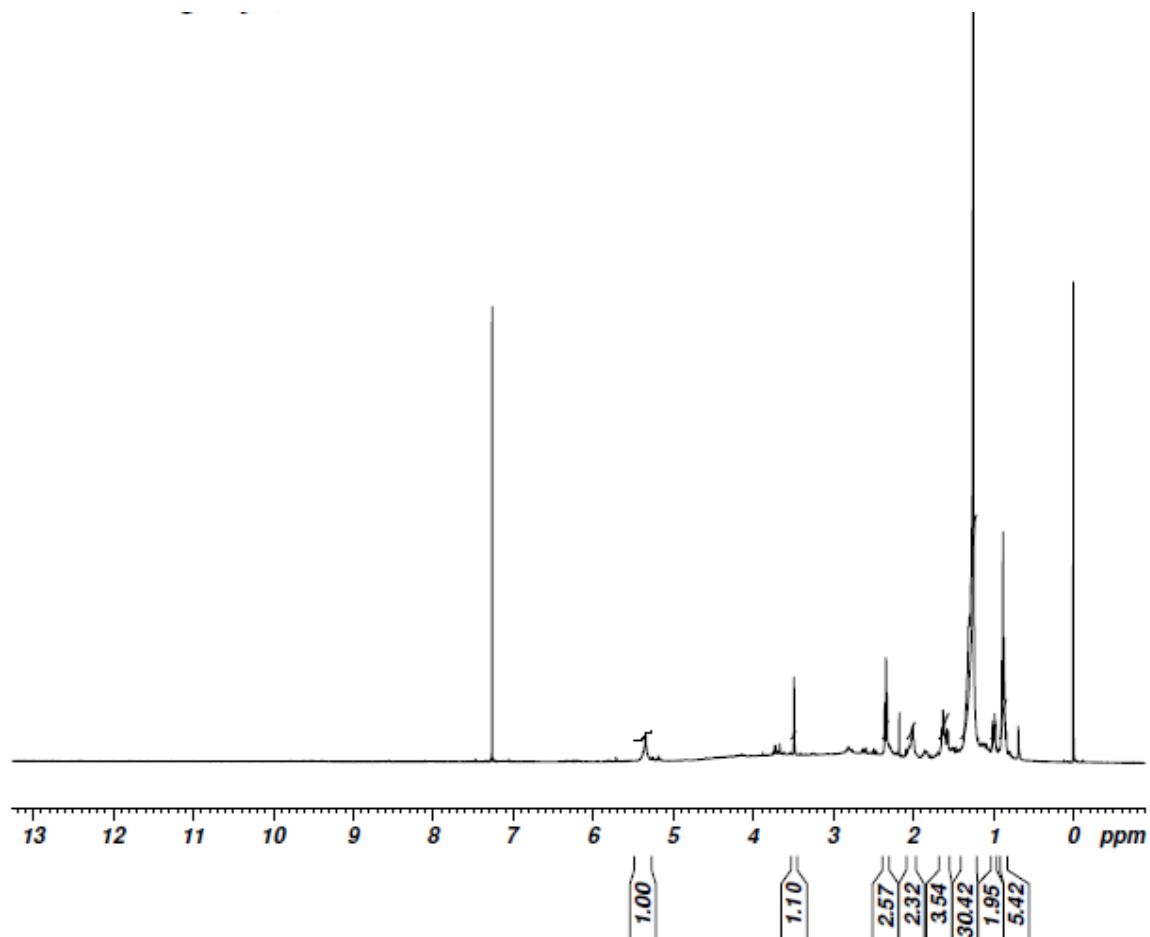


Figure 8. NMR spectral analysis of fucoxanthin

Table 4. NMR results of fucoxanthin extract

No.	Functional group	Peaks (ppm)
1	-(CH ₃ -C) – terminal methyl group	0.866 – 1.009
2	-(CH ₂) _n – backbone CH ₂	1.254 – 1.312
3	-(CH ₂ CH ₂ COOH) – β methylene proton	1.566 – 1.645
4	-(CH ₂ COOH) – α methylene group to acid	2.004 – 2.016
5	-(CH=CH) – olefinic proton	5.341 – 5.351

3.2.3. HPLC analysis of fucoxanthin extract

The fucoxanthin extract from *Padina australis* was analyzed using HPLC (**Figure 9**). The HPLC chromatograms of the crude pigment extract were detected at 450 nm. Two peaks were observed at retention times of 10.08 and 17.22 minutes, which were consistent with the standard fucoxanthin reported in the literature.

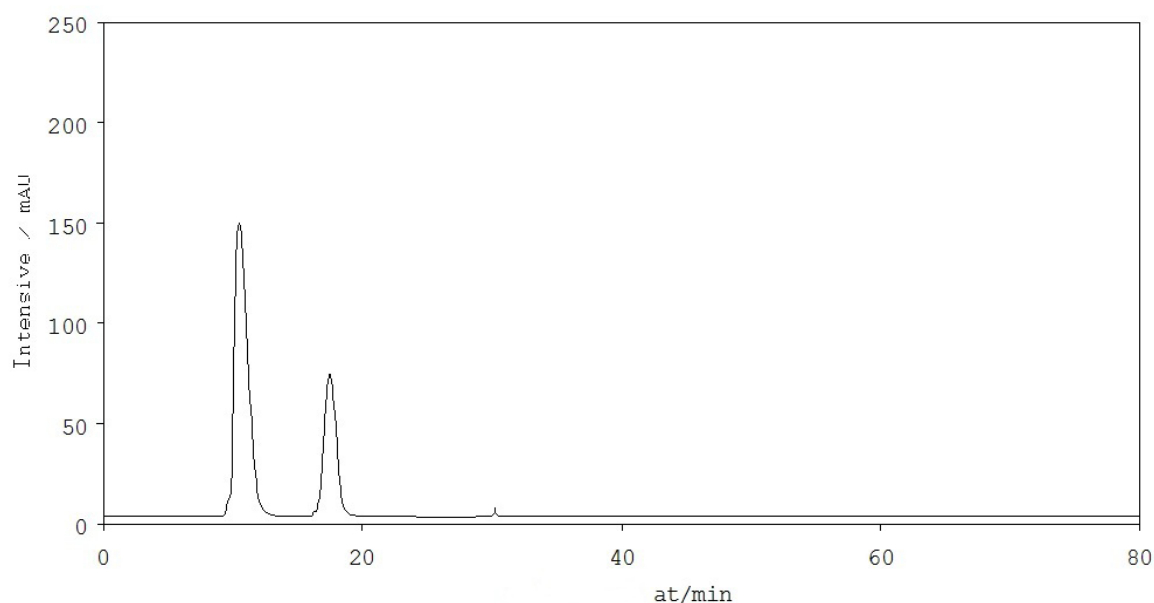


Figure 9. HPLC analysis of fucoxanthin extract

3.3. Studies on the application of fucoxanthin

3.3.1. Determination of antimicrobial activity using well diffusion assay

The extract obtained from *Padina australis* was investigated for its antimicrobial activity. Concentrations of 250 µg, 500 µg, and 750 µg of the algae extract inhibited the growth of microorganisms. The fucoxanthin extracts from *Padina australis* were particularly effective against Gram-positive bacteria, especially *B. subtilis* and *S. aureus*, as well as Gram-negative bacteria, especially *E. coli* and *S. typhi*. The results indicate that the algal extracts are generally more effective against Gram-positive than Gram-negative bacteria (**Figure 10**), likely due to the more complex structure of the Gram-negative bacterial cell wall.

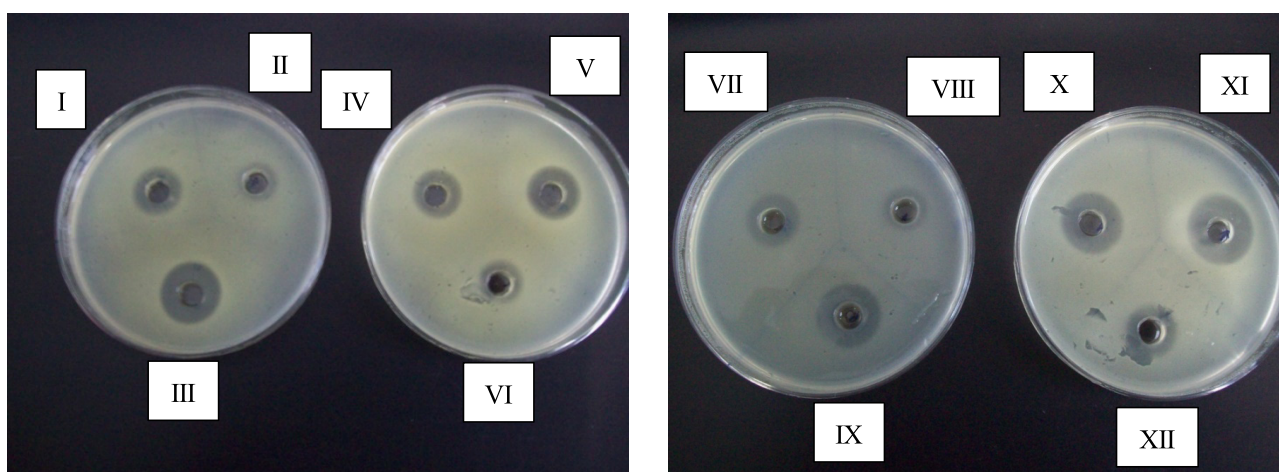


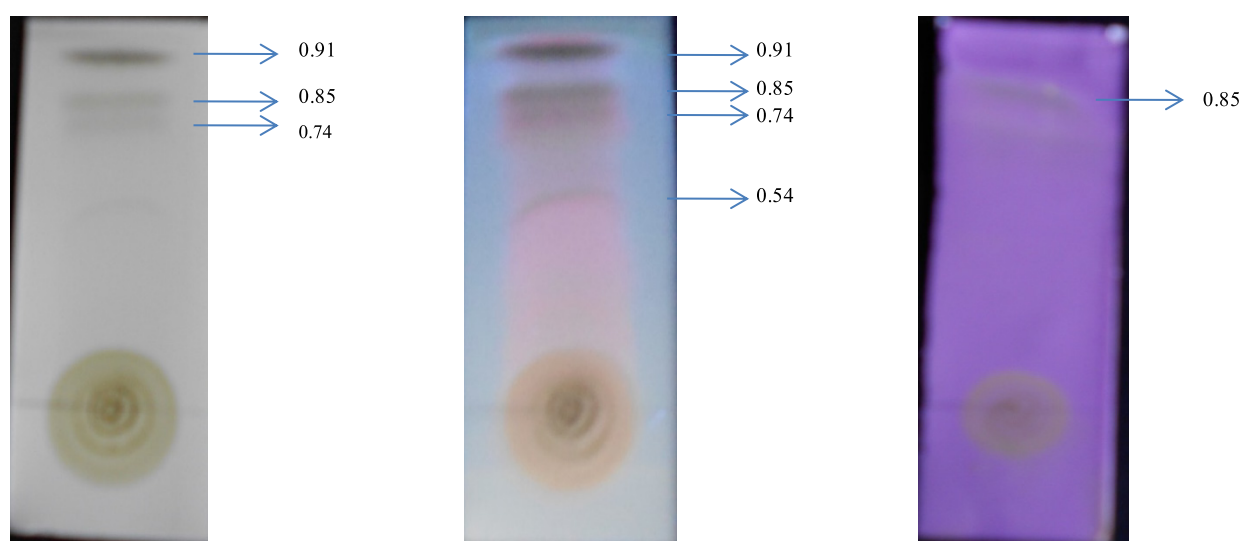
Figure 10. Well diffusion assay

Table 5. Zone of inhibition against different microorganisms

No.	Microorganism	Extract concentration	Inhibition zone (mm)
I	<i>Bacillus subtilis</i>	250 µg	12
II	<i>Bacillus subtilis</i>	500 µg	17
III	<i>Bacillus subtilis</i>	750 µg	18
IV	<i>Staphylococcus aureus</i>	250 µg	12
V	<i>Staphylococcus aureus</i>	500 µg	16
VI	<i>Staphylococcus aureus</i>	750 µg	20
VII	<i>Escherichia.coli</i>	250 µg	12
VIII	<i>Escherichia.coli</i>	500 µg	16
IX	<i>Escherichia.coli</i>	750 µg	18
X	<i>Salmonella typhi</i>	250 µg	15
XI	<i>Salmonella typhi</i>	500 µg	20
XII	<i>Salmonella typhi</i>	750 µg	22

3.4. TLC bioautography for antioxidant property screening

In this study, compounds in the selected crude extract were first separated using analytical TLC. When the chromatographic profile of the crude extract was viewed under light, it revealed three vibrant bands on the TLC plate (**Figure 11 left**). Fucoxanthin was identified as the primary colorful pigment in *Padina australis*, suggesting that this pigment may be responsible for the brown and olive-green bands observed in the TLC. The bands were visible on the analytical TLC at $R_f = 0.91$, 0.85, and 0.74. TLC bioautography was also used to assess the potential biological properties of the separated compounds. To determine antioxidant capacity, the developed plate was sprayed with DPPH[•] reagent. The results showed that only band 2 ($R_f = 0.85$), which turned yellow against a purple background, exhibited antioxidant capacity (**Figure 11 right**). The intensity of the yellow color indicates the quantity and type of radical scavengers present in the sample.

**Figure 11.** TLC bioautography under white light (**left**), under UV (**middle**), and sprayed with 0.2% DPPH (**right**)

3.5. DPPH radical scavenging activity

DPPH is a useful reagent for examining a compound's free radical scavenging ability. In the DPPH test, the extracts were able to convert the stable radical DPPH from purple color to yellow color when neutralized. The method is based on the reduction of an alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant, resulting in the formation of the non-radical DPPH-H. The crude extract of *Padina australis* demonstrated DPPH radical scavenging activity in a concentration-dependent manner.

Table 6. Percentage of antioxidant activity

Extract concentration (µg)	Antioxidant activity (%)
100	9.08
200	14.36
300	17.68

4. Discussion

A method for preparing fucoxanthin from leftover Kombu (*Laminaria japonica*) waste parts was reported by Kazuki *et al.* in 2008. The analysis of Kombu waste components aimed to produce high-quality fucoxanthin. Temperature enhanced fucoxanthin recovery, and further washing with tap water reduced the extract's salt content. After two extractions with three volumes of absolute ethanol, Kombu showed the highest fucoxanthin recovery and the lowest salt content. The extract was subjected to silica gel column chromatography to remove chlorophylls, ultimately yielding 1,490 g of fucoxanthin with an 82% recovery rate from 10 t of Kombu waste. After six months of storage at 4°C, the obtained fucoxanthin remained stable, with only a 2% reduction ^[20]. Therefore, leftover Kombu culture material serves as a valuable bioresource for fucoxanthin production.

In 2011, Noviendri *et al.* isolated and purified fucoxanthin from two species of brown seaweed in Malaysia: *Sargassum binderi* and *Sargassum duplicatum*. HPLC analysis confirmed that fucoxanthin had a purity level of >99%. The total lipid and fucoxanthin contents, along with the fatty acid composition of the seaweeds, indicated that both samples contained significant amounts of these components. *S. duplicatum* had a fucoxanthin content of 21.3 ± 0.10 mg/g dry-weight and a total lipid content of 1.01 ± 0.10 mg/g, while *S. binderi* had lower values of 16.6 ± 4.10 mg/g for total lipids and 0.73 ± 0.39 mg/g for fucoxanthin. Both seaweed varieties also contained significant levels of unsaturated fatty acids. Notably, *S. duplicatum* showed higher concentrations of docosahexaenoic acid, eicosapentaenoic acid, arachidonic acid, linoleic acid, and alpha-linolenic acid (0.76, 2.55, 13.64, 5.81, and 5.35%, respectively) than *S. binderi* (0.70, 1.82, 9.13, 6.37, and 4.39%, respectively). Palmitic acid (C16:0) was found to be the dominant saturated fatty acid in both samples ^[21].

Shang *et al.* found that fucoxanthin extraction efficiency was significantly influenced by temperature and ethanol concentration. The maximum predicted fucoxanthin extraction was 0.42 mg/g at 110°C and 90% ethanol ^[22]. Takeshi *et al.* explored the possibility of producing a high-purity fucoxanthin-rich product using *Cladosiphon okamuranus* as the raw material. Drying and pulverization techniques were optimized to reduce fucoxanthin degradation. The results revealed that 50 µm freeze-dried powder exhibited the best qualities. Additionally, the algal extract demonstrated strong DPPH radical scavenging activity ^[23].

A study was conducted to examine the antioxidant properties of three specific types of Indian brown seaweeds: *Sargassum marginatum*, *Padina tetrastomatica*, and *Turbinaria conoides*. The total phenolic content and reducing power of crude methanolic extracts were analyzed ^[18]. The total antioxidant activity, DPPH radical

scavenging ability, and deoxyribose assay were evaluated for total methanolic extract and five distinct fractions: petroleum ether, ethyl acetate, dichloromethane, butanol, and aqueous. Among these, the ethyl acetate fraction of *S. marginatum* showed significantly higher total antioxidant activity (39.62 mg ascorbic acid equivalent per gram of extract or 0.31 mg ascorbic acid equivalent per gram of seaweed on a dry weight basis). This fraction also exhibited superior DPPH scavenging activity (23.16%), while the petroleum ether fraction of *T. conoides* showed lower scavenging activity. The deoxyribose activity of *T. conoides* was 47.81%, lower than the other samples. The aqueous fraction of *T. conoides* exhibited the highest phenolic content (49.16 mg gallic acid equivalent (GAE) per gram of extract or 0.86 mg GAE per gram of seaweed on a dry weight basis). The *in vitro* antioxidant activity of the methanolic extracts increased with concentration, suggesting a dose-dependent antioxidant property.

In another study by Hii *et al.* ^[24], the stability of fucoxanthin extracted from *S. binderi* was assessed under various storage conditions. The stability was tested across different pH levels, with and without the addition of the antioxidant ascorbic acid, under both light and dark conditions over four weeks. Fucoxanthin exhibited the highest stability in a dark environment. Stability was also greater in an alkaline pH environment compared to neutral or acidic conditions. The addition of 1.0% w/v ascorbic acid further delayed fucoxanthin degradation, particularly under dark conditions, with the pigments showing a high retention rate. In conclusion, fucoxanthin was sensitive to light exposure and acidic pH conditions, but ascorbic acid was effective in stabilizing the pigments.

Algal blooms pose significant threats to ecosystems, disrupting food chains and altering faunal communities. Taylor *et al.* noted that increased algal biomass displaces natural seagrass and higher plant communities ^[25]. Similarly, macroalgal blooms impact the biogeochemical cycles of carbon, nitrogen, phosphorus, and sulfur ^[26]. The macroalgal biomass requires attention for cleanup, making it a useful resource for fucoxanthin extraction. According to Zailanie *et al.*, *P. australis* and *T. conoides* are rich sources of fucoxanthin, making them promising candidates for cultivation as edible brown seaweed ^[27]. Roh *et al.* extracted fucoxanthin from *Undaria pinnatifida* using supercritical carbon dioxide (SCO₂) and ethanol as a co-solvent. The ethanol flow rate was 3.0% (v/v) compared to the SCO₂ flow rate ^[28].

The utilization of marine macroalgae for fucoxanthin extraction offers dual benefits. It serves as a source for fucoxanthin while mitigating environmental impacts. Therefore, the primary aim of this study was to examine and evaluate the levels of fucoxanthin in *P. australis*, *L. japonica*, and *U. pinnatifida*, while exploring their characteristics and potential pharmacological effects.

5. Conclusion

To conclude, the utilization of ultrasound-assisted extraction resulted in a higher yield of fucoxanthin from the marine macroalga *Padina australis*, surpassing the results of other studied extraction techniques. *Padina australis* demonstrated a higher fucoxanthin content compared to *Laminaria japonica* and *Undaria pinnatifida*. The highest yield of fucoxanthin was obtained using a methanol and chloroform mixture in a 1:2 ratio. Under optimal conditions, the maximum fucoxanthin content reached 85.85 mg/g. These conditions included a moisture content of 8.17%, a particle size of 0.063 mm, a mixing intensity of 700 rpm, an extraction temperature of 60°C, an extraction time of 60 minutes, and a solid-to-solvent ratio of 1:12. The extracted fucoxanthin was analyzed through agar well diffusion and DPPH assays, revealing its antimicrobial and antioxidant properties. This confirms the pharmacological potential of fucoxanthin and its suitability for medicinal applications.

Disclosure statement

The authors declare no conflict of interest.

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A Pan-Cancer Analysis of GAPDH as a Common Biomarker for Various Cancers

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Abstract: *Objective:* To investigate the expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and explore its prognostic value across 24 different human cancers. This investigation was conducted using comprehensive bioinformatics and in vitro approaches, incorporating multiple layers of analysis. *Methods:* GAPDH expression and methylation levels were assessed using bioinformatics tools and validated in cell lines through RNA-seq and targeted bisulfite-seq analyses. The potential prognostic significance of GAPDH was evaluated using the KM plotter. Additionally, cBioPortal was employed to investigate genetic alterations associated with this gene. Pathway analysis was conducted using DAVID. Furthermore, a correlation analysis between GAPDH expression and CD8⁺ T immune cells was performed using TIMER and CDT. Finally, a gene-drug interaction network analysis was conducted using Cytoscape to examine the relationship between GAPDH and various drugs. *Results:* GAPDH was found to be commonly upregulated in 24 types of human cancers, with its upregulation significantly correlated with poor relapse-free survival (RFS) and overall survival (OS) in BLCA, CESC, HNSC, KIRP, LIHC, and LUAD. This suggests that GAPDH plays a significant role in the development of these cancers. GAPDH upregulation was also associated with various clinicopathological features in patients with BLCA, CESC, HNSC, KIRP, LIHC, and LUAD. Pathway analysis revealed GAPDH's involvement in diverse pathways. Additionally, notable correlations were observed between GAPDH expression and its promoter methylation level, genetic alterations, and CD8⁺ T immune cell levels. Moreover, several regulatory drugs targeting GAPDH were identified, with the potential to modulate its expression and potentially prevent conditions such as BLCA, CESC, HNSC, KIRP, LIHC, and LUAD. *Conclusion:* Based on our findings, GAPDH emerges as a promising diagnostic and prognostic biomarker for BLCA, CESC, HNSC, KIRP, LIHC, and LUAD.

Keywords: Cancer; Expression; GAPDH; Biomarker

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1. Introduction

Cancer is not a single disease but a collection of various diseases ^[1], characterized by the uncontrolled growth and spread of abnormal cells in the body. It can arise in any part of the body and manifest in numerous forms, such as breast, lung, prostate, and colon cancers ^[2]. Each type of cancer has its unique characteristics, including specific risk factors, symptoms, and treatment approaches. The impact of cancer extends beyond the physical

realm, affecting individuals emotionally, socially, and economically ^[3]. It places a significant burden on patients, their families, and healthcare systems. The development of cancer can be influenced by various factors, including genetic mutations, environmental exposures, lifestyle choices, and certain infections ^[4]. Therefore, cancer can also be described as a disorder of altered gene expression, primarily resulting from variations in the expression of various DNA repair and tumor suppressor genes ^[5]. According to 2019 disease prevalence and mortality statistics, cancer has been declared the second leading cause of death worldwide, with an estimated 9.6 million deaths, or one in six deaths, following cardiovascular diseases ^[6].

Recently, an increasing body of evidence has suggested that regulatory changes resulting in the alteration of gene expression play a critical role in complex traits and disorders, and such genomic changes with regulatory effects are also predicted to participate in the development of cancer ^[7]. Identifying these regulatory alterations and their impact on gene expression levels is crucial for understanding cancer biology. Major cancer subtypes, including breast cancer, colorectal cancer, and leukemia, have typically been profiled for CpG methylation, post-transcriptional, post-translational changes, and mutational analysis of a few DNA repair and tumor suppressor genes to understand the molecular landscape of cancer development ^[8]. However, the effect of such regulatory alterations on the gene expression of several other essential genes remains to be uncovered.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an important enzyme in the human body that catalyzes the redox reaction via the glycolytic pathway ^[9]. GAPDH is a housekeeping enzyme and is thus frequently used as an internal control in various laboratory-based experiments, including Western blotting and RT-PCR ^[10,11]. Earlier evidence linked GAPDH upregulation with cancer development, as initial findings were documented in the Dunning R-3327 rat prostatic adenocarcinoma, where higher expression of GAPDH was observed in cancer cells compared to the ventral prostate tissue of normal rats ^[12]. Additionally, Tang *et al.* ^[13] reported higher GAPDH expression in human colon cancer (CC) tissue compared to controls, with even higher levels in metastatic liver tissue, suggesting that GAPDH may contribute to colon cancer metastasis. In contrast, Seykora *et al.* reported that GAPDH expression might be slightly diminished in melanoma metastases and nodular primary melanomas relative to melanocytic nevi ^[14], while other reports have shown significant upregulation of GAPDH in melanoma tissues compared to normal tissues ^[15]. Furthermore, recent studies have identified point mutations in the GAPDH gene as a novel melanoma tumor antigen, recognized by tumor-infiltrating T-lymphocytes in a patient with metastatic melanoma ^[16].

However, the role of GAPDH as a biomarker in other human cancers has been little reported. In the current study, we analyzed the diagnostic and prognostic potential of GAPDH in 24 human cancer subtypes using a multi-layered bioinformatics and in vitro approach. The novelty of our study lies in the pan-cancer analysis of GAPDH expression across multiple cancer types. To our knowledge, such a comprehensive examination of GAPDH expression in a diverse range of cancers has not been conducted before. By performing this pan-cancer analysis, our study uncovers previously undiscovered trends and associations between GAPDH expression and different cancer types. This broader perspective allows us to identify commonalities and variations in GAPDH expression patterns across various cancers, shedding new light on its potential roles in different oncogenic processes. Therefore, our study adds a unique and valuable dimension to the existing body of research on GAPDH and its implications in cancer biology.

2. Methods

2.1. UALCAN

GAPDH pan-cancer analysis across distinct cancers was performed using the online resource UALCAN.

This archive contains raw data from TCGA cancer projects, including data on expression, methylation, and clinicopathological parameters^[17]. UALCAN is based on data extracted from The Cancer Genome Atlas (TCGA) database, one of the largest and most comprehensive cancer genomics databases in the world. The UALCAN database offers researchers access to processed and normalized transcriptome sequencing data for multiple cancer types, including lung, breast, colorectal, ovarian, and others. One of the most significant benefits of UALCAN is that it bridges the gap between cancer genomics data and easy-to-use visualization and statistical computation tools. The platform provides researchers with an intuitive way to explore cancer transcriptomic data. It incorporates several analytical tools, including gene expression analysis, patient survival analysis, tumor mutation burden analysis, and more^[17]. The gene expression module, a key feature of UALCAN, enables researchers to analyze gene expression levels of their genes of interest. They can compare the expression levels with normal tissue samples and among tumor subgroups. The gene expression tool also provides charts, box plots, and heat maps. For statistical analysis, UALCAN uses a student *t*-test, with a *P*-value < 0.05 considered statistically significant.

2.2. Kaplan-Meier plotter

GAPDH relapse-free survival (RFS) and overall survival (OS) in distinct cancer subtypes were evaluated using the user-friendly online tool, Kaplan-Meier (KM) Plotter^[18]. KM Plotter is a data visualization tool designed to explore the survival rates of cancer patients based on their gene expression profiles. This web-based application allows researchers to create Kaplan-Meier survival plots, perform univariate and multivariate analyses, and identify genes or markers that correlate with disease prognosis. KM Plotter enables users to analyze large cohorts of cancer patients from several public databases, including TCGA and GEO, and investigate the impact of specific genes on patient outcomes. The interface offers various customization options and statistical tools to facilitate data exploration and interpretation^[18]. The outcomes of KM analysis include RFS and OS duration (in weeks) with auto-selected cutoff criteria, *P*-value, and hazard ratios. A *P*-value < 0.05 was considered statistically significant.

2.3. MEXPRESS

MEXPRESS^[19] was utilized in this study to evaluate the Pearson correlation between GAPDH expression and its promoter methylation levels in different cancers. This database provides researchers with multiple analytical methods to interpret RNA sequencing data and identify relevant gene expression profiles. MEXPRESS allows users to explore gene expression data related to sample types, clinical parameters, and cancer subtypes. Users can also access gene-level data, visualizations, and data exports. MEXPRESS is particularly helpful for identifying candidates for new treatments and biomarkers. This platform is free and open to the public, making it an accessible resource for cancer research. MEXPRESS offers a unique combination of user-friendliness, versatility, and reliability, helping researchers gain insights into cancer biology in a straightforward manner^[19]. A *P*-value < 0.05 was considered significant.

2.4. cBioportal

In this study, genetic alterations and copy number variations (CNVs) in GAPDH, as well as their correlation with GAPDH expression levels in distinct cancer subtypes, were evaluated using the cBioPortal database^[20]. cBioPortal is a comprehensive online platform for exploring multi-omics cancer data. It integrates genomic data from public cancer datasets with analysis tools to help researchers gain insights into the molecular mechanisms underlying cancer development and progression. This database provides access to multiple types of data such as

gene expression, mutations, CNVs, protein expression, and clinical information for thousands of cancer patients across numerous cancer types. Researchers can also use a range of algorithms and visualizations to find and investigate potential cancer drivers, mutations, and clinical associations ^[20].

2.5. Co-expressed genes, PPI network, and pathway analysis

The GEPIA database ^[21] was used to identify co-expressed genes with GAPDH. The STRING database ^[22] was utilized to construct a protein-protein interaction (PPI) network for GAPDH using default settings. Subsequently, the PPI network was visualized using Cytoscape software version 3.8.2 ^[23]. Additionally, pathway analysis of the GAPDH-enriched genes was carried out using the online tool DAVID ^[24]. DAVID is a bioinformatics software program used by researchers to identify the biological mechanisms and pathways involved in a set of genes or proteins. The tool offers a range of analytical methods, including functional annotation, gene ontology analysis, pathway analysis, and clustering analysis. Users can upload their own gene lists or use pre-existing ones from publicly available datasets. DAVID bridges the gap between raw data and biological understanding by providing a comprehensive analysis of gene expression data. A *P*-value < 0.05 was considered significant.

2.6. GAPDH and CD8⁺ T cell infiltration levels

The Spearman correlation between GAPDH expression and CD8⁺ T immune markers in BLCA, CESC, HNSC, KIRP, LIHC, and LUAD patients was analyzed using the user-friendly resource, TIMER ^[25]. The TIMER database includes data on 108 cancer types from the TCGA project. TIMER2 offers several functionalities, including differential gene expression analysis, survival analysis, and gene correlations. The platform provides insights into the immune microenvironment of tumors and can aid in the development of immunotherapy strategies. TIMER uses a deconvolution algorithm that integrates multiple immune cell-specific markers to estimate the abundance of immune cells ^[25]. A *P*-value < 0.05 was considered significant.

2.7. Screening of GAPDH regulatory drugs

The CTD database ^[26] was used to identify GAPDH regulatory drugs, including both positive and negative regulators. The CTD is a valuable resource in the fields of toxicology and genomics. It is a comprehensive and curated database that integrates information on gene-disease associations, chemical-gene interactions, and environmental factors related to toxicology. The CTD database plays a crucial role in understanding complex interactions among genes, chemicals, and diseases, ultimately contributing to the development of safer and more effective strategies for risk assessment and environmental health protection. The identified drugs were later visualized using Cytoscape version 3.8.2.

3. Results

3.1. Expression level analysis of GAPDH

The UALCAN platform was used to analyze the TCGA expression profile of GAPDH in tumor samples and their corresponding normal tissues. This analysis aimed to identify any differences in GAPDH expression between tumor and normal tissues ^[17]. The results revealed statistically significant overexpression of GAPDH (*P* < 0.05) in various human cancer samples, including BLCA, CESC, HNSC, KIRP, LIHC, and LUAD when compared to normal controls (**Figure 1**).

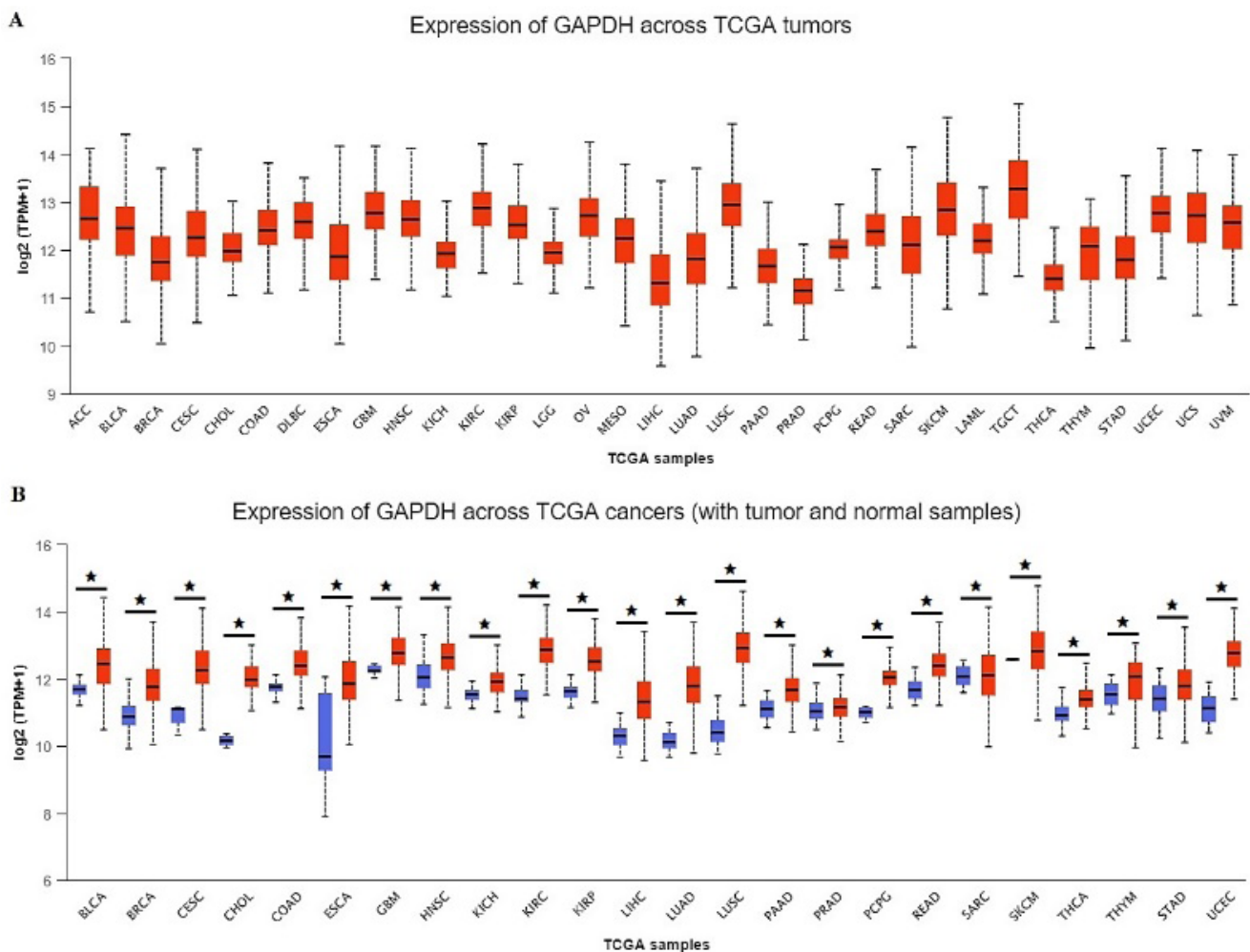


Figure 1. Profile of GAPDH expression across 24 different human cancers. **(A)** Expression profile of GAPDH in cancer samples exclusively. **(B)** Expression profile of GAPDH in both cancer samples and normal controls. A P -value < 0.05 indicates significance.

3.2. GAPDH prognostic potential

Prognostic analysis of GAPDH in 24 cancer types was conducted using the KM Plotter online tool. This analysis aimed to assess the relationship between GAPDH expression and prognosis, in terms of RFS and OS, across 24 cancer types. It was observed that overexpressed GAPDH was significantly associated with decreased RFS and OS duration in BLCA (HR = 1.41, 95% CI: 1.05–1.9, P = 0.02; HR = 1.93, 95% CI: 1.16–3.19, P = 0.0095), CESC (HR = 1.97, 95% CI: 1.24–3.15, P = 0.0035; HR = 1.81, 95% CI: 0.83–3.95, P = 0.013), HNSC (HR = 1.63, 95% CI: 1.17–2.29, P = 0.0038; HR = 1.54, 95% CI: 0.73–3.29, P = 0.026), KIRP (HR = 3.44, 95% CI: 2.01–6.6, P = 5.2×10^{-6} ; HR = 1.84, 95% CI: 0.82–4.14, P = 0.013), LIHC (HR = 2.43, 95% CI: 1.69–3.51, P = 8.7×10^{-7} ; HR = 1.75, 95% CI: 1.25–2.46, P = 0.001), and LUAD (HR = 1.92, 95% CI: 1.43–2.58, P = 1.2×10^{-5} ; HR = 1.55, 95% CI: 0.96–2.49, P = 0.0069) patients (**Figure 2**). However, in other cancer types, elevated GAPDH expression did not show a significant correlation with adverse outcomes in terms of RFS and OS. Collectively, these findings suggest that increased GAPDH expression is specifically linked to reduced RFS and OS in BLCA, CESC, HNSC, KIRP, LIHC, and LUAD cancer types. Therefore, the next part of this study will focus on the unique role of GAPDH in these six types of human cancers.

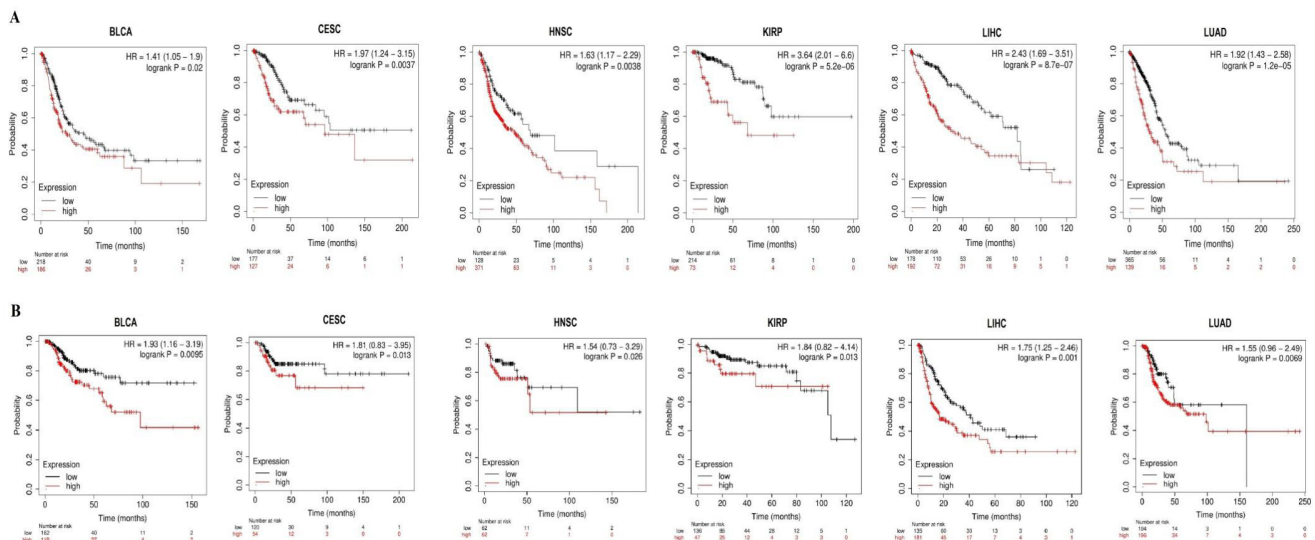


Figure 2. Elevated GAPDH linked to adverse RFS and OS in various cancer subtypes patients. **(A)** Association of GAPDH with RFS in BLCA, CESC, HNSC, KIRP, LIHC, and LUAD. **(B)** Association of GAPDH with OS in BLCA, CESC, HNSC, KIRP, LIHC, and LUAD. A P -value < 0.05 indicates significance.

3.3. Association between GAPDH expression and clinicopathological characteristics

An analysis of GAPDH expression across different clinicopathological features—including cancer stages, patient races, and nodal metastasis statuses—in BLCA, CESC, HNSC, KIRP, LIHC, and LUAD patients revealed significant up-regulation of GAPDH ($P < 0.05$) relative to normal controls (**Figures 3–5**). The Student's t -test was used to compare GAPDH expression between groups, with a P -value < 0.05 indicating statistical significance.

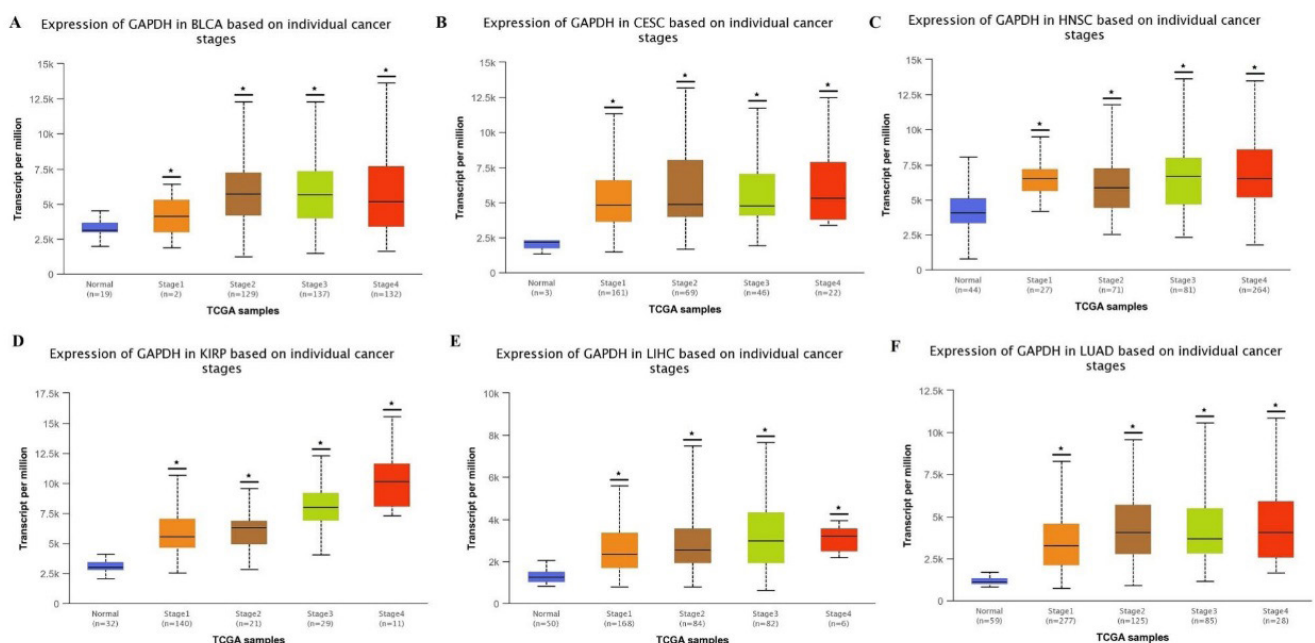


Figure 3. Cancer stage-specific expression patterns of GAPDH in various human cancers. **(A)** BLCA, **(B)** CESC, **(C)** HNSC, **(D)** KIRP, **(E)** LIHC, and **(F)** LUAD. A P -value < 0.05 was deemed statistically significant.

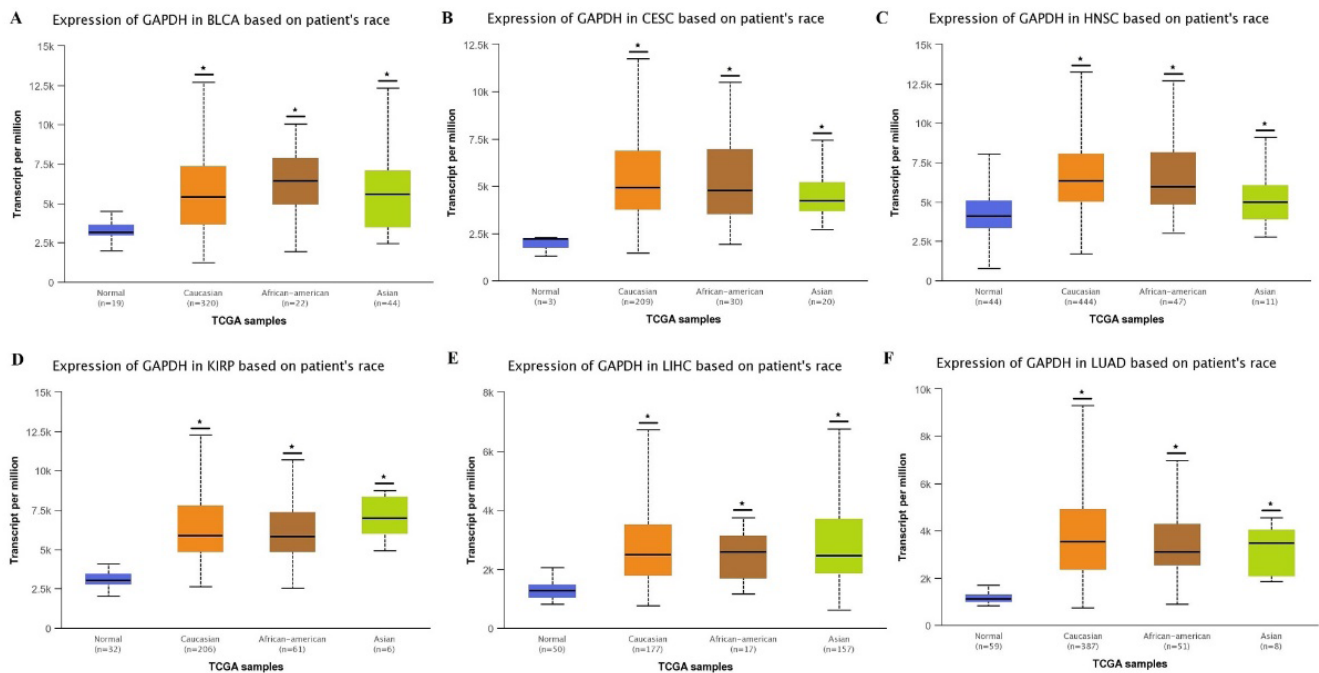


Figure 4. Race-specific expression patterns of GAPDH in various human cancers. (A) BLCA, (B) CESC, (C) HNSC, (D) KIRP, (E) LIHC, and (F) LUAD. A P -value < 0.05 was considered indicative of statistically significant results.

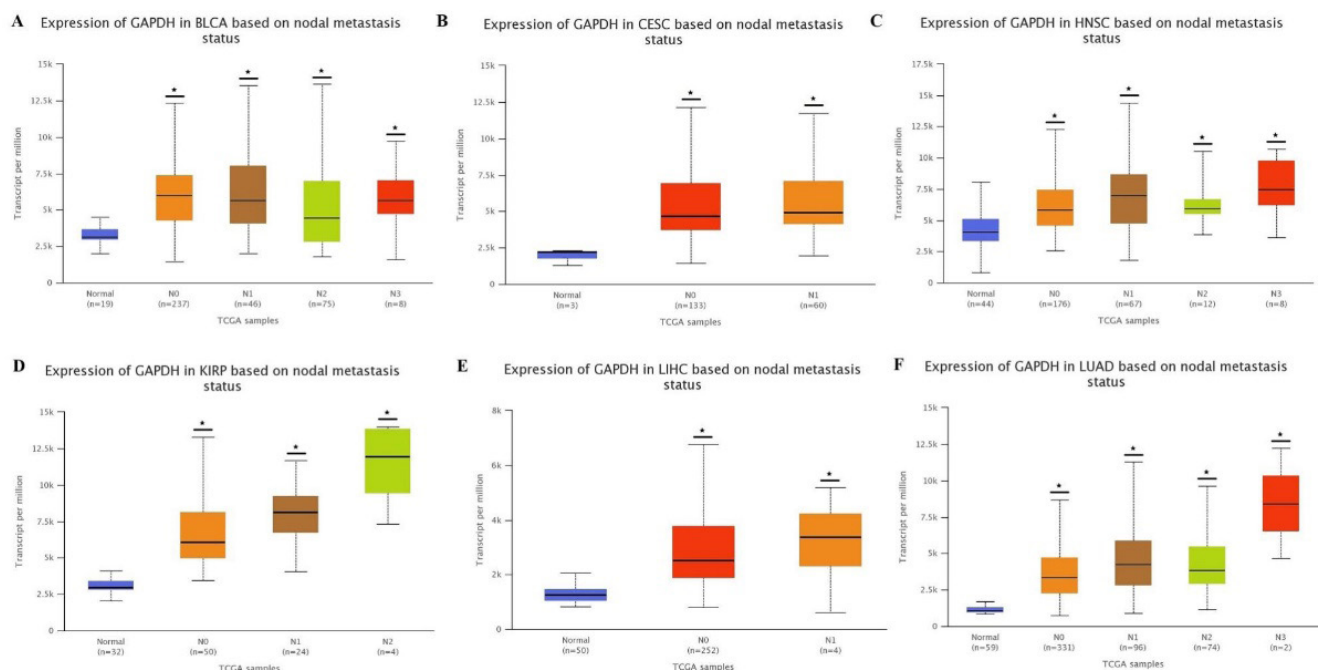


Figure 5. GAPDH expression profiles specific to nodal metastasis in various human cancers. (A) BLCA, (B) CESC, (C) HNSC, (D) KIRP, (E) LIHC, and (F) LUAD. A P -value < 0.05 was considered statistically significant.

Based on the observed trends in GAPDH expression across various cancer stages, it is evident that GAPDH expression is associated with distinct patterns in different cancer types. For instance, in BLCA, GAPDH expression significantly increases in stages 2 and 3, suggesting a potential role in disease progression during these stages. In CESC, GAPDH expression reaches its highest levels in stage 4, possibly indicating involvement in advanced disease states. Conversely, in HNSC, GAPDH expression is more pronounced in stage

1, possibly reflecting its role in early-stage cancer. In KIRP, GAPDH expression notably increases in stage 4, suggesting its significance in late-stage disease. In LIHC, GAPDH expression is most pronounced in stage 4, possibly indicating a role in advanced liver cancer. Finally, in LUAD, GAPDH expression is higher in stage 2, suggesting involvement in this particular stage of lung cancer. These findings underscore the complexity of GAPDH expression regulation across different cancer stages. The variations in GAPDH expression patterns may reflect its multifaceted roles in cellular processes and highlight its potential as a marker for specific stages of cancer.

Regarding GAPDH expression in patients of different racial backgrounds, the following patterns emerged: In BLCA patients, African-American individuals exhibited higher GAPDH expression levels compared to their Caucasian and Asian counterparts. Among CESC patients, GAPDH expression was elevated in Caucasian patients relative to African-American and Asian patients. Similarly, in HNSC patients, GAPDH expression was higher in Caucasian individuals compared to African-American and Asian patients. In KIRP patients, Asian individuals displayed higher GAPDH expression compared to African-American and Caucasian patients. In LIHC patients, African-American patients demonstrated higher GAPDH expression levels than Asian and Caucasian patients. Lastly, in LUAD patients, GAPDH expression was higher in Caucasian patients compared to Asian and African-American patients (**Figure 4**).

Concerning GAPDH expression in cancer patients with varying nodal metastasis statuses, distinct trends emerged across different cancer types: In BLCA patients, those with N0 status exhibited higher GAPDH expression levels compared to those with N1–N3 status. In CESC patients, those with N1 status displayed elevated GAPDH expression in comparison to those with N0 status. Among HNSC patients, those with N3 status demonstrated higher GAPDH expression levels than those with N0–N2 status. In KIRP patients, individuals with N2 status showed increased GAPDH expression relative to those with N0 and N1 statuses. In LIHC patients, those with N1 status exhibited higher GAPDH expression compared to those with N0 status. Finally, in LUAD patients, those with N4 status displayed elevated GAPDH expression compared to those with N0–N3 statuses (**Figure 5**).

Elevated GAPDH expression points to a metabolic shift known as the Warburg effect, commonly observed in cancer cells, where glycolysis is enhanced for rapid energy production and cell proliferation. This phenomenon not only signifies a potential diagnostic tool for early cancer detection but also underscores the aggressive nature of these cancers. Furthermore, these findings may pave the way for innovative therapeutic strategies targeting the glycolytic pathway, including GAPDH, to disrupt cancer cell metabolism and improve treatment outcomes.

3.4. Promoter methylation

The MEXPRESS database was utilized to examine the correlation between GAPDH promoter methylation and its expression in BLCA, CESC, HNSC, KIRP, LIHC, and LUAD. The analysis revealed a significant ($P < 0.05$) negative correlation between GAPDH promoter methylation levels and its expression in these cancer types (**Figure 6**).

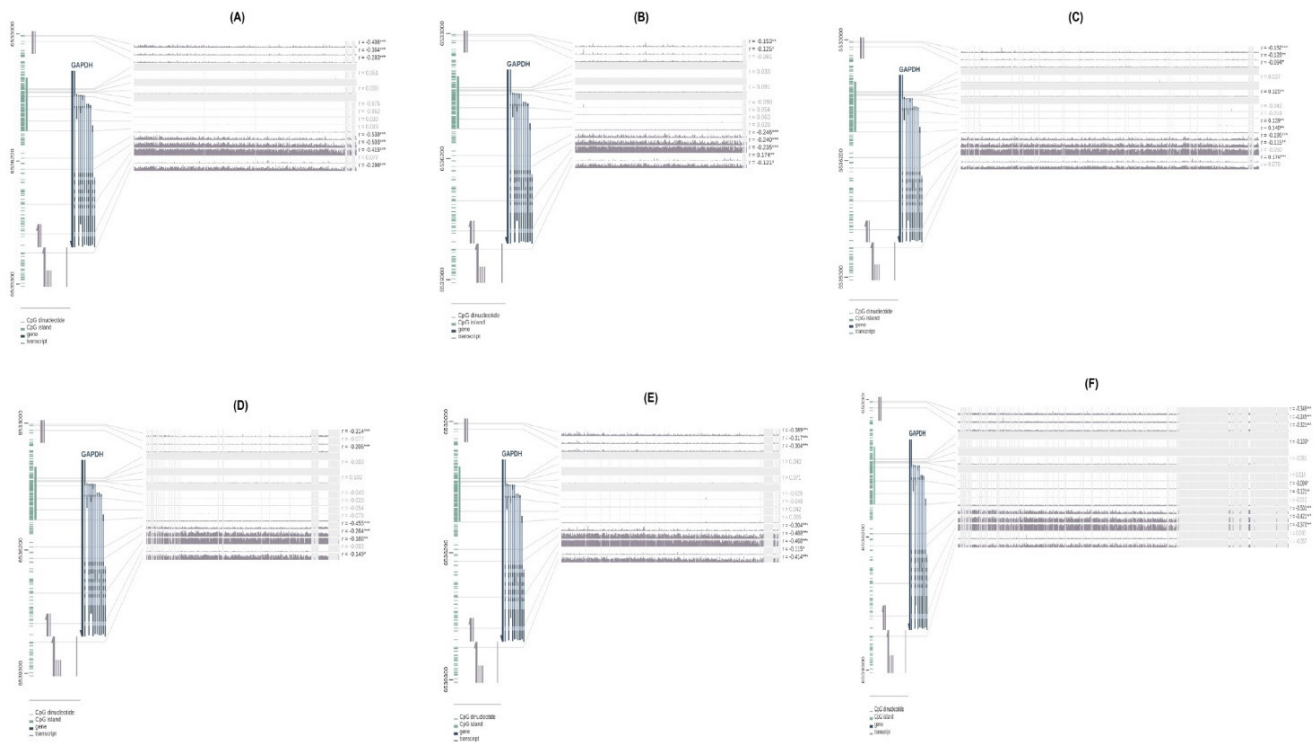


Figure 6. A correlation analysis between GAPDH expression and its promoter methylation level in different cancer subtypes via MEXPRESS. (A) In BLCA, (B) In CESC, (C) In HNSC, (D) In KIRP, (E) In LIHC, and (F) In LUAD. A P -value < 0.05 was considered to indicate the statistically significant results.

3.5. Genomic analysis

Genetic alterations in GAPDH within BLCA, CESC, HNSC, KIRP, LIHC, and LUAD were analyzed using various TCGA datasets, including “Bladder Urothelial Carcinoma (TCGA, Firehose Legacy, consisting of 413 cancerous samples), Cervical Squamous Cell Carcinoma (TCGA, PanCancer Atlas, consisting of 297 cancerous samples), Head and Neck Squamous Cell Carcinoma (TCGA, Firehose Legacy, consisting of 530 cancerous samples), Kidney Renal Clear Cell Carcinoma (TCGA, Firehose Legacy, consisting of 538 cancerous samples), Liver Hepatocellular Carcinoma (TCGA, Firehose Legacy, consisting of 379 cancerous samples), and Lung Adenocarcinoma (TCGA, Firehose Legacy, consisting of 586 cancerous samples).” The results revealed that GAPDH harbors genetic alterations in 2.4% of BLCA cases, with deep amplification being the most common alteration; 1.4% of CESC cases, with missense mutations being the most common; 2.4% of HNSC cases, with deep amplification; 0.5% of KIRP cases, with deep amplification; 0.4% of LIHC cases, with missense mutations; and 4% of LUAD cases, with deep deletions (**Figure 7A–F**). Moreover, GAPDH expression was assessed in two distinct groups: one comprising BLCA, CESC, HNSC, KIRP, LIHC, and LUAD samples without mutations in GAPDH, and the other consisting of samples from the same cancer types but with mutations in GAPDH. The results indicated no significant disparity in overall gene expression, including GAPDH expression, between these two groups (**Figure 7G**). These findings suggest that mutations in GAPDH do not influence its expression in the studied cancers.

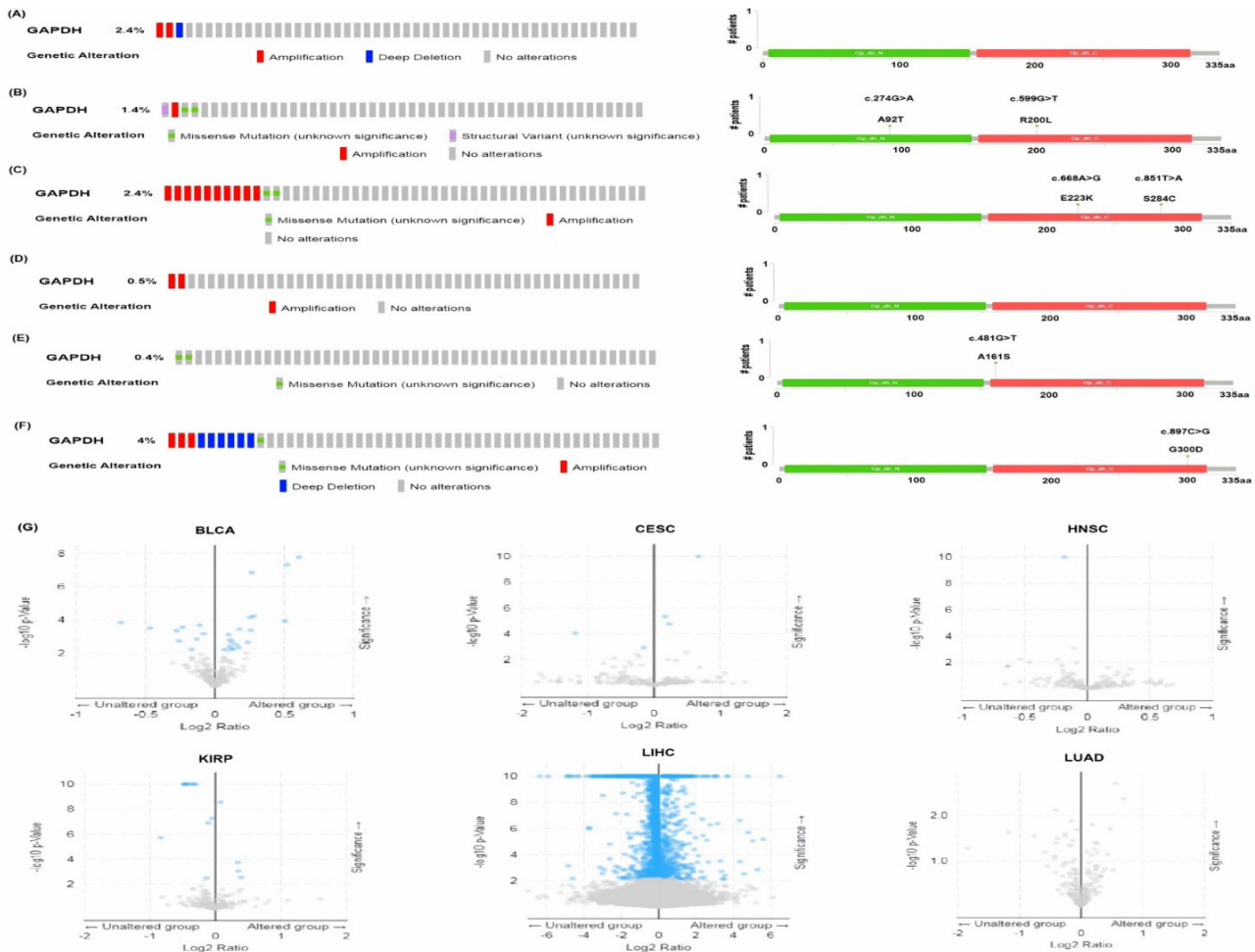


Figure 7. Analysis of genetic alterations in GAPDH across different TCGA datasets. (A) BLCA dataset, (B) CESC dataset, (C) HNSC dataset, (D) KIRP dataset, (E) LIHC dataset, and (F) LUAD dataset.

3.6. Co-expressed genes, PPI network, and pathway analysis

Firstly, GEPIA was used to identify GAPDH co-expressed genes in BLCA, CESC, HNSC, KIRP, LIHC, and LUAD. The analysis showed that TPI1, GAPDHP1, PHB2, ALDOA, YBX3, ENO1, PKM, MRPL51, USP5, and NCAPD2 are the top nine co-expressed genes with GAPDH (**Figure 8A**). These GAPDH-enriched genes were further processed for pathway analysis using the DAVID tool. The results revealed that GAPDH-enriched genes are significantly involved in various pathways, including “Fructose and Mannose Metabolism,” “Glycolysis/Gluconeogenesis,” “Biosynthesis of Amino Acids,” and “Carbon Metabolism” (**Figure 8B**).

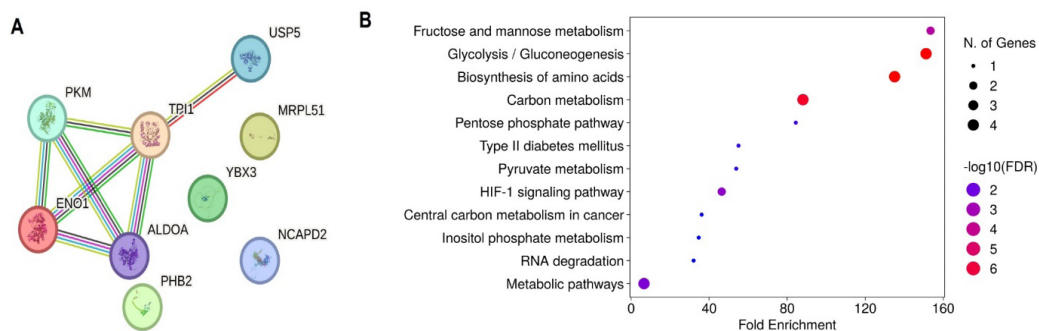


Figure 8. Construction of a protein-protein interaction (PPI) network and pathway analysis of genes enriched with GAPDH. (A) PPI network displaying the interactions among genes enriched with GAPDH, and (B) Pathway analysis illustrating the functional pathways associated with genes enriched with GAPDH.

3.7. GAPDH and infiltration level of CD8⁺ T cells

CD8⁺ T immune cells play a crucial role in the success of current cancer immunotherapies [29]. Therefore, maintaining an appropriate level of CD8⁺ T immune cells in cancer tissues is of utmost importance. In this study, TIMER was used to calculate the Spearman correlation between GAPDH expression and CD8⁺ T cell levels. The results demonstrated a significant ($P < 0.05$) positive correlation between GAPDH expression and CD8⁺ T cell levels in BLCA, while a significant ($P < 0.05$) negative correlation was observed in CESC, HNSC, KIRP, LIHC, and LUAD (Figure 9).

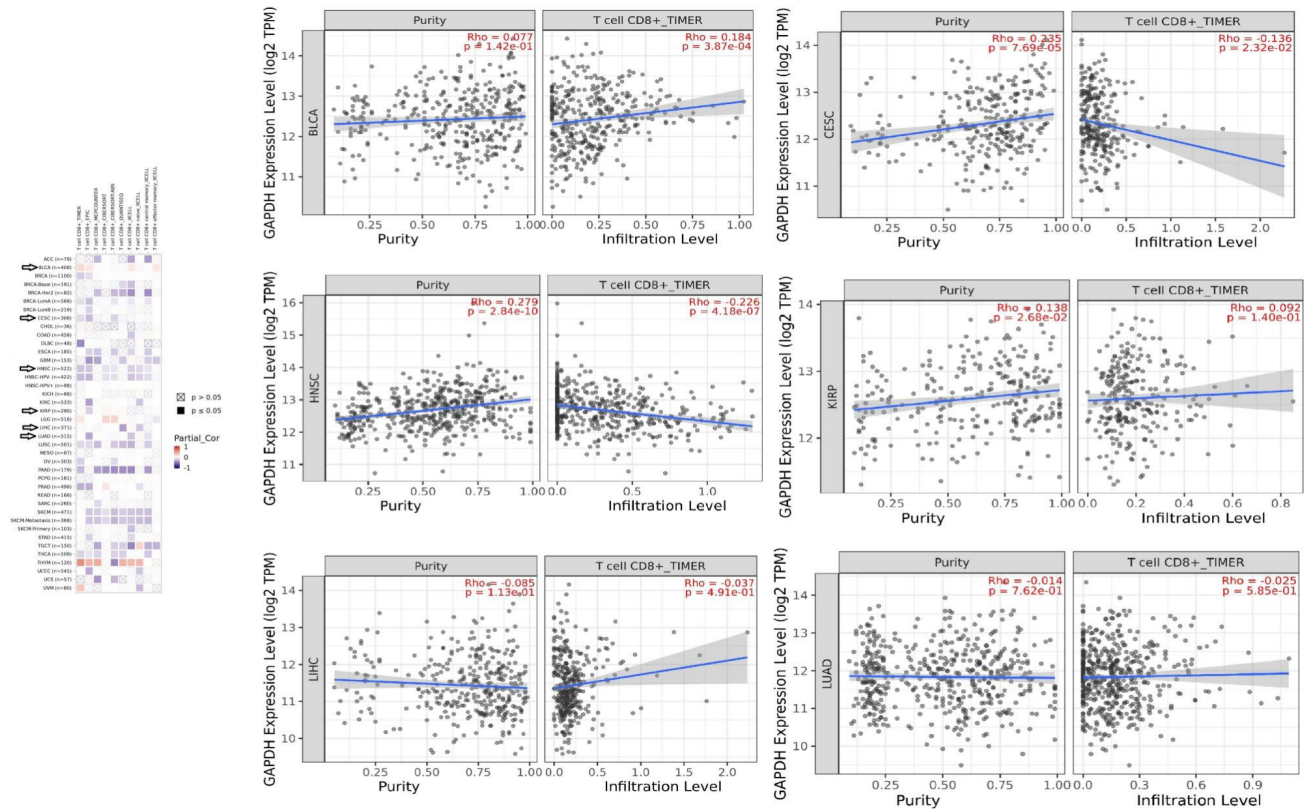


Figure 9. Correlational analysis of GAPDH expression and levels of CD8⁺ T cells in BLCA, CESC, HNSC, KIRP, LIHC, and LUAD using TIMER. A P -value < 0.05 was considered indicative of statistically significant results.

3.8. GAPDH-associated drugs

A gene-drug interaction network analysis was conducted to identify drugs associated with GAPDH using the CTD database. The analysis revealed several drugs that may regulate GAPDH expression. For instance, bisphenol A and tretinoin were found to increase GAPDH expression levels, while ethinyl estradiol and carbon tetrachloride were associated with a decrease in GAPDH expression levels (Figure 10). These findings suggest that these drugs may impact GAPDH expression and provide insights into potential therapeutic interventions.

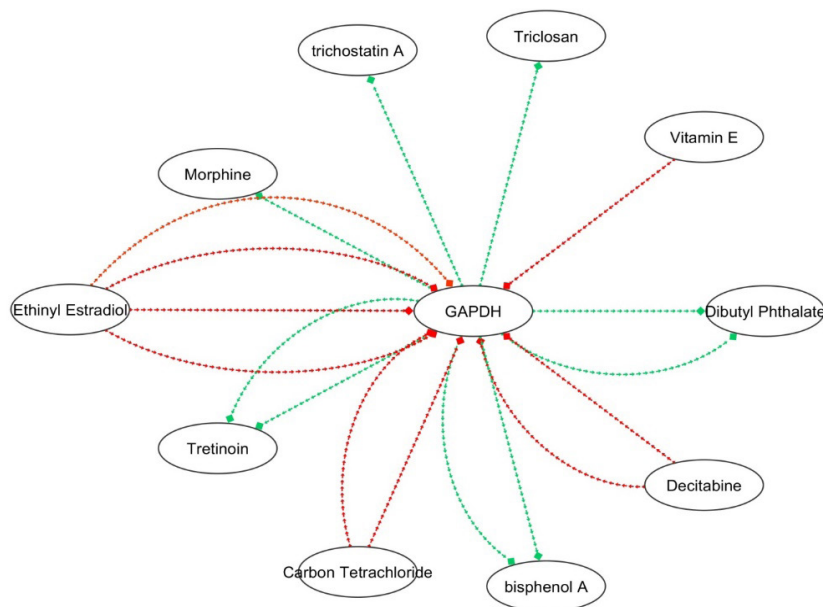


Figure 10. Network of drugs targeting GAPDH. The red color indicates drugs that can increase GAPDH expression, and the green arrows represent drugs that can decrease GAPDH expression. The numbers of arrows denote the reference count of selected drugs.

4. Discussion

Under normal physiological conditions, healthy cells primarily rely on oxidative phosphorylation, a more energy-efficient process, to generate adenosine triphosphate (ATP) ^[30]. However, in stark contrast, cancer cells often exhibit a metabolic shift known as the Warburg effect. This effect refers to the active utilization of glycolysis—the breakdown of glucose—for ATP production, even in the presence of sufficient oxygen. Despite the availability of oxygen, cancer cells demonstrate a preference for glycolysis, leading to increased glucose uptake and lactate production ^[30]. The Warburg effect is a hallmark of many cancer types and provides cancer cells with metabolic advantages, such as increased biosynthetic precursors and reduced dependence on oxygen for energy production. Understanding the mechanisms behind the Warburg effect is crucial for developing targeted therapeutic approaches that exploit the metabolic vulnerabilities of cancer cells while sparing normal cells ^[30].

GAPDH, a glycolytic enzyme, specifically catalyzes the conversion of glyceraldehyde-3-phosphate (G-3-P) to 1,3-diphosphoglycerate ^[31]. Additionally, GAPDH contributes to numerous other cellular functions, such as the export of nuclear tRNA, DNA repair, DNA replication, exocytosis, endocytosis, cytoskeletal organization, carcinogenesis, and cell death ^[32,33]. Although GAPDH is commonly used as an internal control, its expression variations have also been documented in various human cell lines ^[34]. Remarkably decreased expression of GAPDH has been observed in breast cancer, glioma, prostate cancer, liver cancer, colorectal cancer, pancreatic cancer, gastric cancer, melanoma, and bladder cancer ^[35]. Conversely, an increased level of GAPDH has been confirmed as a pro-apoptotic agent by Nakajima *et al.* ^[36]. Such variation in the expression of GAPDH across different cancer subtypes suggests its inconsistent role in determining cell fate ^[35].

To the best of our knowledge, no previous study has investigated the expression profile of GAPDH in different human cancer subtypes and its correlation with various clinicopathological features such as RFS, OS,

promoter methylation status, genetic alterations, CNVs, and CD8⁺ T cell levels. Hence, this study aimed to examine the expression pattern of GAPDH across 24 types of human cancers and its association with diverse parameters, including RFS, OS, promoter methylation status, genetic alterations, CNVs, and CD8⁺ T cell levels. By exploring these relationships, this study aimed to enhance the understanding of the potential role of GAPDH in cancer development and its significance as a biomarker in different cancer types.

GAPDH was observed to be upregulated in 24 major human cancers and its overexpression was significantly associated with decreased RFS and OS in patients with BLCA, CESC, HNSC, KIRP, LIHC, and LUAD. These findings suggest a crucial role for GAPDH in the development of these specific cancer subtypes. Therefore, this study focused primarily on BLCA, CESC, HNSC, KIRP, LIHC, and LUAD. Furthermore, GAPDH was found to be significantly overexpressed ($P < 0.05$) in patients with these cancers across different clinicopathological features, including cancer stages, patient races, and nodal metastasis status, compared to normal controls. The increased expression of GAPDH throughout all stages of these cancers suggests its involvement not only in glycolysis-related processes but also in non-glycolytic mechanisms during tumor development. Moreover, the observed elevated expression of GAPDH in patients of different races with BLCA, CESC, HNSC, KIRP, LIHC, and LUAD highlights the potential for race-independent treatment strategies in these patient populations. Additionally, the increased GAPDH expression in patients with different nodal metastasis statuses implies that GAPDH may also affect the prognosis of these patients.

To investigate the factors contributing to the overexpression of GAPDH in BLCA, CESC, HNSC, KIRP, LIHC, and LUAD, a correlation analysis was performed using the MEXPRESS database to examine the relationship between GAPDH expression and its promoter methylation levels. The results revealed a significant negative correlation between GAPDH expression and promoter methylation levels, suggesting that hypomethylation may significantly influence GAPDH expression in these cancers. However, further experimental studies on a larger scale are necessary to validate and expand upon these findings.

Several biomarkers have been identified for the diagnosis and prognosis of BLCA, CESC, HNSC, KIRP, LIHC, and LUAD. For example, Brisuda *et al.* recently identified circulating tumor DNA (ctDNA) as a novel biomarker for BLCA [37]. Berger *et al.* identified various mutated genes as novel biomarkers for CESC by analyzing Gene GEO datasets [38]. Song *et al.* developed a long noncoding RNA-microRNA-mRNA network in CESC using GEO datasets, providing novel insights into CESC biology [39]. Sawyers *et al.* published a review article highlighting the role of novel HNSC molecular biomarkers, including EGFR, CCND1, Bcl-2, Kip1, VEGF, and p53 [40]. Similarly, various KIRP-related diagnostic and prognostic biomarkers have been identified, including VHL [41], VEGF [42], CAIX [43], and HIF-1 α /2 α [44]. Furthermore, the diagnostic and prognostic potential of different genes, including TTF-1, p63, CK5/6, napsin-A, SPATS2, and ST6GALNAC1, has been well-established in LUAD by previous studies [45]. However, there is a lack of generalization of any biomarkers in patients with BLCA, CESC, HNSC, KIRP, LIHC, and LUAD with diverse clinicopathological features. In this study, we observed a significant upregulation of GAPDH expression in these patients compared to the control group. Additionally, our analysis of GAPDH promoter methylation levels, as well as the assessment of RFS and OS, supports its potential as a novel biomarker for BLCA, CESC, HNSC, KIRP, LIHC, and LUAD patients.

CD8⁺ T cells play a pivotal role in the immune response against cancer [46]. This study revealed intriguing correlations between GAPDH expression and CD8⁺ T cell levels in BLCA, CESC, HNSC, KIRP, LIHC, and LUAD. These findings suggest that GAPDH might modulate the immune response and contribute to the development of these cancers.

In this study, pathway analysis of GAPDH-enriched genes revealed their involvement in several KEGG pathways, including “Fructose and Mannose Metabolism,” “Glycolysis/Gluconeogenesis,” “Biosynthesis of

Amino Acids,” and “Carbon Metabolism,” among others. Additionally, a few potential drugs that could help prevent BLCA, CESC, HNSC, KIRP, LIHC, and LUAD were identified by controlling GAPDH expression.

5. Conclusion

In summary, this study has identified the diagnostic significance of GAPDH in patients with BLCA, CESC, HNSC, KIRP, LIHC, and LUAD across diverse clinicopathological features. The prognostic value of GAPDH was also assessed, thereby establishing correlations with its expression that could potentially aid in predicting the prognosis of patients with these cancers. However, further experimental investigations are warranted before translating these findings into clinical applications.

Disclosure statement

The author declares no conflict of interest.

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ATM is a Prognostic Biomarker of Survival in Head and Neck Squamous Cell Carcinoma Patients

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Abstract: This review examines the role of *ATM* expression in head and neck squamous cell carcinoma (HNSCC). Analysis revealed significant overexpression of *ATM* in HNSCC cells compared to normal control samples, suggesting its involvement in cancer proliferation. *ATM* expression was notably upregulated across various clinical parameters, including different stages of cancer, racial groups, genders, and age groups, highlighting its role in cancer progression. Validation using the GEPIA2 tool confirmed strong *ATM* expression throughout all four stages of HNSCC, with the highest levels in stage II and the lowest in stage I. Promoter methylation analysis of *ATM* showed distinct patterns across different demographics and cancer stages, reinforcing its significance. The study also explored the relationship between *ATM* expression and patient outcomes using the KM plotter tool, finding that high *ATM* expression was associated with better overall survival (OS), while low *ATM* expression correlated with better disease-free survival (DFS). Genetic mutation analysis via cBioPortal identified minimal *ATM* mutations in HNSCC, including in-frame, splice, truncating, and missense mutations, suggesting their role in *ATM* dysregulation. The STRING tool was used to construct a protein-protein interaction (PPI) network, revealing that the *ATM* gene interacts with ten key genes (*NBN*, *ATR*, *CHEK2*, *MDC1*, *MSH2*, *MSH6*, *MRE11*, *TP53*, *TP53BP1*, *BRCA1*), indicating its involvement in various biological functions. Functional annotation of differentially expressed genes (DEGs) through the DAVID web server revealed their participation in critical biological processes, including double-strand break repair, cellular response to DNA damage, and DNA damage checkpoints. KEGG pathway analysis further linked DEGs to cellular senescence, platinum drug resistance, homologous recombination, p53 signaling, and the cell cycle, underscoring *ATM*'s multifaceted role in HNSCC.

Keywords: Head and neck squamous cell carcinoma; Diagnosis; Treatment; Biomarker

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) poses a significant global health challenge, ranking as the sixth most common cancer worldwide ^[1]. Projections suggest a 30% increase in HNSCC cases by 2030, with an estimated 1.08 million new diagnoses annually ^[2]. HNSCC develops from the mucosal epithelium of the oral cavity, pharynx, and larynx and remains the most common cancer in these regions. Despite substantial efforts, survival rates for HNSCC patients only improved marginally from 55% to 66% between 1992–1996

and 2002–2006 ^[3]. Notably, HNSCC survivors have the second-highest suicide risk among cancer survivors, often due to psychological distress and reduced quality of life ^[4]. In 2020 alone, approximately 0.88 million new cases and 0.44 million deaths were reported globally, making HNSCC the eighth most prevalent cancer ^[5]. Despite comprehensive treatment strategies, including chemotherapy and surgery, the median overall survival (OS) of HNSCC patients remains less than one year, highlighting the urgent need for reliable biomarkers for diagnosis, prognosis, and treatment ^[6]. Previous studies have identified several genes and pathways associated with HNSCC mutation rates, which play critical roles in the disease's pathogenesis, progression, metastasis, and outcomes ^[7]. These statistics underscore the urgent need to identify biomarkers that can improve patient outcomes in HNSCC ^[8]. Biomarkers can guide personalized treatment approaches, such as targeted therapies, enhancing treatment efficacy while minimizing toxicity. Therefore, future research must prioritize the identification and validation of biomarkers to improve HNSCC patient outcomes.

Over the past thirty years, *ATM* (ataxia telangiectasia mutated) has played a central role in advancing our understanding of the mammalian DNA damage response, cancer initiation and progression, and redox signaling pathways. Recent publications have highlighted ATM's diverse roles in cellular processes such as growth, metabolism, energy production, oxidative homeostasis, chromatin remodeling, and genomic integrity, all of which are crucial in cancer development and progression. The *ATM* gene, an onco-suppressor located on chromosome 11q23, encodes a 350-KDa protein composed of 3056 amino acids ^[9]. It belongs to the phosphatidylinositol 3-kinase-related protein kinase (PIKK) superfamily, which includes six serine/threonine kinases with sequence similarities to phosphatidylinositol 3-kinases (PI3Ks), such as ATR (ATM-and RAD3-related), DNA-PKcs (DNA-dependent protein kinase catalytic subunit), and mTOR (mammalian target of rapamycin). ATM is involved in DNA repair and activates DNA damage response pathways ^[10]. HEAT motifs are crucial for ATM's interaction with and recruitment of various proteins to DNA damage sites for repair ^[11-14]. ATM is a key initiator of the DNA damage response in mammalian cells through the Mre11/Rad50/Nbs1 (MRN) complex at DNA lesion sites ^[15-18]. Mutations in the *ATM* gene result in deficiencies in the DNA damage response, leading to ataxia telangiectasia, a rare autosomal recessive disorder with a frequency of 1 in 40,000 to 300,000 in Caucasians ^[19]. ATM's primary cancer-suppressing mechanisms include inducing apoptosis and cell cycle arrest by activating p53, SIRT1, CHK1, CHK2, DBC1, RAIDD, and other downstream targets ^[20]. Consequently, cancer cells can employ various mechanisms to downregulate ATM. For example, in breast cancer cells, miRNA-18a can reduce ATM expression ^[21]. Enhancing ATM signaling and expression may enable cancer cells to resist chemotherapy and radiation, metastasize, and survive ^[22]. Epidemiological studies of families affected by both AT and BC suggest that heterozygous *ATM* mutation carriers have a two- to thirteen-fold increased risk of developing breast cancer, with a higher relative risk under 50 years of age ^[23-26].

In this review, we explore *ATM* mutations, expression levels, prognostic outcomes on survival, and functional perspectives within the context of HNSC through bioinformatics analysis. We also examine the relationship between *ATM* expression and promoter methylation levels. To achieve this, we utilized various databases, including The Cancer Genome Atlas (TCGA) dataset, the UALCAN portal, the Kaplan-Meier tool, Gene Expression Profiling Interactive Analysis (GEPIA2), cBioPortal, STRING for predicting protein-protein interactions (PPI), and the Database for Annotation, Visualization, and Integrated Discovery (DAVID). DAVID provides a comprehensive set of functional annotation tools to understand the biological significance of large gene lists. The primary aim of this study was to assess the *ATM* expression pattern in HNSC and determine its potential importance in cancer development, treatment, and prognosis.

2. Materials and methods

2.1. Expression and promoter methylation analysis of ATM

To investigate *ATM* expression, we utilized the UALCAN online database. UALCAN is a comprehensive, user-friendly, and visually appealing web resource for analyzing cancer genomics data ^[27]. It draws on extensive data from The Cancer Genome Atlas (TCGA) to facilitate in-depth analyses of gene expression, protein abundance, and patient survival across various cancer types. UALCAN's intuitive interface allows researchers to explore and visualize gene expression patterns across different cancer stages, molecular subtypes, and patient demographics. In this study, the UALCAN dataset was used to assess *ATM* expression across different stages of cancer development, where this gene shows significant dysregulation and a strong correlation with poorer overall survival (OS). For the assessment of *ATM* promoter methylation levels in HNSC, we also used the UALCAN dataset. Additionally, promoter methylation data were analyzed across various clinical parameters, including patient age, gender, and race.

2.2. Validation analysis of ATM

GEPIA2 is a widely used online tool for the analysis of gene expression and survival in genomic data ^[28]. With 198,619 isoforms and 84 cancer subtypes, GEPIA2 has extended gene expression quantification from the gene level to the transcript level and supports the examination of specific cancer subtypes and comparisons between them. Since cancers often consist of heterogeneous subtypes with distinct prognoses, GEPIA2 allows users to tailor their investigations to focus on all 84 cancer subtypes and to compare across different subtypes. Moreover, as single-cell sequencing becomes more prevalent, new standards of analysis have emerged. The differences in *ATM* expression and prognosis (OS and DFS) in HNSCC patients were obtained from the GEPIA2 dataset. In this study, GEPIA2 was employed to explore the association between *ATM* expression and prognosis (OS and DFS) in HNSCC patients.

2.3. Survival analysis of ATM

The Kaplan-Meier (KM) plotter is an essential online tool for survival analysis ^[29]. This platform leverages extensive clinical data to examine the impact of specific genes on patient survival across various cancer types. Researchers can easily explore the prognostic value of gene expressions, identifying potential prognostic biomarkers. KM Plotter's user-friendly interface offers Kaplan-Meier survival curves, providing insights into how gene expression correlates with patient outcomes. In this study, the KM plotter tool was used to assess the impact of *ATM* dysregulation on the overall survival (OS) of HNSCC patients.

2.4. Mutational analysis of ATM

cBioPortal is a crucial online platform for cancer genomics analysis ^[30]. It provides an intuitive platform for exploring large-scale cancer genomic datasets, enabling researchers to delve into genetic alterations, pathways, and clinical significance across various cancer types. With user-friendly visualization tools, it simplifies the analysis of complex genomic data, making it accessible to a wide range of researchers. This database was utilized in the present study to perform a mutational analysis of *ATM* in HNSCC.

2.5. PPI development and gene enrichment analysis of ATM

The STRING database is an essential resource for elucidating protein-protein interactions (PPIs) ^[31]. It aggregates a wealth of information to help researchers unravel complex networks of protein interactions. In this study, we used STRING to construct the *ATM* protein interaction network. Among all databases, STRING is well-known for its comprehensive coverage, data abundance, and high-quality control of PPI information ^[32-34].

STRING contains PPIs from both experimental and computational methods and provides a combined quality score for each interaction by integrating data from various sources such as literature and gene expression profiles. For functional annotation of Gene Ontology (GO) terms and analysis of KEGG pathway enrichment, we utilized the online DAVID tool ^[35]. DAVID is a significant resource for the functional evaluation of high-throughput gene expression profiles.

3. Results

3.1. Expression analysis of ATM in HNSCC

To examine *ATM* expression in HNSCC and typical control samples, we used the UALCAN dataset (**Figure 1**). Upon analysis, we found a significant upregulation of *ATM* expression in HNSCC cancer cells compared to normal control samples. This overexpression indicated a strong association between *ATM* expression and the proliferation of HNSCC cancerous cells. This observation suggests that *ATM* may play a crucial role as a regulator of proliferation in HNSCC, highlighting its potential as a therapeutic target or diagnostic marker in this cancer type.

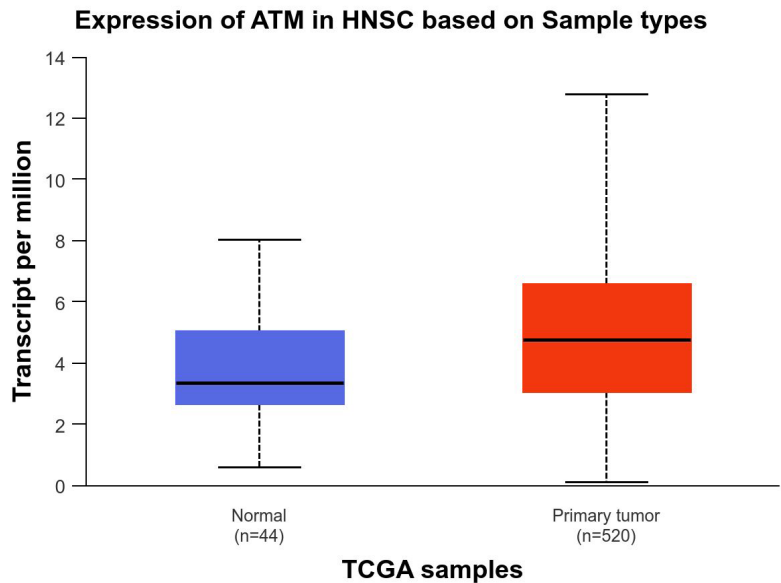


Figure 1. Expression profiling of *ATM* in HNSCC and normal tissue samples

3.2. Expression analysis of ATM in HNSCC stratified by clinical parameters

We further evaluated *ATM* expression in HNSCC samples across various clinical parameters, including individual cancer stages, patient race, gender, and age (**Figure 2**). Initially, we analyzed *ATM* expression across different tumor stages and observed significant overexpression of *ATM* in HNSCC at all stages compared to normal control samples (**Figure 2A**). Next, we investigated *ATM* expression in HNSCC patients across racial groups, revealing significant upregulation of *ATM* expression in Caucasian, African-American, and Asian patients compared to normal control samples (**Figure 2B**). Additionally, we examined *ATM* expression in HNSCC patients stratified by gender, showing notable upregulation of *ATM* in both male and female patients compared to normal controls (**Figure 2C**). Finally, we explored the association between *ATM* expression and patient age in HNSCC, revealing overexpression of *ATM* across different age groups among HNSCC patients (**Figure 2D**). These findings underscore the significance of *ATM* in HNSCC and its potential as a valuable biomarker for diagnosis and therapeutic targeting.

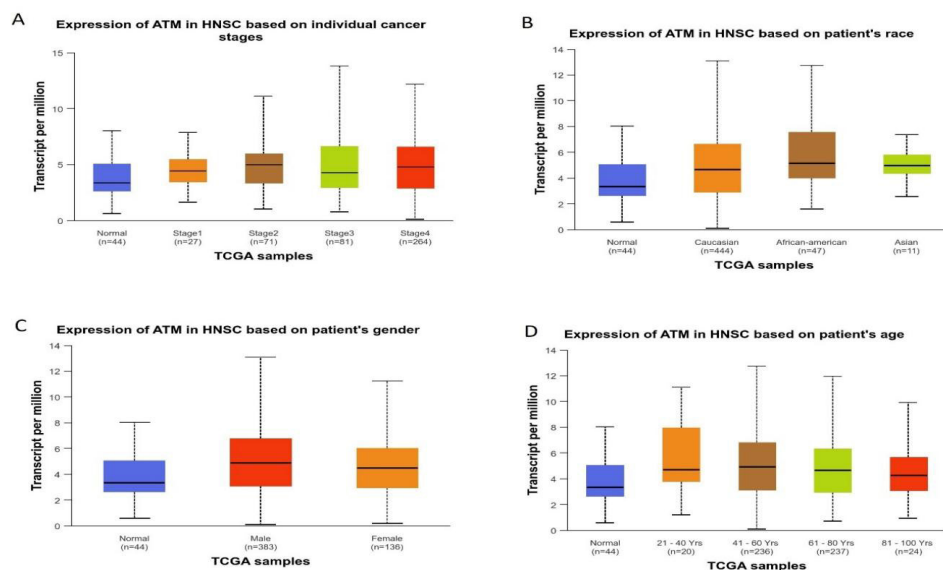


Figure 2. Expression of *ATM* across different clinical parameters

3.3. Prognostic analysis of *ATM* expression in HNSCC

To investigate *ATM* expression between HNSCC cells and normal control samples, we utilized GEPIA2. The results showed that *ATM* expression was significantly higher in HNSCC compared to normal control samples (Figure 3A). We further analyzed the association between *ATM* expression and different cancer stages using the GEPIA2 dataset. The outcomes revealed a strong correlation between *ATM* expression and the stages of HNSCC patients, with the highest expression observed in stage II and the lowest in stage I (Figure 3B).

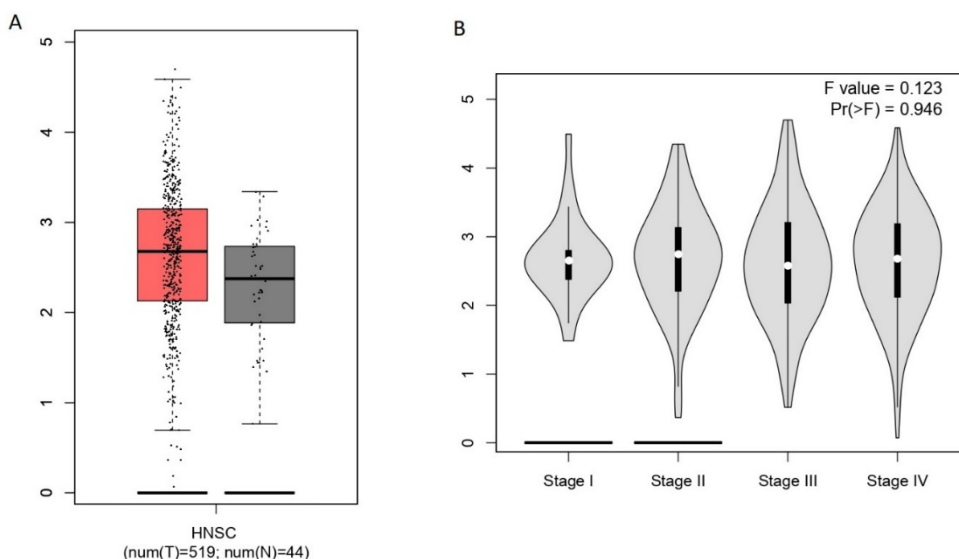


Figure 3. Validation of *ATM* expression across different stages of HNSCC

3.4. Promoter methylation of *ATM* in HNSCC and normal control samples

We conducted an analysis of the promoter methylation levels of *ATM* in HNSCC and normal control samples using the UALCAN online database. Our findings revealed that *ATM* was hypomethylated in HNSCC samples compared to normal control samples (Figure 4). This observation suggests a negative correlation between *ATM*

expression and promoter methylation in HNSCC. Such a correlation highlights the therapeutic potential of *ATM* in the pathogenesis of HNSCC, suggesting its role as a potential target for therapeutic interventions in this cancer type.

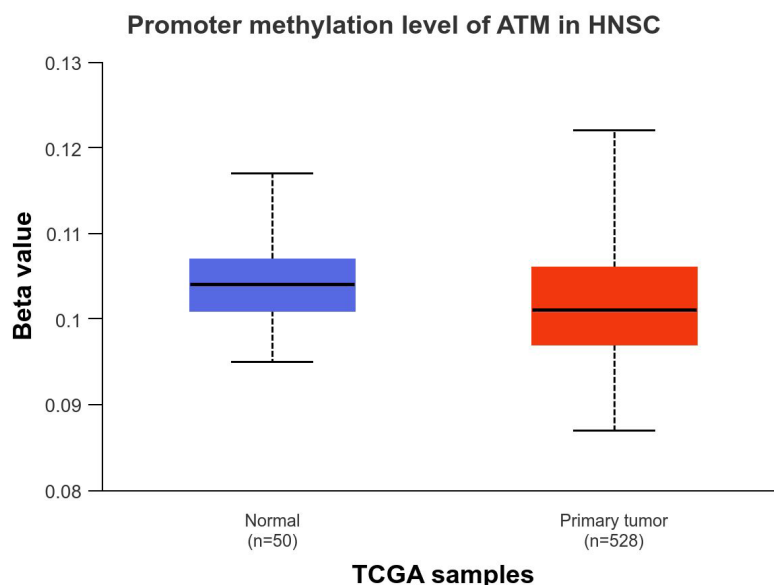


Figure 4. Promoter methylation pattern of *ATM* in HNSCC and normal control samples

3.5. Promoter methylation of *ATM* in HNSCC stratified by clinical parameters

To further elucidate the promoter methylation of *ATM* in HNSCC, we explored different clinical parameters (**Figure 5**). We examined *ATM* promoter methylation across different HNSCC cancer stages compared to normal control samples, revealing significant variations among stages, with all four stages showing prominent hypomethylation compared to normal controls (**Figure 5A**). We also examined *ATM* promoter methylation in HNSCC patients stratified by race, finding hypomethylation in the *ATM* promoter region across all three racial groups—Caucasian, African-American, and Asian—compared to normal controls (**Figure 5B**). Additionally, the assessment of *ATM* promoter methylation by gender revealed hypomethylation in both male and female patients (**Figure 5C**). Finally, we explored *ATM* promoter methylation concerning patient age, revealing varying methylation levels across different age groups (**Figure 5D**). These comprehensive assessments highlight the strong association between *ATM* promoter methylation and various clinical parameters in HNSCC, consistently showing a pattern of hypomethylation in *ATM*, emphasizing its potential significance in the pathogenesis of HNSCC.

3.6. Survival analysis of *ATM*

To further investigate the role of *ATM* gene expression in HNSCC, we conducted an evaluation of overall survival (OS) and disease-free survival (DFS) using the KM plotter tool. The analysis revealed a significant association between *ATM* gene expression and patient survival outcomes in the current study. Specifically, HNSCC patients with high *ATM* expression exhibited favorable OS compared to those with low *ATM* expression levels (**Figure 6A**). Additionally, in DFS analysis, HNSCC patients with low *ATM* expression experienced better DFS compared to those with high *ATM* expression. These findings underscore the critical role of *ATM* in influencing survival outcomes in HNSCC patients, highlighting its potential clinical significance as a prognostic marker in HNSCC management and suggesting its involvement in the progression and development of HNSCC.

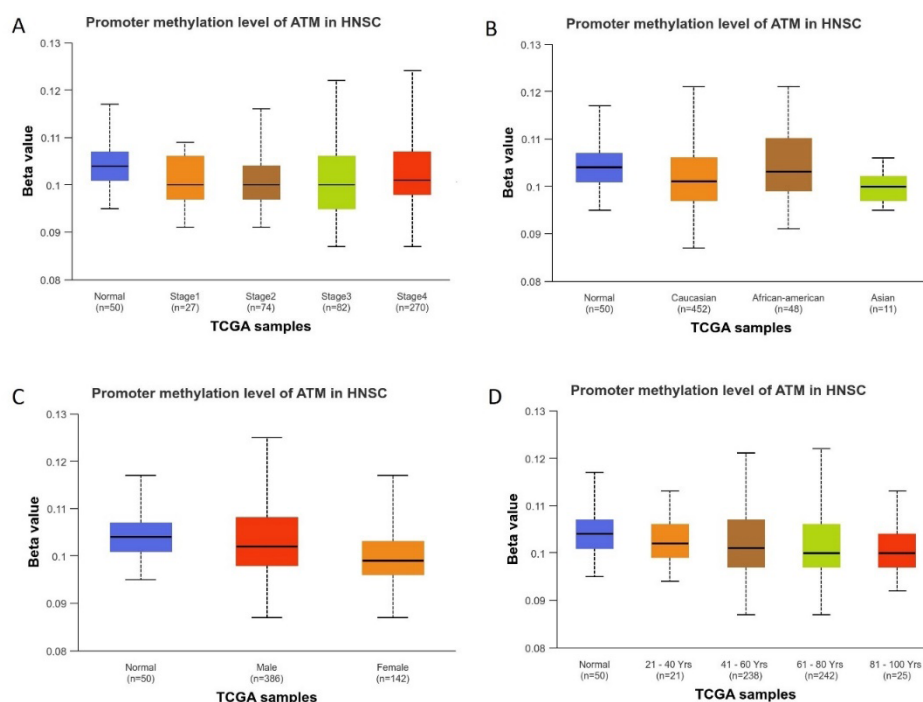


Figure 5. *ATM* promoter methylation pattern across different clinical parameters

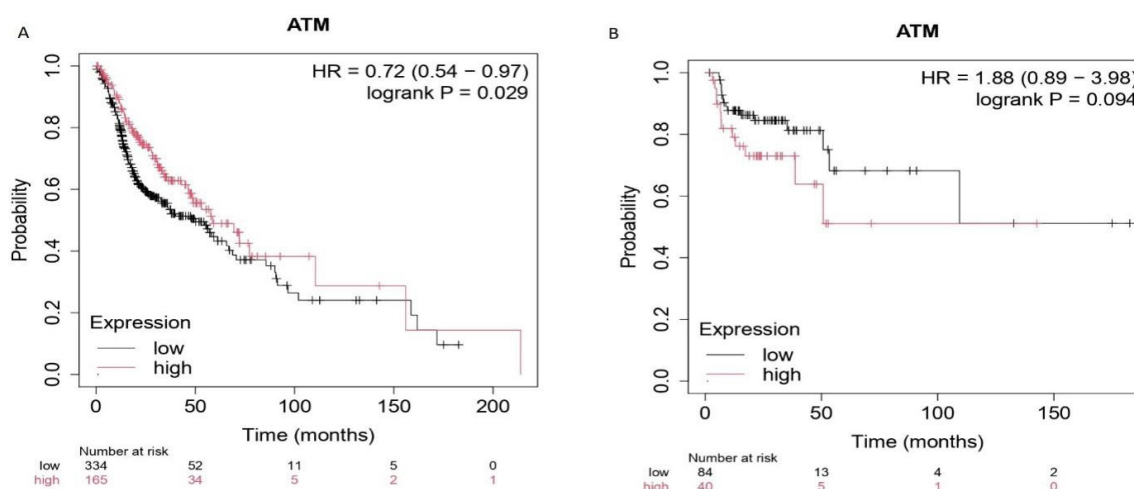


Figure 6. KM survival curve (OS, DFS) of *ATM* in HNSCC patients

3.7. Prognostic analysis of ATM in HNSCC

The GEPIA2 dataset was used to explore the prognostic value of *ATM* expression in HNSCC tumor progression. We stratified HNSCC patients into low and high-expression groups based on *ATM* expression levels. In HNSCC, high *ATM* expression was associated with favorable OS compared to low *ATM* expression (Figure 7A). Furthermore, low *ATM* expression was linked to better DFS in HNSCC compared to the high *ATM* expression group (Figure 7B). These findings suggest the vital role of the *ATM* gene in the progression and development of HNSCC cancer.

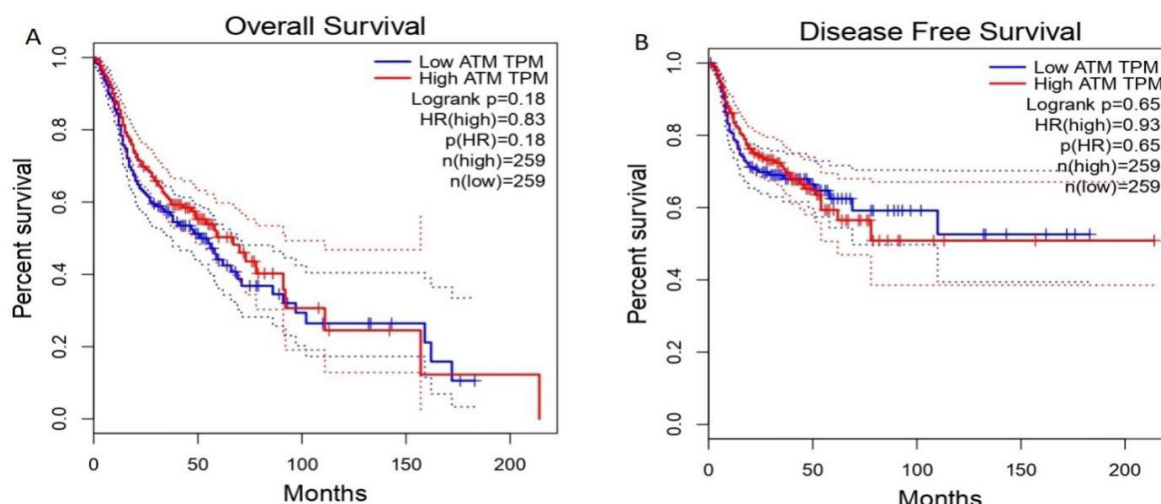


Figure 7. Survival curve (OS, DFS) of *ATM* in HNSCC patients

3.8. Mutational analysis of ATM in HNSCC

We further investigated the genetic mutations of *ATM* in HNSCC patients using cBioPortal. Our findings revealed that only 10% of HNSCC samples exhibited genetic mutations in *ATM*. The mutations identified in HNSCC included in-frame mutations, splice mutations, truncating mutations, and missense mutations (**Figure 8**). These findings suggest that while genetic mutations in *ATM* are relatively rare in HNSCC, the observed mutations may play a crucial role in the dysregulation of *ATM* in this cancer type.



Figure 8. Oncoplot of *ATM* in HNSCC cancer

3.9. Establishment of PPI networks of ATM

The physical and functional relationships among the proteins of differentially expressed genes (DEGs) of *ATM* were analyzed using the STRING tool. The construction of PPI networks showed that the *ATM* hub gene is interconnected with ten genes: *NBN*, *ATR*, *CHEK2*, *MDC1*, *MSH2*, *MSH6*, *MRE11*, *TP53*, *TP53BP1*, and *BRCA1*, highlighting the versatile nature of the *ATM* gene (**Figure 9**). This suggests that *ATM* plays a crucial role in various biological functions and strongly interacts with interconnected genes.

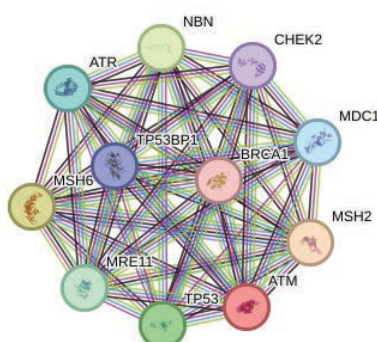


Figure 9. Protein-protein interactions of *ATM*

3.10. DAVID enrichment analysis

For the functional annotation of DEGs, the DAVID online server was utilized. To identify KEGG pathway-enriched genes and potential Gene Ontology (GO) terms related to biological processes, molecular functions, and cellular components, KEGG pathways were analyzed. By analyzing biological processes (BP), we found that DEGs from the complex PPI network were enriched in double-strand break repair (GO:0006302), cellular response to DNA damage stimulus (GO:0006974), DNA damage checkpoint (GO:0000077), replicative senescence (GO:0090399), and intra-S DNA damage checkpoint (GO:0031573) (**Table 1**). The cellular component (CC) analysis revealed that DEGs from the PPI network were enriched in the chromosome, telomeric region (GO:0000781), chromosome (GO:0005694), nucleoplasm (GO:0005654), site of double-strand break (GO:0035861), and PML body (GO:0016605) (**Table 2**). The molecular function (MF) analysis showed that DEGs were involved in damaged DNA binding (GO:0003684), MutLalpha complex binding (GO:0032405), DNA binding (GO:0003677), p53 binding (GO:0002039), and single thymine insertion binding (GO:0032143) (**Table 3**). Additionally, the KEGG pathway analysis revealed the involvement of DEGs in cellular senescence (hsa04218), platinum drug resistance (hsa01524), homologous recombination (hsa03440), p53 signaling pathway (hsa04115), and the cell cycle (hsa04110). The annotated results were tabulated in **Table 4**.

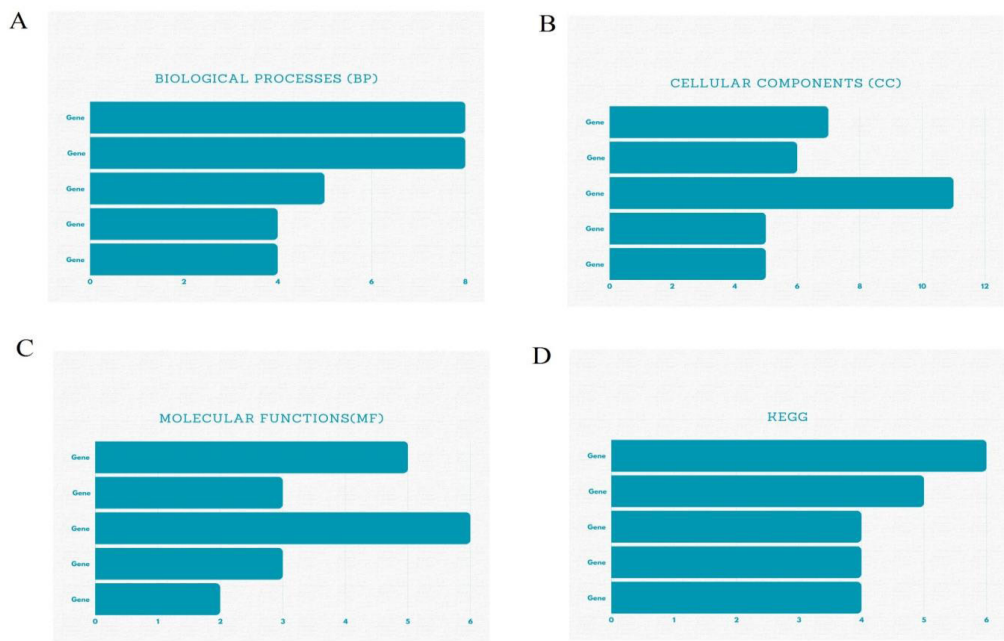


Figure 10. GO and KEGG analysis of ATM by DAVID tool

Table 1. Gene enrichment analysis (BP)

BP			
Gene term	Gene count	Genes	P-value
GO:0006302~double-strand break repair	08	MRE11, MSH2, CHEK2, ATM, BRCA1, NBN, TP53, ATR	1.4670891505118574E-15
GO:0006974~cellular response to DNA damage stimulus	08	MDC1, MRE11, CHEK2, ATM, BRCA1, TP53BP1, TP53, ATR	2.180060502472521E-11
GO:0000077~DNA damage checkpoint	05	CHEK2, ATM, TP53BP1, NBN, ATR	2.9898564405423035E-9
GO:0090399~replicative senescence	04	CHEK2, ATM, TP53, ATR	5.6279469976638965E-8
GO:0031573~intra-S DNA damage checkpoint	04	CHEK2, ATM, TP53, ATR	6.832071558594709E-8

Table 2. Gene enrichment analysis (CC)

CC			
Gene term	Gene count	Genes	P-value
GO:0000781~chromosome, telomeric region	07	MRE11, MSH2, CHEK2, ATM, TP53BP1, NBN, ATR	6.720471932650596E-11
GO:0005694~chromosome	06	MRE11, MSH2, CHEK2, ATM, BRCA1, TP53BP1	7.095074850862256E-8
GO:0005654~nucleoplasm	11	MSH6, MDC1, MRE11, MSH2, CHEK2, ATM, BRCA1, TP53BP1, NBN, TP53, ATR	7.313672569344439E-8
GO:0035861~site of double-strand break	05	MDC1, MRE11, TP53BP1, NBN, TP53	8.129255107445212E-8
GO:0016605~PML body	05	MRE11, CHEK2, NBN, TP53, ATR	1.6009955981982125E-7

Table 3. Gene enrichment analysis (MF)

MF			
Gene term	Gene count	Genes	P-value
GO:0003684~damaged DNA binding	05	MSH6, MSH2, ATR, BRCA1, TP53BP1, TP53	3.371818308101678E-8
GO:0032405~MutLalpha complex binding	03	MSH6, MSH2, ATR	3.7820216211091084E-6
GO:0003677~DNA binding	06	MRE11, MSH2, ATM, BRCA1, TP53, ATR	3.4458833683515824E-4
GO:0002039~p53 binding	03	BRCA1, TP53BP1, TP5	6.151526152403066E-4
GO:0032143~single thymine insertion binding	02	MSH6, MSH2	0.001058901316322019

Table 4. Gene enrichment analysis (KEGG)

KEGG			
Gene term	Gene count	Genes	P-value
hsa04218:Cellular senescence	06	MRE11, CHEK2, ATM, NBN, TP53, ATR	2.112854669096672E-7
hsa01524:Platinum drug resistance	05	MSH6, MSH2, ATM, BRCA1, TP53	5.666650804938227E-7
hsa03440:Homologous recombination	04	MRE11, ATM, BRCA1, NBN	8.107663061909261E-6
hsa04115:p53 signaling pathway	04	CHEK2, ATM, TP53, ATR	4.846197604830809E-5
hsa04110:Cell cycle	04	MRE11, ATM, TP53, ATR	4.52909326389761E-4

4. Discussion

In this review article, we utilized various online bioinformatics tools to assess *ATM* expression, prognosis, methylation, survival, mutations, and gene enrichment in HNSCC. Furthermore, differentially expressed significant data in HNSCC were validated using OS and DFS. The findings demonstrated the crucial impact of *ATM* expression on the human body and suggested a possible association between *ATM* expression and the development of HNSCC, indicating *ATM* expression as a potential regulator in the pathogenesis of HNSCC.

Currently, there is a lack of solid prognostic biomarkers for HNSCC patients' survival. The discovery of these novel biomarkers will be useful for creating innovative, customized therapeutic strategies that meet the emerging needs of precision medicine ^[36,37]. TCGA HNSCC data have recently been utilized to study patterns

from both progressors and non-progressors from a network perspective. HNSCC presents a significant challenge for humanity. Conventional prognostic models based on single clinical parameters have limited predictive power. Integrating bioinformatics and clinical data offers a promising approach to improving prediction accuracy. Indeed, gene signatures predicting the prognosis of HNSCC have been established in previous studies. For instance, an NK cell-related gene signature has been reported to perform well in assessing the prognosis of HNSCC patients ^[38]. An oxidative stress-related gene signature could predict prognosis in HNSCC patients ^[39], and a prognostic signature based on autophagy, apoptosis, and pyroptosis-related genes was constructed ^[40]. However, these current signatures were developed by analyzing a small number of specific genes. As we are probably aware, genes with particular functions (e.g., angiogenesis ^[41], metabolism ^[42], immune escape ^[43]) have recently been implicated in cancer development, rather than a specific group of genes with explicit functions.

Located on chromosome 11q23, the *ATM* gene is a tumor suppressor that produces a 350-kDa protein with 3,056 amino acids. The *ATM* gene, which is generally involved in telomere maintenance, oxidative stress, gene regulation, cell cycle control, and apoptosis, is dysregulated in many cancers, including breast cancer (BC). Numerous *ATM* mutations have been identified and linked to a moderate risk of developing BC ^[44]. Previous research has highlighted the substantial correlation between *ATM* mutations and the likelihood of developing BC. The D1853V, L546, and S707P isoforms are linked to the lowest chance of developing BC, while the V2424G variant carries the highest risk ^[45]. Additionally, the COSMIC database indicates that *ATM* is one of the most aberrant genes in sporadic cancer. Moreover, loss of heterozygosity in the *ATM* region has been found in about 40% of human sporadic BC cases, according to next-generation sequencing (NGS) analysis ^[46,47]. In larger-scale studies including solid cancers, 5% of patients showed *ATM* aberrations (either mutation or deletion). According to the description, 8% of patients with lung cancer had *ATM* mutations, which were mainly mutually exclusive with those of TP53. More recently, it has been discovered that patients with colorectal cancer (CRC) who have both stable and unstable microsatellite tumors also have *ATM* alterations. Targeted next-generation sequencing of prostate cancer has revealed an 8% incidence of *ATM* mutations. Between 1% and 5% of endometrial, kidney, liver, esophageal, ovarian, salivary gland, gastric, thyroid, and urinary tract tumors were found to have *ATM* mutations ^[45]. The *ATM* protein function is of significant relevance in cancer research because it plays a critical role in DNA repair by activating enzymes that fix broken strands ^[48]. In *ATM*, pathogenic mutations are frequent. Specifically, an *ATM* mutation is present in about 0.35% of the population, and there is a substantial correlation between *ATM* mutations and cancer. Researchers validated the previously identified two-fold invasive ductal BC in patients with an *ATM* mutation and found a four-fold increased risk for pancreatic cancer, a three-fold increased risk for stomach cancer, and a two- to three-fold increased risk for prostate cancer. Additionally, they discovered a low to moderate increase in the incidence of melanoma, colorectal cancer, ovarian cancer, and breast cancer in males. *ATM* is a large gene with thousands of potential sites for mutations. Compared to other *ATM* mutations, a frequent variant called c.7271T>G is associated with a notably higher risk of BC (about four times) ^[49]. Asian patients are more likely than Caucasian patients to have a high association of *ATM* variants with BC, mostly because of racial disparities in lifestyle and environmental conditions. Recently, 60,466 specimens from BC patients and 53,461 samples were examined using a panel of 34 potential susceptibility genes created by the Breast Cancer Association Consortium. Furthermore, *ATM* proved to be potentially useful for genetic counseling ^[50]. As was previously indicated, sporadic BC also exhibits *ATM* mutations that result in *ATM* gene inactivation, but the underlying mechanisms are still unknown. Various mutations have been reported, including allelic loss ^[51].

The UALCAN database was utilized in the current evaluation to determine *ATM* expression in HNSCC. Upregulation of *ATM* expression was observed in various cancer stages, specific cancer development types,

age groups, genders, and racial groupings. Regarding tumor progression, the findings demonstrated that HNSCC tissues had significantly higher *ATM* expression levels than normal control samples. Additionally, our analysis using the KM plotter tool revealed that, compared to low and high *ATM* expression, HNSCC patients with high *ATM* expression had better overall survival, while HNSCC patients with low *ATM* expression had better disease-free survival. Furthermore, STRING and DAVID tool analysis depicted the diverse nature of the *ATM* gene, showing that *ATM* is interconnected with other genes and plays a crucial role in various biological processes and pathways. In our investigation, we found that *ATM* expression level in tissue was an independent poor prognostic factor. Further evaluations should explore the prognostic value of *ATM* expression in cancer development.

5. Conclusion

Our research concludes that promoter methylation, genetic alterations, and poor overall survival are strongly associated with *ATM* overexpression in HNSCC. By effectively utilizing multiple public databases such as UALCAN, TCGA, cBioPortal, STRING, DAVID, and KM plotter, we have highlighted the diagnostic, predictive, and potentially therapeutic roles of *ATM* in HNSCC. To further test and corroborate these results and investigate the underlying mechanisms causing *ATM* dysregulation in HNSCC, more research is necessary. These findings may eventually contribute to the development of better therapeutic approaches and diagnostic tools for HNSCC patients.

Disclosure statement

The author declares no conflict of interest.

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A Case of *Lautropia mirabilis* Infection in a Lung Transplant Patient and a Review of the Literature

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Abstract: *Lautropia mirabilis* is an opportunistic pathogen that typically causes intestinal and oral infections when the body's immune system is compromised or the microbial flora is imbalanced. Respiratory infections caused by *Lautropia mirabilis* are extremely rare. The symptoms and severity of *Lautropia mirabilis* infection may vary depending on individual differences and the site of infection. Through a review of relevant literature and this case study, it has been observed that *Lautropia mirabilis* may also cause pulmonary infectious diseases, and in immunocompromised patients, it can lead to severe infections, potentially resulting in death.

Keywords: *Lautropia mirabilis*; Lung transplant; Infection; Literature review

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1. Introduction

Lautropia mirabilis consists of coccoid bodies at various developmental stages, aggregated on a common surface layer, and is described as Gram-negative irregular spherical cells ^[1]. Currently, there are few studies on this pathogen, both domestically and internationally. This study presents a case of *Lautropia mirabilis* infection in a lung transplant patient treated at the hospital, along with a review of the literature.

2. Case information

The patient, Mr. Lai, male, 58 years old, was admitted to the hospital due to “cough and sputum production for more than 10 days, worsening for 2 days.” Over six years ago, the patient underwent a right single-lung transplant for “interstitial lung disease” at another hospital and has been on post-operative oral anti-rejection medication (tacrolimus 2 mg orally four times a day, CellCept 4 tablets orally four times a day).

Temperature: 36.5°C, pulse: 88 bpm, respiratory rate: 20 breaths/min, blood pressure: 118/70 mmHg. The patient appeared lethargic, with signs of acute illness, rapid breathing, mild cyanosis of the lips, and no jugular

vein distension. The chest was symmetrical, with slightly coarse breath sounds in both lungs. Noticeable moist rales were heard in the left lung, especially at the bases of both lungs. No wheezing was detected, and there was no edema in the lower limbs.

C-reactive protein: 41.88 mg/L, white blood cell count: $3.14 \times 10^9/L$, neutrophil percentage: 80.30%, creatinine: 155 $\mu\text{mol/L}$. Arterial blood gas analysis: partial pressure of carbon dioxide (PCO_2): 30 mmHg, partial pressure of oxygen (PO_2): 88 mmHg. Chest computed tomography (CT): scattered, patchy ground-glass opacities in both lungs, with a high suspicion of viral pneumonia. Interstitial changes in the left lung. Slightly enlarged cardiac silhouette, small pericardial effusion, and bilateral pleural thickening (**Figure 1**). Antiviral treatment with Azvudine and anti-infective treatment with piperacillin-tazobactam were initiated.

Five days later, the patient's respiratory distress showed little improvement. A repeat arterial blood gas test revealed: pH: 7.42, PCO_2 : 32 mmHg, PO_2 : 63 mmHg (with nasal cannula oxygen). Antibiotics were switched to meropenem for anti-infective therapy. However, the condition did not improve significantly, and non-invasive mechanical ventilation was initiated [inspiratory positive airways pressure (IPAP): 16.0, expiratory positive airways pressure: 6.0, respiratory rate: 20 breaths/min, the maximum time it will spend in IPAP: 1.4 s, the minimum time it will spend in IPAP: 0.5 s, rise time: 300 ms, tidal volume: 480–500 mL, peripheral oxygen saturation: 95%]. Despite treatment, the patient continued to experience pronounced dyspnea, and signs of septic shock, such as blood pressure drops, appeared. A repeat chest CT showed increased patchy ground-glass opacities in both lungs, highly suggestive of viral pneumonia, with interstitial changes in the left lung. Compared to the chest CT on January 1, 2023, the lesions had significantly increased, and a small amount of new left pleural effusion was noted. The cardiac silhouette was slightly enlarged, with a small pericardial effusion and bilateral pleural thickening. There was also evidence of an old right rib fracture (**Figure 2**).

White blood cell count: $30.11 \times 10^9/L$. Both Gram-negative bacilli and Gram-positive cocci were detected, and bedside lavage was performed. BALF-NGS indicated *Lautropia mirabilis* with a sequence count of 8912 (**Table 1**). The patient was immediately intubated and placed on invasive mechanical ventilation. A bedside chest X-ray was repeated (**Figure 4**), and antibiotics were switched to a combination of imipenem-cilastatin, cefoperazone-sulbactam, and voriconazole for anti-infective treatment. Norepinephrine was administered to maintain blood pressure, and the patient continued on invasive mechanical ventilation. On the 15th day of admission, the patient experienced cardiac arrest and was pronounced dead.

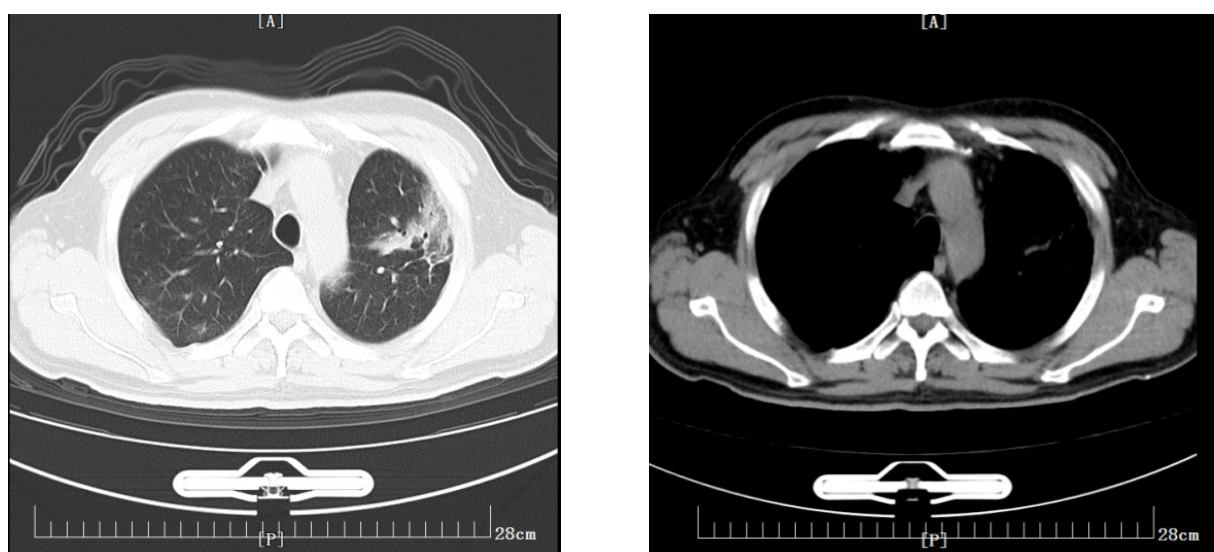


Figure 1. Chest CT upon admission. (**Left**) Lung window: consolidation and ground-glass opacities in the upper lobe of the left lung; (**Right**) Mediastinal window: consolidation visible

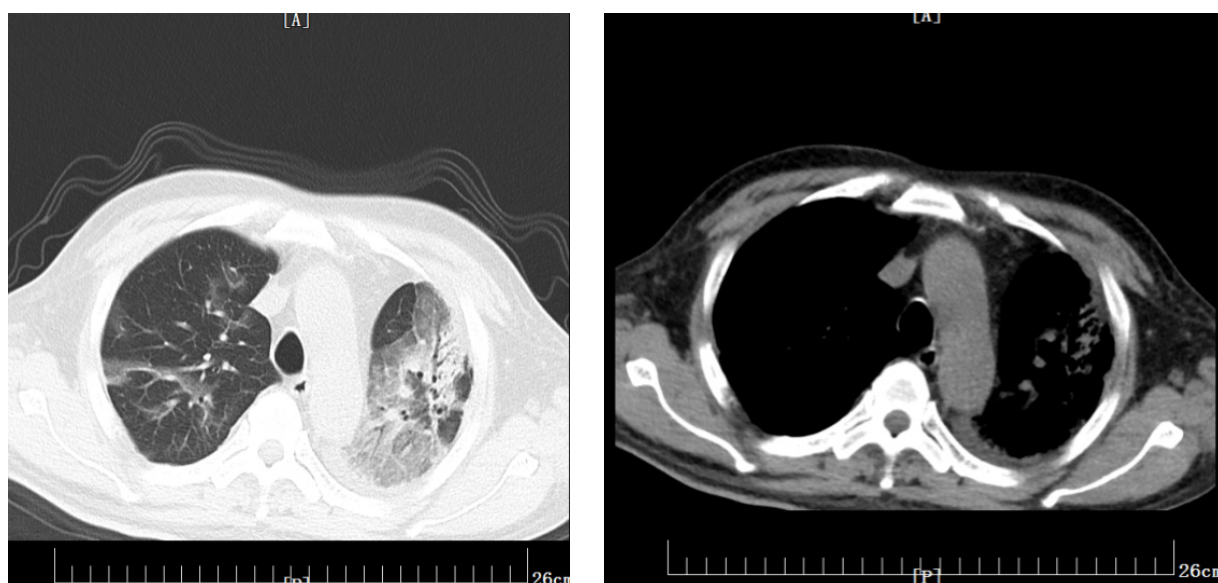


Figure 2. Repeat chest CT. **(Left)** Lung window: increased consolidation and ground-glass opacities in the upper lobe of the left lung; **(Right)** Mediastinal window: increased consolidation

Table 1. BALF-NGS indicated *Lautropia mirabilis*

Bacteria						
Genus				Species		
Type	Name	Sequence number	Relative abundance %	Name	Sequence number	Relative abundance %
G -	Lautropia	8912	80.98	Lautropia mirabilis	8912	80.98



Figure 3. ICU repeat bedside chest X-ray: multiple consolidations and ground-glass opacities in both lungs

3. Discussion

Lautropia mirabilis consists of coccoid bodies at various stages, aggregated on a common surface layer, and is characterized by irregular spherical Gram-negative cells. Under Gram staining, the morphology is different from common bacteria, presenting as relatively large spherical cells that are not easily emulsified and tend to aggregate, often leading to mistaken identification as impurities. This bacterium is positive for oxidase, catalase,

urease, glucose, and maltose, but negative for lactose, sucrose, phenylalanine deaminase, and indole. Preliminary identification can be made based on morphology and key biochemical reactions in routine laboratory work. *Lautropia mirabilis* is widely distributed in nature, as well as in the intestines of humans and animals ^[2]. It is an opportunistic pathogen, meaning that under normal circumstances, it does not cause disease in the human intestines. However, when the immune system is compromised or microbial flora becomes imbalanced, it can cause intestinal or oral infections, although respiratory infections caused by this bacterium are extremely rare ^[3]. The symptoms and severity of *Lautropia mirabilis* infections can vary depending on individual differences and the infection site. In some cases, it may present without noticeable symptoms, while in specific populations or infection sites, it may cause significant illness. This case shows that *Lautropia mirabilis* can also lead to pulmonary infectious diseases, and in immunocompromised patients, it can cause severe infections and even death.

Regarding the pathogens responsible for infections in lung transplant patients, literature reports that the long-term prognosis and survival rates at 1, 3, and 5 years post-transplant are 66.1%, 56.3%, and 36.2%, respectively. During follow-up, five fatal infections were confirmed, three occurring within 30 days post-transplant, one between 2 and 12 months, and one after 1 year. Of these fatal pulmonary infections, two involved Gram-negative bacterial coinfections with septic shock, two were fungal infections with associated candidemia, and one case was CMV pneumonia ^[4]. To date, there have been no reported cases of *Lautropia mirabilis* infections leading to death. However, in this case, the patient, who had undergone a right single-lung transplant six years earlier, died from *Lautropia mirabilis* infection. There have been reports of *Lautropia mirabilis* breaching the oral mucosal barrier and causing oral mucositis, even leading to bloodstream dissemination and sepsis in immunocompromised patients on extensive antibiotic therapies, such as glycopeptides and β -lactams ^[5]. This patient had been on long-term immunosuppressants following the lung transplant, compounded by left lung IPF, which led to repeated infections and hospitalizations. Before this current infection, the patient had been hospitalized multiple times for lung infections and treated with piperacillin-tazobactam and other β -lactam antibiotics, which may have contributed to the *Lautropia mirabilis* infection ^[1].

Lautropia mirabilis was first described in 1994 by Gerne-Smidt ^[1], and in 1997, it was detected twice in the sputum of a patient with cystic fibrosis. In a screening of 500 sputum samples from cystic fibrosis patients, 12% tested positive for *Lautropia mirabilis*. Large quantities of *Lautropia mirabilis* have been isolated from the oral cavities of children infected with HIV, and its role in oral and periodontal diseases in HIV-infected individuals requires further investigation. The bacterium has also been isolated from blood and sterile body fluids, suggesting that it may cause invasive diseases ^[4]. *Lautropia mirabilis*-induced oral mucositis typically presents as painful oral ulcers, and when systemic infection occurs, patients may develop high fever, chills, and, in some cases, septic shock. In this case, the infection primarily presented with respiratory symptoms, including dyspnea and the production of scant, white sputum. Laboratory findings showed elevated white blood cell counts, severe hypoxemia, and later-stage acute heart failure, with symptoms including orthopnea and bilateral leg edema. Blood pressure gradually decreased, and the patient developed septic shock. Imaging findings on CT mainly showed consolidation and exudative changes, with rapid progression of pulmonary imaging, although the radiological features were not distinctive.

The post-lung transplant infection rate is 74% (37/50), with 9 cases (24.3%) of single-pathogen infections and 28 cases (75.7%) of mixed infections involving two or more pathogens. The main causative pathogens are Gram-negative bacteria (*Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, and *Klebsiella pneumoniae*), with resistance rates for *Pseudomonas aeruginosa* to cephalosporins, fluoroquinolones, and trimethoprim-sulfamethoxazole ranging from 66.7% to 94.1%. *Acinetobacter baumannii* exhibited high resistance to multiple antibiotics, with resistance rates of 72.7% to 100%. The resistance rate of *Stenotrophomonas maltophilia* to ceftazidime was 50%, and *Klebsiella pneumoniae* showed resistance rates of 44.4% to 71.4% against

carbapenems, β -lactams, cephalosporins, and fluoroquinolones. Gram-positive bacteria, such as *Staphylococcus aureus*, *Streptococcus hemolyticus*, and *Enterococcus faecalis*, were also isolated, with good sensitivity to linezolid, daptomycin, tigecycline, and vancomycin, which can be considered as first-line treatments ^[6].

Lautropia mirabilis consists of coccoid bodies at different stages, aggregated on a common surface layer, and presents as irregular spherical Gram-negative cells. Regarding the treatment of *Lautropia mirabilis*, the literature suggests that antibiotics such as ampicillin or cefotaxime may be effective. For patients allergic to these drugs, aminoglycosides or chloramphenicol may be alternatives. In severe infections, combination therapy may be required. In this case, the patient received anti-infective treatment with cefoperazone-sulbactam, voriconazole, and imipenem-cilastatin, but the outcome was poor.

Infections with *Lautropia mirabilis* are rare in lung transplant patients. Due to immunosuppression and concurrent COVID-19 infection, early diagnosis and treatment are crucial, as many patients had already received β -lactam antibiotics before their hospital visits. Immunocompromised individuals may be more susceptible to *Lautropia mirabilis*. To prevent infection, maintaining good personal hygiene, such as frequent handwashing and avoiding sharing personal items, is important. Additionally, regular physical exercise and a healthy lifestyle can help boost immunity and reduce the risk of infection. Clinicians should raise awareness of this bacterium, as early detection, diagnosis, and treatment are closely related to patient outcomes in lung transplant recipients infected with *Lautropia mirabilis*.

Disclosure statement

The authors declare no conflict of interest.

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Clinical Application Study of Super-Mini Percutaneous Nephrolithotomy in the Treatment of Kidney Stones

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Abstract: *Objective:* To observe the clinical efficacy and complication rates of different stone fragmentation techniques in the treatment of kidney stones. *Methods:* This study selected 100 patients with urinary stones treated at the Third Division General Hospital from 2021 to November 2023 as subjects. The control group ($n = 50$) received conventional percutaneous nephrolithotomy (PCNL) for stone fragmentation, while the research group ($n = 50$) received super-mini percutaneous nephrolithotomy (SMP) treatment. Surgical parameters, stone clearance rates, recurrence rates, and complication rates were compared between the two groups. *Results:* After treatment, the surgical parameters in the research group were significantly better than those in the control group. The research group had a higher stone clearance rate and lower rates of stone recurrence and complications ($P < 0.05$). *Conclusion:* Compared with conventional PCNL, SMP shows better clinical outcomes for patients with kidney stones. It improves surgical parameters, increases stone clearance rates, and reduces both stone recurrence and complication rates, making it a valuable technique for clinical reference.

Keywords: Super-mini percutaneous nephrolithotomy; Kidney stones; Stone clearance; Clinical application

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1. Introduction

Kidney stones are a common surgical condition, primarily referring to stones located in the renal pelvis and calyces ^[1]. These stones are usually composed of calcium oxalate, calcium phosphate, and other compounds. Kidney stones are a prevalent condition, often associated with metabolic dysfunction, chronic nephritis, and the long-term consumption of high-calcium foods ^[2]. Clinically, patients present with severe lower back pain, soreness, and in some cases, stones that have migrated to the ureter may cause intense renal colic, greatly affecting the patient's daily life.

Currently, the primary treatment for kidney stones is surgical, aimed at alleviating symptoms and improving quality of life. With the advent of EMS ultrasonic lithotripsy, percutaneous nephrolithotomy (PCNL) has established a standard 20–24 F access channel, which is suitable for treating larger individual stones.

However, for more complex staghorn calculi, conventional access may not meet the practical requirements for lithotripsy [3]. Super-mini percutaneous nephrolithotomy (SMP) is a method that involves accessing the kidney through a small incision to visually locate and fragment the stones. This technique has demonstrated favorable outcomes in clinical practice [4].

This study selected 100 patients with urinary stones treated at the Third Division General Hospital from 2021 to November 2023 to explore the clinical efficacy of different nephroscopy techniques for kidney stone treatment. The results may provide useful insights for future clinical practice.

2. Materials and methods

2.1. General information

The study selected 100 patients with urinary stones who were treated at the Third Division General Hospital between 2021 and November 2023. Based on the different treatment methods, the patients were divided into a control group ($n = 50$) and a research group ($n = 50$). There were no statistically significant differences in the general data between the two groups ($P > 0.05$), making them comparable (see **Table 1**).

Table 1. Comparison of general data between the two groups

Groups	Number of cases	Gender		Age (years)	Average age (years)	Stone type (cases)	
		Male	Female			Left-sided stones	Right-sided stones
Control group	50	26	24	19–70	51.25 ± 3.15	27	23
Research group	50	25	25	20–70	51.27 ± 3.14	26	24
χ^2 / t			0.040	-	0.032		0.040
P			0.841	-	0.975		0.841

Inclusion criteria: (1) All patients met the relevant diagnostic criteria for kidney stones; (2) The American Society of Anesthesiologists (ASA) score [5] was between grades 1–2; (3) Patients had surgical indications; (4) Age < 70 years; (5) All surgeries were assisted by medical staff from the Third Division General Hospital.

Exclusion criteria: (1) Patients with severe renal insufficiency; (2) Those with contraindications to surgery; (3) Patients undergoing immunotherapy or with hematological disorders; (4) Patients with concurrent renal tuberculosis; (5) Patients with mental disorders.

2.2. Methods

The control group received conventional PCNL. After preoperative preparations, patients were placed in the lithotomy position. A ureteroscope was inserted into the bladder through the urethra, and a 0.35 mm ultra-smooth guide wire was placed into the ureter of the affected side. An F5 ureteral catheter was inserted into the renal pelvis along the guide wire, and a urethral catheter was fixed in place. The patient was then placed in the prone position, and ultrasound was used to determine the puncture site. An 18G interventional needle was used to puncture the target renal calyx. After a successful puncture, a zebra guide wire was placed into the renal pelvis, and the tract was dilated to F20. The nephroscope was then introduced into the renal pelvis along the tract, and a holmium laser lithotripsy system was used to completely remove the stones. After surgery, an F5 double-J stent and a nephrostomy tube were routinely placed.

The research group received treatment with SMP. After intubation anesthesia, a rigid ureteroscope was used to place a 5 F ureteral catheter, and a 16 F urinary catheter was inserted into the bladder. After confirming

no abnormalities, 10 mL of 0.9% saline was injected into the catheter, and the ureteral catheter was secured in place. The patient was placed in the prone position, and after disinfection and draping, the location of the stones was determined using X-ray and ultrasound. A contrast agent (iohexol) was injected into the renal pelvis and calyces, and based on the imaging results, a puncture point was selected, and a zebra guide wire was inserted into the renal collecting system. After confirming the position of the guide wire with an X-ray, a fascial dilator was used to expand the tract to F12. An F12 metal sheath was then placed, followed by a 3.3/7 F SMP nephroscope. The renal calyces and pelvis were inspected for stones, and a 200 μ m–365 μ m holmium laser fiber was used for lithotripsy, adjusting the frequency to 20–40 Hz and energy to 0.5–2.0 J. Stones were fragmented starting from the edges to avoid damage to the renal pelvis mucosa. The stones were pulverized into particles smaller than 3 mm and promptly suctioned out. After successful lithotripsy, an F5 double-J stent was placed.

2.3. Observation indexes

- (1) Comparison of surgical indicators: The two groups were compared in terms of operative time, intraoperative blood loss, and length of hospital stay;
- (2) Comparison of stone clearance rates: Bilateral kidney CT scans were used to observe stone clearance. If residual stones were found, the surgery was deemed unsuccessful; otherwise, it was considered successful. Secondary stone clearance success rate: If the first stone removal was unsuccessful, and medication proved ineffective, any residual stones during the second stone removal attempt would result in a failed outcome, while complete removal was deemed successful;
- (3) Comparison of recurrence rates: The two groups were followed for 6 months postoperatively, and the recurrence rates were recorded;
- (4) Comparison of complication rates: Complications included ureteral injury, infection, and others. The total complication rate was calculated as (ureteral injury + infection + others) / total cases \times 100%.

2.4. Statistical analysis

Data processing was conducted using SPSS 25.00 software. Continuous variables were described using the mean \pm standard deviation (SD), and intergroup comparisons were conducted using the *t*-test. Categorical variables were described using [*n* (%)], and comparisons were made using the chi-squared (χ^2) test. A *P* value $<$ 0.05 was considered statistically significant.

3. Results

3.1. Comparison of surgical indicators between the two groups

Table 2 shows that the research group had significantly shorter operative time and length of hospital stay as well as lesser intraoperative hemorrhage as compared to the control group (*P* $<$ 0.05).

Table 2. Comparison of surgical indicators between the two groups (mean \pm SD)

Groups	<i>n</i>	Operative time (min)	Intraoperative hemorrhage (mL)	Length of hospital stay (d)
Control group	25	80.25 \pm 12.15	53.25 \pm 5.92	10.25 \pm 2.33
Research group	25	60.03 \pm 10.18	39.43 \pm 4.28	6.62 \pm 1.21
<i>t</i>		9.020	13.377	9.777
<i>P</i>		$<$ 0.001	$<$ 0.001	$<$ 0.001

3.2. Comparison of stone clearance rates between the two groups

After treatment, the stone clearance rate in the control group was 80.0% (20/25), while the stone clearance rate in the study group was 96.0% (24/25). The study group had a higher stone clearance rate than the control group ($\chi^2 = 12.121$, $P < 0.05$).

3.3. Comparison of recurrence rates between two groups

After treatment, the recurrence rate in the control group was 20.0% (5/25), while the recurrence rate in the study group was 4.0% (1/25). The recurrence rate in the study group was lower than that of the control group ($\chi^2 = 10.653$, $P < 0.05$).

3.4. Comparison of complication rates between the two groups

As shown in **Table 3**, the research group had only one adverse event (infection), which is significantly lower than the control group, which had 8 adverse events (2 ureteral injuries, 2 infections, and 1 other; $P < 0.05$).

Table 3. Comparison of complication rates between the two groups [n (%)]

Groups	<i>n</i>	Ureteral injury	Infection	Other	Total incidence
Control group	25	2 (8.0)	2 (8.0)	1 (4.0)	5 (20.0)
Research group	25	0 (0.0)	1 (4.0)	0 (0.0)	1 (4.0)
χ^2		-	-	-	10.653
<i>P</i>		-	-	-	< 0.001

4. Discussion

Kidney stones are a common clinical disease, and once the kidney becomes diseased, urine abnormalities often occur. Studies have shown that calcium plaques exist within the renal papilla, and 19.6% of 1,154 patients had calcium plaques, 65 of which showed plaque growth, indicating that calcium plaques are the foundation of kidney stones [6]. When the stone moves within the kidney or enters the ureter, it can cause symptoms such as severe pain in the lower rib area, radiating pain to the lower abdomen and groin, difficulty urinating, pink-colored urine, frequent urination, as well as symptoms of infection such as high fever and chills, and acute renal failure symptoms like oliguria or anuria, severely impacting the patient's daily life. Therefore, performing timely surgical treatment is crucial for improving the patient's clinical symptoms and enhancing their quality of life.

With the continuous development and maturation of PCNL technology in China, it has been widely used in kidney stone treatment and has become one of the primary methods for treating urinary stones. SMP, guided by ultrasound, allows real-time observation of the layers being punctured, achieving precise puncturing. The use of a micro nephroscope facilitates intraoperative handling without the need for large tract dilation, which reduces surgical trauma and blood loss [7]. Related research [8] shows that compared to traditional lithotripsy techniques, applying SMP in lithotripsy for kidney stone patients achieves better outcomes, which is consistent with the results of this study. The results of this study showed that, after treatment, the surgical indicators in the study group were significantly better than those in the control group, and the stone clearance rate was higher in the study group. This indicates that SMP has less impact on the patient's kidney function. During the procedure, guided by ultrasound, a 10–12 F peel-away sheath with suction functionality can be used. The advantage of this peel-away sheath is that it can remove the stone from the body in the shortest time, improving the stone

clearance rate^[9]. The results of this study also indicate that the complication rate in the study group was significantly lower than that in the control group, suggesting that SMP can significantly reduce postoperative infections and other complications. For patients with larger stones, the nephroscope can be used to clear the stones, overcoming the problem of frequent postoperative complications associated with traditional lithotripsy techniques^[10].

In conclusion, applying SMP in the clinical treatment of kidney stone patients provides excellent results, improving surgical indicators, increasing stone clearance rates, reducing stone recurrence, and lowering the incidence of complications, making it worthy of clinical application.

Disclosure statement

The authors declare no conflict of interest.

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Expression and Prognostic Potential of *ESR1* in Breast Cancer

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Abstract: This study aims to explore the potential of *ESR1* as a biomarker in breast cancer (BRCA) using bioinformatics analysis tools. The up-regulation of *ESR1* expression in BRCA was investigated using UALCAN and GEPIA2, illustrating its role in BRCA progression. Furthermore, analyses based on various variables such as gender, age, race, and pathological stages of BRCA patients revealed a consistent up-regulation of *ESR1*, emphasizing its role in the development and progression of BRCA. Additionally, an analysis of *ESR1* promoter methylation levels across various parameters revealed hypomethylation, affirming the inverse correlation between methylation and *ESR1* expression. Prognostic analysis further indicated that overexpression of *ESR1* is associated with poor overall survival, highlighting its potential as a prognostic biomarker in BRCA. Moreover, genetic mutation analysis using cBioPortal disclosed a minor role of *ESR1* genetic mutations in BRCA, with only 2.5% of genetic alterations observed. The STRING and DAVID tools were utilized to conduct pathway enrichment analysis, revealing diverse biological functions of *ESR1* and its 10 interconnected genes. Altogether, these results underscore the significance of understanding *ESR1* up-regulation in BRCA and demonstrate its potential as a therapeutic, diagnostic, and prognostic biomarker.

Keywords: Breast cancer; *ESR1*; Biomarker; Bioinformatics analysis

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1. Introduction

Cancer is a predominant medical problem worldwide. In 2022, approximately 20 million new cases and 9.7 million cancer-related deaths were reported globally. Among the numerous types of cancer, breast cancer (BRCA) is the second most common, accounting for 2.3 million cases and representing the leading cause of cancer-related mortality in women worldwide^[1-3]. The incidence of breast cancer continues to rise, with the majority of cases occurring in developed and industrialized nations^[4,5]. Major risk factors associated with breast cancer include alcohol consumption, aging, hormonal status, family history, nutrition, obesity, and genetic mutations^[6]. The World Health Organization (WHO) recognizes 18 histological types of BRCA, with invasive breast cancer being the most prevalent, comprising 40%–80% of cases^[6,7]. Additionally, four molecular subtypes of BRCA have been identified: Luminal, HER2-enriched, Basal-like, and Normal Breast-like^[8,9]. Common treatments

for BRCA include surgery, radiotherapy, chemotherapy, endocrine therapy, and targeted therapy^[10]. However, a 30% recurrence rate in early-stage BRCA, along with treatment resistance and medication side effects, remains a significant obstacle to successful treatment^[11]. Metastatic BRCA, in particular, has a poor prognosis, with a 5-year tumor-specific survival rate of only 29%^[12,13]. Given these challenges, the identification of effective diagnostic, therapeutic, and prognostic biomarkers for BRCA is crucial.

The estrogen receptor 1 (*ESR1*) gene encodes the estrogen receptor- α (ER α), a ligand-activated transcription factor. ER α is expressed in BRCA, and 75% of tumors are estrogen receptor-positive (ER⁺), which is associated with high mortality. The Luminal A and Luminal B subtypes of BRCA are ER⁺, with Luminal B being a highly metastatic and recurrent subtype^[14-17]. Endocrine therapy (ET) is highly recommended and effective for treating ER⁺ BRCA. However, a significant number of early-stage tumors relapse even after ET, with 15%–20% of metastatic BRCA cases showing resistance to this therapy^[18]. The transcriptional activity of ER α is regulated by specific domains within *ESR1*. Various mechanisms alter ER expression, such as when ligands bind to the receptor, allowing ER to regulate critical tumorigenesis genes by interacting with specific DNA sequences and coregulatory proteins. Signaling pathways are activated when ER interacts with tyrosine kinase receptors and signaling proteins, with growth factors further stimulating ER's transcriptional function. Alterations in these pathways contribute to ET resistance^[19-23]. Furthermore, mutations in *ESR1* are associated with a worse prognosis^[24].

Given the potential of *ESR1* in BRCA, extensive research has been conducted globally. However, to date, bioinformatics analysis of *ESR1* as a diagnostic, therapeutic, and prognostic biomarker in BRCA has not been fully explored. Therefore, this study aims to conduct a comprehensive bioinformatics analysis of *ESR1* in BRCA.

2. Methodology

2.1. UALCAN

UALCAN is an accessible and effective online database based on TCGA data, widely employed to analyze gene expression in cancer^[25]. In this study, UALCAN was used to elucidate *ESR1* expression in BRCA. Additionally, UALCAN contributed significantly to analyzing the promoter methylation levels of *ESR1* in BRCA. This database allowed for the investigation of *ESR1* methylation and expression across various variables.

2.2. Kaplan-Meier plotter

Kaplan-Meier (KM) Plotter is a web-based tool that plays a pivotal role in determining the impact of genes on the overall survival (OS) of cancer patients^[26]. In this study, KM Plotter was used to perform a survival analysis of *ESR1* in BRCA. The hazard ratio was calculated with a 95% confidence interval, and the *P*-value was set at 0.05.

2.3. GEPIA2

Gene Expression Profiling Interactive Analysis 2 (GEPIA2) is an online tool based on TCGA and GTEx datasets, which enables comprehensive analysis of gene expression and survival data^[27]. In this study, GEPIA2 was utilized to assess the survival analysis of *ESR1* in BRCA. GEPIA2 was also used to evaluate *ESR1* expression in BRCA samples and across different cancer stages.

2.4. cBioPortal

cBioPortal is a user-friendly and robust web-based tool widely used to evaluate genetic mutations in various cancers^[28]. This study employed cBioPortal to analyze genetic alterations of *ESR1* in BRCA.

2.5. STRING

The Search Tool for the Retrieval of Interacting Genes (STRING) is a web-based tool used to construct Protein-Protein Interaction (PPI) networks of genes ^[29]. In this study, STRING was used to construct a PPI network for *ESR1* and to reveal interactions with related genes.

2.6. DAVID

To examine the enrichment analysis of specific genes and their interlinked counterparts, the bioinformatics tool DAVID was employed ^[30]. In this study, DAVID was used to perform pathway enrichment analysis, evaluating the biological significance of *ESR1* and its interconnected genes.

3. Results

3.1. Analysis of *ESR1* expression in BRCA and normal samples

The UALCAN database was employed to analyze *ESR1* expression in BRCA and normal control samples. An increase in *ESR1* expression was observed in BRCA samples compared to normal samples (**Figure 1**). Previous studies have suggested that significantly upregulated genes are involved in the progression of cancers ^[31,32]. The significant overexpression of *ESR1* in primary tumor samples indicates that *ESR1* plays a role in the progression and development of BRCA.

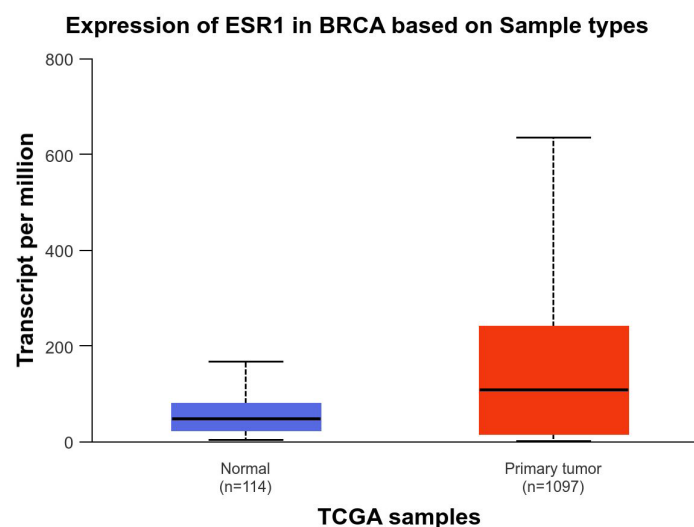


Figure 1. Expression analysis of *ESR1* in BRCA and normal samples using UALCAN

3.2. Analysis of *ESR1* expression in BRCA categorized by various parameters

Further investigation was conducted to assess *ESR1* expression in BRCA across various parameters. First, *ESR1* expression was analyzed based on individual cancer stages, showing a significant upregulation across all stages of BRCA (**Figure 2A**). Next, *ESR1* expression was assessed by age group, revealing upregulation with variation between groups; the highest expression was observed in patients aged 81–100 years compared to those aged 21–40 years (**Figure 2B**). *ESR1* expression was then analyzed based on race, showing upregulation in Caucasians and dysregulation in African-Americans and Asians (**Figure 2C**). Gender-based analysis revealed upregulation in both male and female BRCA patients, with higher expression in males (**Figure 2D**). Overall, the observed variation, dysregulation, and upregulation of *ESR1* expression suggest its involvement in the progression and development of BRCA.

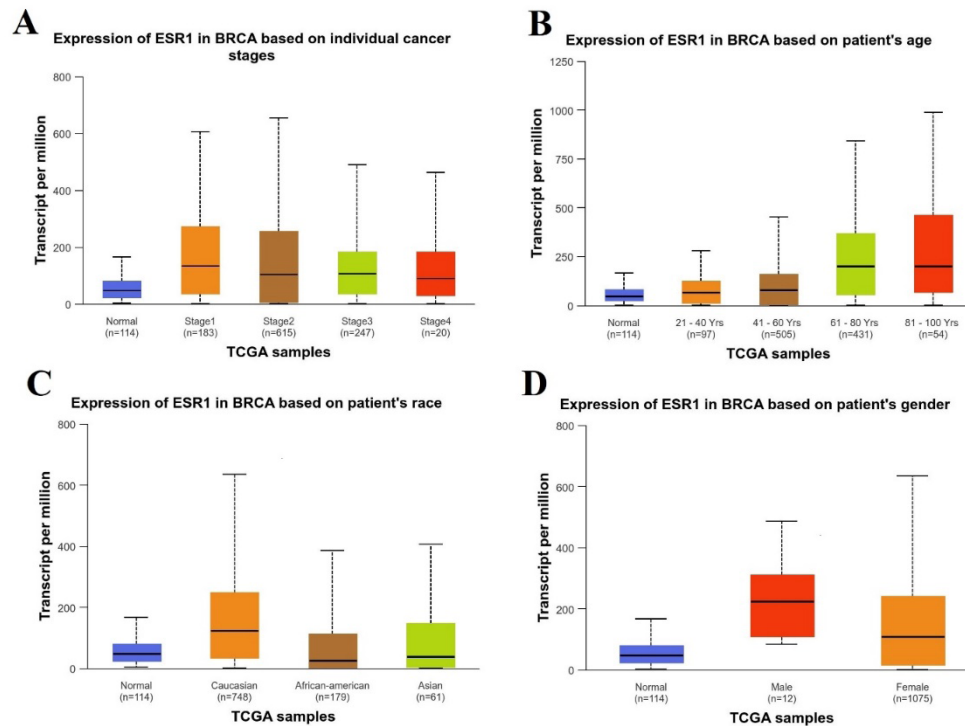


Figure 2. *ESR1* expression in various parameters. (A) Analysis of *ESR1* expression in BRCA based on pathological stages; (B) Analysis of *ESR1* expression in BRCA based on patients' age; (C) Analysis of *ESR1* expression in BRCA based on patients' race; (D) Analysis of *ESR1* expression in BRCA based on patients' gender

3.3. Analysis of promoter methylation of *ESR1* in BRCA and normal samples

To further the research, the promoter methylation level of *ESR1* in BRCA and normal control samples was analyzed using UALCAN. Significant hypomethylation of *ESR1* was observed in BRCA samples compared to normal samples (Figure 3). Previous research has indicated an inverse relationship between gene methylation and gene expression^[33]. Thus, the hypomethylation of *ESR1* suggests its overexpression and role in BRCA progression.

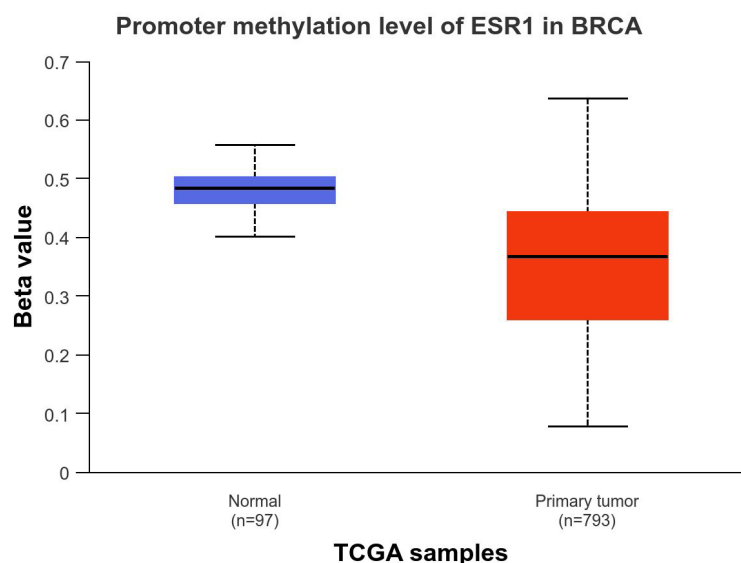


Figure 3. Analysis of promoter methylation level of *ESR1* in BRCA using UALCAN

3.4. Analysis of promoter methylation levels of *ESR1* in BRCA categorized by various parameters

Next, promoter methylation levels of *ESR1* in BRCA were analyzed across various parameters, including patient age, gender, race, and cancer stage. First, hypomethylation of *ESR1* was observed across all individual cancer stages (**Figure 4A**). *ESR1* was also hypomethylated in BRCA patients of different races (**Figure 4B**). Further analysis revealed variation in *ESR1* methylation levels across age groups, with the highest hypomethylation observed in patients aged 81–100 years, compared to those aged 21–40 years (**Figure 4C**). Similarly, gender-based analysis showed that *ESR1* was more highly hypomethylated in male patients than in female patients (**Figure 4D**). Collectively, these results suggest that *ESR1* hypomethylation contributes to BRCA progression across different demographic parameters.

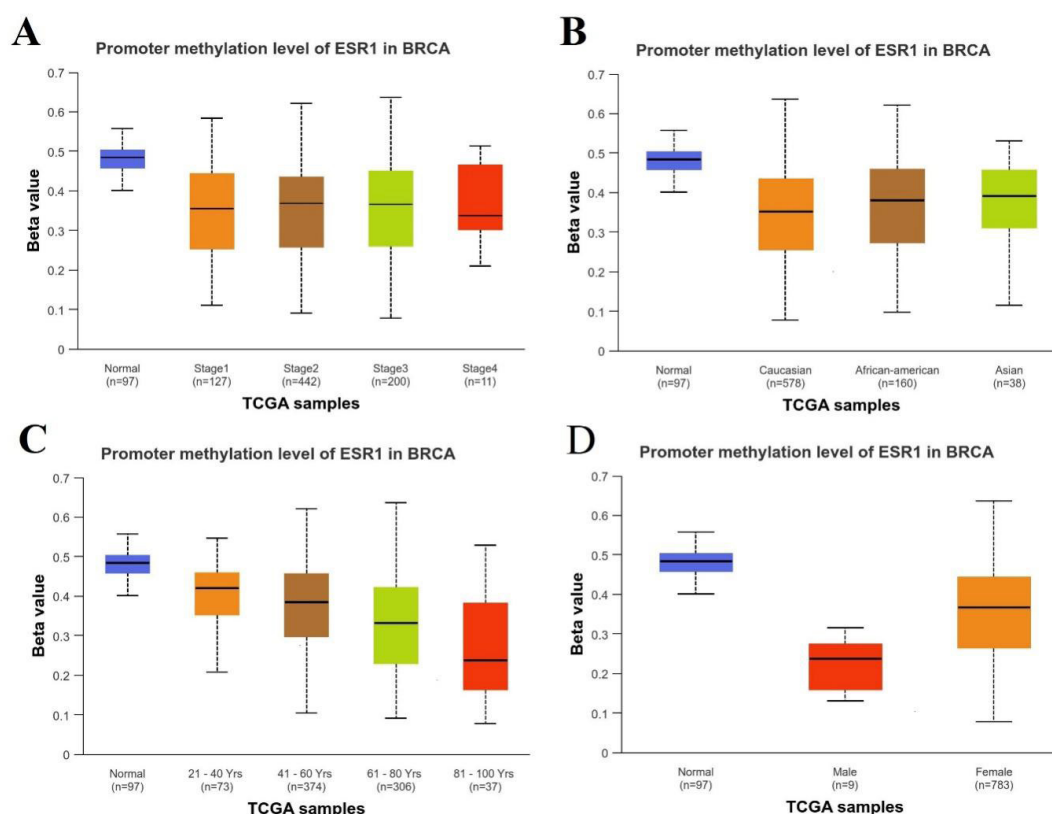


Figure 4. Promoter methylation levels of *ESR1* in various parameters. **(A)** Analysis of *ESR1* promoter methylation levels in BRCA based on pathological stages; **(B)** Analysis of *ESR1* promoter methylation levels in BRCA based on patients' race; **(C)** Analysis of *ESR1* promoter methylation levels in BRCA based on patients' age; **(D)** Analysis of *ESR1* promoter methylation levels in BRCA based on patients' gender

3.5. Prognostic analysis of *ESR1* in BRCA

The KM plotter was employed to examine the role of *ESR1* in the OS of BRCA patients. It was found that BRCA patients with overexpression of *ESR1* had lower OS, whereas those with lower expression showed better OS (**Figure 5**). However, the difference was not statistically significant, as the *P*-value was 0.1. These results suggest that *ESR1* expression impacts the OS of BRCA patients. The observed overexpression of *ESR1* in BRCA samples correlates with a higher mortality rate, highlighting its potential as a prognostic biomarker in BRCA.

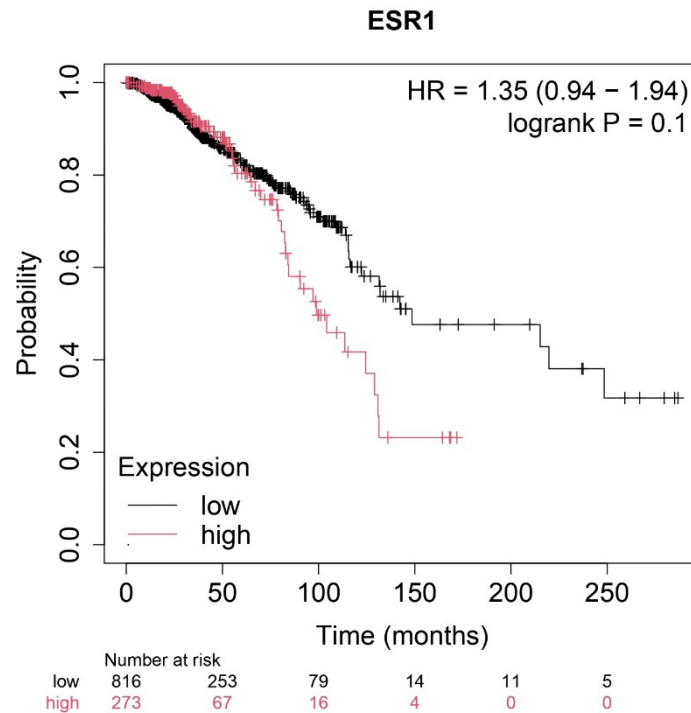


Figure 5. Prognostic analysis of ESR1 in BRCA using KM plotter

3.6. Verification of survival analysis and ESR1 expression in BRCA

To validate the findings related to *ESR1* expression and its effect on the OS of BRCA patients, GEPIA2 was utilized. *ESR1* expression in BRCA samples was first compared to normal samples, revealing that *ESR1* was upregulated in BRCA, consistent with previous findings (**Figure 6A**). Subsequently, the stage plot module of GEPIA2 was used to analyze *ESR1* expression in individual cancer stages, showing that *ESR1* was upregulated across all stages of BRCA (**Figure 6B**). These results confirm the earlier conclusion that *ESR1* plays a role in BRCA progression.

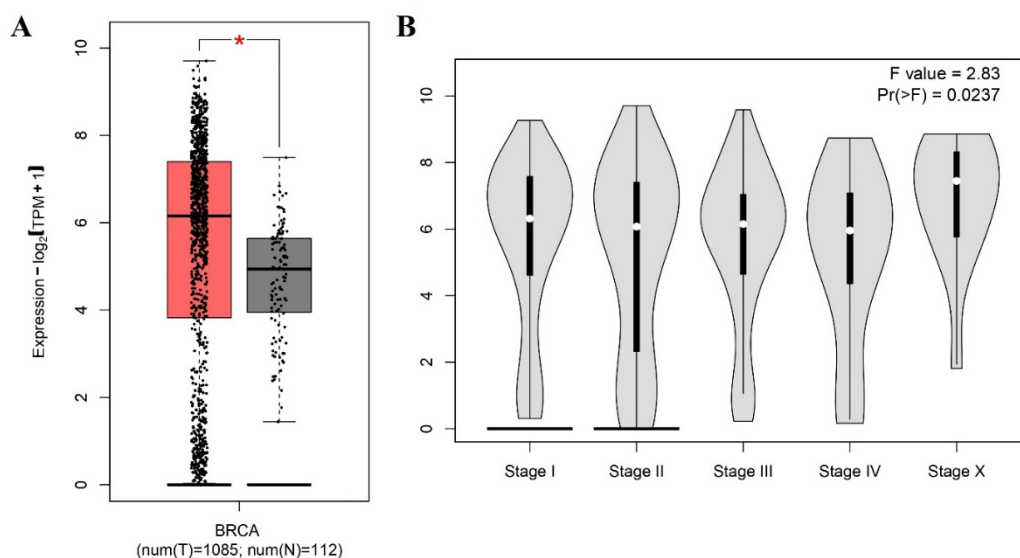


Figure 6. (A) Expression analysis of *ESR1* in BRCA and normal control samples using GEPIA2; **(B)** Expression analysis of *ESR1* in BRCA based on pathological stages using GEPIA2

The survival analysis module of GEPIA2 was then employed to evaluate the role of *ESR1* expression in the OS of BRCA patients. The analysis revealed that lower *ESR1* expression was associated with better OS, while elevated *ESR1* expression correlated with worse OS (**Figure 7**). The observed *P*-value of 0.52 indicates a marginal difference between the groups. These results align with previous findings and suggest that *ESR1* contributes to the development and progression of BRCA.

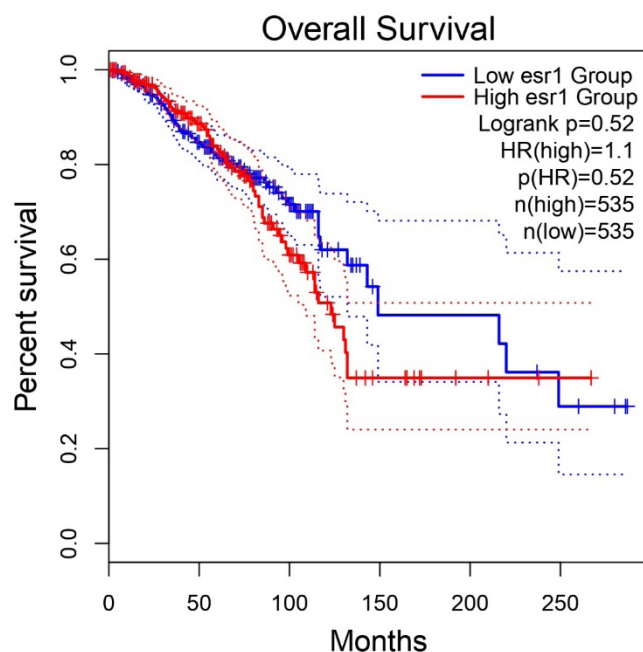


Figure 7. Survival analysis of *ESR1* in BRCA using GEPIA2

3.7. Genetic mutations of *ESR1* in BRCA

To evaluate the genetic alterations of *ESR1* in BRCA and their impact on BRCA progression, cBioPortal was utilized. A low frequency of *ESR1* mutations was observed, with only 2.5% of BRCA cases showing alterations, including amplification and deep deletion (**Figure 8**). These findings suggest that *ESR1* mutations play a minor role in BRCA proliferation, yet provide valuable insights into the genetic landscape of *ESR1* in BRCA.



Figure 8. Genetic mutations of *ESR1* in BRCA using cBioPortal

3.8. Gene enrichment analysis

STRING and DAVID tools were used to conduct a comprehensive examination, including PPI network construction, Gene Ontology (GO) analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses, to explore the biological functions of *ESR1* (**Table 1**). A PPI network of *ESR1* was constructed using the STRING tool, revealing interactions with 10 genes (**Figure 9**), which shed light on the functional role of *ESR1*.

Table 1. Gene enrichment analysis

Gene term	Gene count	Genes	P-value
BP			
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	8	<i>NCOA1, NCOA2, JUN, SPI, NCOA3, NRIP1, FOS, ESR1</i>	4.316633463367988E-7
GO:0032570~response to progesterone	3	<i>NCOA1, NCOA2, FOS</i>	1.3497253797827575E-4
GO:0071392~cellular response to estradiol stimulus	3	<i>NCOA3, NRIP1, ESR1</i>	1.689493584985378E-4
GO:0032870~cellular response to hormone stimulus	3	<i>NCOA1, NCOA2, NCOA3</i>	1.8735062934716631E-4
GO:0045893~positive regulation of transcription, DNA-templated	5	<i>NCOA1, JUN, SPI, FOS, ESR1</i>	3.362932589262899E-4
CC			
GO:0000785~chromatin	9	<i>NCOA1, NCOA2, JUN, NCOR1, SPI, NCOA3, NRIP1, FOS, ESR1</i>	2.6594084651584337E-9
GO:0005654~nucleoplasm	11	<i>NCOA1, NCOA2, HSP90AA1, JUN, NCOR1, SRC, SPI, NCOA3, NRIP1, FOS, ESR1</i>	7.313672569344439E-8
GO:0090575~RNA polymerase II transcription factor complex	4	<i>NCOA1, NCOA2, JUN, FOS</i>	2.5663102058774573E-5
GO:0005634~nucleus	10	<i>NCOA1, NCOA2, HSP90AA1, JUN, NCOR1, SPI, NCOA3, NRIP1, FOS, ESR1</i>	1.1814041714380625E-4
GO:0005667~transcription factor complex	4	<i>NCOA1, NCOA2, JUN, ESR1</i>	1.45523934603438E-4
MF			
GO:0016922~ligand-dependent nuclear receptor binding	5	<i>NCOA1, NCOA2, NCOR1, NCOA3, NRIP1</i>	4.845351136764623E-9
GO:0030331~estrogen receptor binding	4	<i>NCOA1, SRC, NRIP1, ESR1</i>	1.1281165912027505E-6
GO:0042826~histone deacetylase binding	4	<i>HSP90AA1, NCOR1, SPI, NRIP1</i>	3.609105102658606E-5
GO:0061629~RNA polymerase II sequence-specific DNA binding transcription factor binding	4	<i>JUN, NCOR1, SPI, FOS</i>	1.0554759656852471E-4
GO:0000978~RNA polymerase II core promoter proximal region sequence-specific DNA binding	6	<i>NCOA2, JUN, SPI, NRIP1, FOS, ESR1</i>	1.7546914941193677E-4
KEGG			
hsa04915:Estrogen signaling pathway	9	<i>NCOA1, NCOA2, HSP90AA1, JUN, SRC, SPI, NCOA3, FOS, ESR1</i>	2.8282454696593787E-14
hsa01522:Endocrine resistance	7	<i>JUN, NCOR1, SRC, SPI, NCOA3, FOS, ESR1</i>	1.4689368682759732E-10
hsa04919:Thyroid hormone signaling pathway	6	<i>NCOA1, NCOA2, NCOR1, SRC, NCOA3, ESR1</i>	5.900138396894648E-8
hsa05224:Breast cancer	6	<i>NCOA1, JUN, SPI, NCOA3, FOS, ESR1</i>	1.5689863645171283E-7
hsa05200:Pathways in cancer	7	<i>NCOA1, HSP90AA1, JUN, SPI, NCOA3, FOS, ESR1</i>	3.6991021891065767E-6

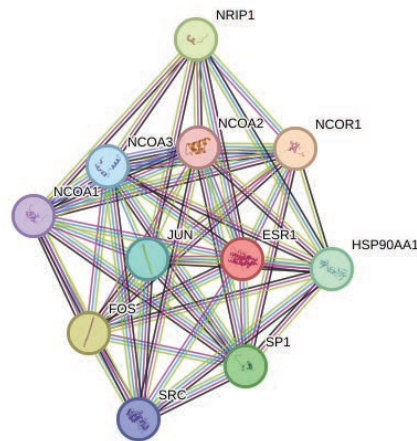


Figure 9. PPI network of *ESR1* using STRING tool

Further, the top five terms from the GO and KEGG analyses were observed using the DAVID tool (**Figure 10**). KEGG pathway analysis identified terms such as the Estrogen signaling pathway, Endocrine resistance, Thyroid hormone signaling pathway, Breast cancer, and Pathways in cancer (**Figure 10A**). GO analysis of biological processes (BP) revealed links to positive regulation of transcription from RNA polymerase II promoter, response to progesterone, cellular response to estradiol stimulus, cellular response to hormone stimulus, and DNA-templated positive regulation of transcription (**Figure 10B**).

Additionally, GO analysis of cellular components (CC) indicated enrichment in chromatin, nucleoplasm, RNA polymerase II transcription factor complex, nucleus, and transcription factor complex (**Figure 10C**). In terms of molecular function (MF), enrichment was observed in ligand-dependent nuclear receptor binding, estrogen receptor binding, histone deacetylase binding, RNA polymerase II sequence-specific DNA-binding transcription factor binding, and RNA polymerase II core promoter proximal region sequence-specific DNA binding (**Figure 10D**). These findings provide valuable insights into the functional role of *ESR1* and its associated genes in various pathways and biological processes.



Figure 10. KEGG and GO analysis of *ESR1* in BRCA using the DAVID tool

4. Discussion

Cancer is a heterogeneous and deadly disease, and comprehensive research has been conducted for many years. Cancer patients continue to experience high mortality rates because early diagnosis remains a challenge^[34-36]. Breast cancer, as a diverse disease, is the second most common type of cancer, with millions of cases and deaths. The number of BRCA cases varies by region, with more than 50% occurring in developed countries^[37,38]. Major risk factors associated with BRCA include alcohol consumption, aging, hormonal status, family history, nutrition, obesity, and genetic mutations^[39]. Treatments for BRCA typically include surgery, radiotherapy, chemotherapy, endocrine therapy, and targeted therapies. Approximately 75% of BRCA cases are ER⁺, making ET a highly recommended and effective treatment. Although most early-stage BRCA cases respond well to ET, the cancer often reappears later. While ET is beneficial for metastatic BRCA, 15%–20% of these cases show resistance to the therapy.

The *ESR1* gene codes for ER, and mutations in the ligand-binding domains, activation of signaling pathways, or stimulation by growth factors can lead to *ESR1* mutations. Studies suggest that these *ESR1* mutations contribute to poor prognosis in BRCA and lead to resistance to ET^[40,41]. Therefore, it is essential to assess the potential of *ESR1* as a diagnostic, prognostic, and therapeutic biomarker. The present study conducted a bioinformatics analysis of *ESR1* in BRCA.

Initially, expression analysis of *ESR1* in BRCA was performed using the UALCAN database. The results demonstrated a significant upregulation of *ESR1* in BRCA samples compared to normal samples, suggesting that *ESR1* plays a role in BRCA progression. Following this, expression analysis of *ESR1* was conducted across various parameters, including age, gender, race, and cancer stage, revealing the upregulation of *ESR1* in all categories. These findings suggest that *ESR1* acts as an oncogene and has potential as a diagnostic marker. Additionally, promoter methylation analysis of *ESR1* in BRCA and normal samples, also using UALCAN, indicated hypomethylation of *ESR1* in BRCA samples. Further assessment of promoter methylation based on parameters such as age, race, gender, and cancer stage also revealed hypomethylation of *ESR1*. Given that methylation is inversely correlated with gene expression, hypomethylation of *ESR1* indicates its upregulation and role in BRCA progression.

KM plotter was employed to analyze the survival impact of *ESR1* in BRCA. The results revealed that high *ESR1* expression was associated with poor prognosis, while low expression corresponded with better outcomes. These findings are consistent with the observation that *ESR1* is highly expressed in BRCA, contributing to poor prognosis. Genetic mutation analysis showed a 2.5% mutation rate in *ESR1*, including amplification and deep deletion. Previous studies have noted that *ESR1* amplification is a significant cause of ET resistance, leading to poor prognosis^[41]. To verify these results, GEPIA2 was used to conduct a box plot, stage plot, and survival analysis of *ESR1* in BRCA, further confirming that *ESR1* is upregulated and a major factor in poor prognosis. These results emphasize the potential of *ESR1* as a biomarker in BRCA.

Pathway enrichment analysis was also performed to evaluate the biological role of *ESR1*. The construction of a PPI network using the STRING tool revealed 10 genes that interact with *ESR1* (**Figure 9**). Further, KEGG and GO analyses using the DAVID tool indicated pathways linked with *ESR1*. GO analysis of biological processes highlighted positive regulation of transcription from RNA polymerase II promoter, response to progesterone, cellular response to estradiol stimulus, and DNA-templated transcription. Cellular component analysis revealed enrichment in chromatin, nucleoplasm, RNA polymerase II transcription factor complex, and transcription factor complex. Molecular function analysis showed enrichment in ligand-dependent nuclear receptor binding, estrogen receptor binding, histone deacetylase binding, and RNA polymerase II core promoter proximal region sequence-specific DNA binding. These findings provide valuable insights into the functional

role of *ESR1* and its associated genes in various pathways and biological processes. Altogether, these results underscore *ESR1*'s potential as a prognostic, diagnostic, and therapeutic biomarker in BRCA.

5. Conclusion

This study highlighted the diagnostic and prognostic significance of *ESR1* in BRCA by investigating its expression pattern, methylation level, and genetic mutation using various bioinformatics tools. These results support *ESR1* as a potential biomarker in BRCA, and further research in this direction is crucial.

Disclosure statement

The author declares no conflict of interest.

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Study on the Expression, Prognosis, and Clinical Features of HOXA6 in Liver Cancer

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Abstract: *Objective:* To study and analyze the expression level of homeobox A6 (HOXA6) in patients with liver cancer and its correlation with prognosis. *Methods:* From January 2020 to July 2021, 43 patients with liver cancer who underwent surgery without prior radiotherapy or chemotherapy were selected. Liver cancer tissues and adjacent tissues were collected, and the expression levels of HOXA6 mRNA and protein were measured using RT-qPCR and Western blot. The expression level of HOXA6 in tumor tissues (without radiotherapy and chemotherapy) was compared with that in adjacent tissues. Additionally, the prognosis and clinical characteristics of patients with low HOXA6 expression were compared to those with high HOXA6 expression, and the factors influencing high HOXA6 expression in liver cancer patients were analyzed. *Results:* HOXA6 mRNA and protein expression levels in tumor tissues were significantly higher than in adjacent tissues ($P < 0.05$). There was no significant difference in the 1-year and 2-year survival rates between the high HOXA6 expression group and the low HOXA6 expression group ($P > 0.05$). However, the 3-year survival rate was lower in the high HOXA6 expression group compared to the low HOXA6 expression group ($P < 0.05$). There were no statistically significant differences between the two groups in terms of gender, age, tumor diameter, alpha-fetoprotein levels, or hepatitis B virus DNA levels ($P > 0.05$). However, significant differences were found in the number of tumor lesions, degree of differentiation, and the proportion of tumor metastasis between the two groups ($P < 0.05$). Multivariate logistic regression analysis revealed that the number of tumor lesions, degree of differentiation, and tumor metastasis were influencing factors for high HOXA6 expression in liver cancer patients ($P < 0.05$). *Conclusion:* HOXA6 expression levels are abnormally elevated in liver cancer patients, and higher HOXA6 expression is associated with a worse 3-year survival rate. The factors influencing HOXA6 expression include the number of tumor lesions, degree of differentiation, and tumor metastasis.

Keywords: Liver cancer; HOXA6; Expression level; Clinical features; Influencing factors

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1. Introduction

Hepatocellular carcinoma (HCC) is a malignant tumor that occurs in hepatocytes or intrahepatic bile duct epithelial cells. It arises from multiple factors, including hepatitis virus, alcohol, high-fat diet, cirrhosis, and environmental influences. In the early stages, clinical symptoms are not specific, while in the middle and late stages, symptoms such as wasting, fatigue, poor appetite, abdominal distension, and hepatic pain become

more prominent, leading to a low survival rate among patients. Early diagnosis is crucial in enabling timely interventions. Analyzing the gene expression profiles of cancer cells plays a significant role in cancer diagnosis and treatment.

HOX genes are essential regulators in vertebrate embryonic development and organ formation, with a role in regulating transcription^[1,2]. Homeobox A6 (*HOXA6*), an important member of the *HOX* family, not only encodes and regulates gene expression but also governs transcription factors involved in cell differentiation. It plays a key role in cell differentiation, proliferation, and invasion^[3]. Studies have shown that *HOXA6* is implicated in glioma and other cancer types, with overexpression observed in tumor tissues of cancer patients, which can promote tumor cell proliferation^[4]. However, the expression of *HOXA6* in liver cancer and its impact on prognosis remains unclear. Identifying tumor markers related to liver cancer development and prognosis is crucial for understanding its pathogenesis, guiding treatment plans, and evaluating patient outcomes.

In this study, 43 liver cancer patients were selected, and the diagnosis and treatment were conducted at our hospital between January 2020 and July 2021. RT-qPCR and Western blot were employed to detect the expression levels of *HOXA6* mRNA and protein, aiming to analyze the expression of *HOXA6* in liver cancer and its correlation with patient prognosis.

2. Materials and methods

2.1. General information

A total of 43 patients with liver cancer were selected for the study. The male-to-female ratio was 24:19, with a mean age of 48.36 ± 3.26 years. The study protocol was approved by the Medical Ethics Committee.

Inclusion criteria: (1) Liver cancer was pathologically confirmed, meeting the diagnostic criteria outlined in the Code for Diagnosis and Treatment of Primary Liver Cancer (2019 Edition)^[5]; (2) Patients had surgical indications and voluntarily underwent hepatocellular carcinoma resection; (3) Patients were fully informed about the study protocol; (4) Patients cooperated with all required examinations.

Exclusion criteria: (1) Patients who received immunotherapy, radiotherapy, or chemotherapy before surgery; (2) Patients with other malignant wasting diseases; (3) Patients with incomplete clinical or follow-up data; (4) Patients with inflammation in adjacent tissues; (5) Patients with uncontrolled infectious lesions.

2.2. Methods

2.2.1. Immunohistochemistry of paraffin-embedded tissue

Liver cancer tissues and adjacent normal tissues were collected, fixed in formalin, and embedded in paraffin. After sectioning, the samples were dewaxed, and hydrated, and antigen retrieval was performed using sodium citrate buffer (boiling for 15 minutes). After cooling to room temperature, the samples were incubated with 3% hydrogen peroxide for 15 minutes to remove endogenous peroxidase, followed by sealing with goat serum for 40 minutes. The primary antibody (1:150) was incubated overnight at 4°C and then rewarmed. The secondary antibody was incubated at 37°C for 1 hour. Following color development using DAB, the tissues were restained with hematoxylin, dehydrated, and sealed. Scoring of the staining depth was independently conducted by two pathologists. A score of 2–3 indicated high *HOXA6* expression, while a score of 0–1 indicated low *HOXA6* expression.

2.2.2. RT-qPCR method

Total RNA was extracted from liver cancer tissues and adjacent tissues using the Trizol RNA extraction

kit. cDNA was synthesized using a reverse transcription kit. After reverse transcription into cDNA, qRT-PCR was performed. HOXA6 primers: upstream 5'-TACACGCGCTACCagAC-3', downstream 5'-GCGTGGAAATTGATGAGCTTGTTT-3'. The reaction system was as follows: upstream and downstream primers (0.5 μ L each), 10 \times amplification buffer and dNTP (2 μ L total), 0.2 μ g template DNA, Taq DNA polymerase (0.2 μ L), Mg²⁺ (1.5 μ L, 1.5 mmol/L), and 20 μ L double-distilled water. Reaction conditions: 94°C for 10 minutes, followed by 30 cycles of 94°C for 15 seconds, and annealing/extension at 60°C for 32 seconds. The CT values for each sample were calculated, and the expression level of HOXA6 mRNA was determined using the 2^{- $\Delta\Delta$ CT} method.

2.2.3 Western blot

Total protein was extracted from liver cancer and adjacent tissues using the RIPA method, and protein concentration was determined using the BCA method. A 20 μ g protein sample was subjected to SDS-PAGE, transferred to a PVDF membrane, and blocked overnight with 50g/L skim milk powder at 4°C. The primary antibody was incubated overnight at 4°C. After incubation with the secondary antibody at room temperature for 2 hours, strip scanning was performed following development with an ECL luminescence kit. ImageJ software was used for analysis.

2.3. Observation indicators

(1) The prognosis of patients with low HOXA6 expression was compared with that of patients with high HOXA6 expression. The 1-year, 2-year, and 3-year survival rates were compared between the two groups.

(2) Clinical characteristics, including gender, age, number of tumor lesions, tumor diameter, degree of differentiation, tumor metastasis, and the expression levels of alpha-fetoprotein (AFP) and hepatitis B virus (HBV DNA), were compared between the low and high HOXA6 expression groups.

(3) Factors influencing high HOXA6 expression in liver cancer patients were analyzed.

2.4. Statistical analysis

SPSS 25.0 software was used for data analysis. The results were expressed as [*n* (%)] and mean \pm standard deviation (SD). Factors influencing high HOXA6 expression in liver cancer patients were analyzed using χ^2 test data and multivariate logistic regression. A *P*-value < 0.05 indicated statistical significance.

3. Results

3.1. HOXA6 expression levels in tumor tissues versus adjacent tissues

Compared to adjacent non-cancerous tissues, both HOXA6 mRNA and protein expression levels were significantly higher in tumor tissues of liver cancer patients, with statistical significance (*P* < 0.05). See **Table 1**.

Table 1. Comparison of HOXA6 expression levels between tumor tissues and adjacent tissues (mean \pm SD)

Groups	Number of cases	HOXA6 mRNA	HOXA6 protein
Adjacent tissues	43	157.36 \pm 20.13	4.25 \pm 1.03
Tumor tissues	43	183.19 \pm 21.04	6.37 \pm 0.98
<i>t</i>		5.817	9.778
<i>P</i>		0.000	0.000

3.2. Prognosis comparison between low and high HOXA6 expression groups

No significant differences were observed in 1-year and 2-year survival rates between the low and high HOXA6 expression groups ($P > 0.05$). However, the 3-year survival rate was significantly lower in the high HOXA6 expression group compared to the low expression group ($P < 0.05$). See **Table 2**.

Table 2. Prognosis comparison between low and high HOXA6 expression groups [n (%)]

Groups	Number of cases	1-year survival rate	2-year survival rate
Low HOXA6 expression group	20	18 (90.00)	15 (75.00)
High HOXA6 expression group	23	21 (91.30)	13 (56.52)
χ^2		0.022	1.608
P		0.883	0.205

3.3. Comparison of clinical features between low and high HOXA6 expression groups

There were no statistically significant differences in gender, age, tumor diameter, AFP, or HBV DNA levels between the high and low HOXA6 expression groups ($P > 0.05$). However, statistically significant differences were found in the number of tumor lesions, degree of differentiation, and tumor metastasis between the two groups ($P < 0.05$). See **Table 3**.

Table 3. Comparison of clinical features between low and high HOXA6 expression groups [n (%)]

Outcome measures		HOXA6 high expression group ($n = 23$)	HOXA6 low expression group ($n = 20$)	χ^2	w
Gender	Male	13 (56.52)	11 (55.00)	0.010	0.920
	Female	10 (43.48)	9 (45.00)		
Age (years)	< 60	14 (60.87)	10 (50.00)	0.513	0.474
	≥ 60	9 (39.13)	10 (50.00)		
Number of tumor lesions	Single	14 (60.87)	5 (25.00)	5.581	0.018
	Multiple	9 (39.13)	15 (75.00)		
Tumor diameter (cm)	≤ 5	9 (39.13)	8 (40.00)	0.003	0.954
	> 5	14 (60.87)	12 (60.00)		
Degree of differentiation	Low differentiation	5 (21.74)	14 (70.00)	10.103	0.001
	Medium-high differentiation	18 (78.26)	6 (30.00)		
Tumor lesion metastasis	Yes	6 (26.09)	12 (60.00)	5.055	0.025
	No	17 (73.91)	8 (40.00)		
AFP (ng/mL)	≤ 400	10 (43.48)	11 (55.00)	0.568	0.451
	> 400	13 (56.52)	9 (45.00)		
HBV DNA (IU/mL)	≤ 400	6 (26.09)	9 (45.00)	01.685	0.194
	> 1000	17 (73.91)	11 (55.00)		

3.4. Factors influencing high HOXA6 expression in liver cancer patients

Using variables that showed statistical significance in univariate analysis, a multivariate logistic regression was performed. The results indicated that the number of tumor lesions, degree of differentiation, and tumor metastasis were factors influencing the high expression of HOXA6 in liver cancer patients ($P < 0.05$). See **Table 4**.

Table 4. Influencing factors of HOXA6 expression in liver cancer patients

Influencing factors	β	SE	Wald	<i>P</i>	OR	95% CI
Number of tumor lesions	0.711	0.322	4.887	0.027	2.037	1.084–3.828
Degree of differentiation	0.631	0.315	4.004	0.045	1.879	1.013–3.485
Tumor focus metastasis	1.046	0.510	4.211	0.040	2.845	1.048–7.723

4. Discussion

Hepatocellular carcinoma is among the most prevalent malignant tumors worldwide, with hepatocellular carcinoma being the most common form of primary liver cancer. Its clinical course is highly variable, and its pathological process mirrors embryonic mechanisms. The *HOX* gene plays a crucial role in early liver development, and mutations in *HOX* genes may significantly impact the progression of liver cancer^[6]. The *HOX* gene family is largely characterized by gene clusters, with the *HOXA* gene cluster located on chromosome 7. This cluster contains six long-chain non-coding RNAs and 11 protein-coding genes, which are essential in the development of vertebrate organs, limbs, axons, and the central nervous system. Abnormal gene regulation may play a pivotal role in the onset of malignant tumors^[7,8]. The *HOX* gene can act as either an oncogene or a tumor suppressor, and its expression level is prone to alterations in cancers such as colon cancer, lung cancer, brain tumors, breast cancer, leukemia, and others^[9,10].

HOXA6 encodes transcription factors and plays a regulatory role in cell differentiation and gene expression. However, its role varies across different cancers^[11]. Research has shown that *HOXA6* is hypermethylated in certain malignant tumors, and its expression is downregulated in breast cancer tissues^[12,13]. In this study, *HOXA6* mRNA and protein expression levels in liver cancer tumor tissues were significantly higher than in adjacent non-cancerous tissues ($P < 0.05$), suggesting that *HOXA6* plays an important role in the onset and progression of liver cancer. Patients with high *HOXA6* expression levels had a lower 3-year survival rate compared to those with low *HOXA6* expression levels ($P < 0.05$). Detecting *HOXA6* expression levels could provide valuable insights for clinical evaluation of patient prognosis and survival prediction.

The *HOX* family plays an important role in tumor regulation. Studies have demonstrated that *HOXA6* is highly expressed in various malignant tumors, correlating with cell invasion, proliferation, and chemoresistance^[11]. For instance, *HOXA6* has been shown to promote the proliferation, invasion, and metastasis of colorectal cancer cells^[3,14]. In this study, multivariate logistic regression analysis indicated that the number of tumor lesions, the degree of differentiation, and tumor metastasis were the key factors influencing the high expression of *HOXA6* in liver cancer patients ($P < 0.05$). Clinically, *HOXA6* expression in liver cancer patients can be assessed by observing the number of tumor lesions, the degree of tumor differentiation, and the presence of metastasis^[15].

In conclusion, *HOXA6* expression is abnormally elevated in liver cancer patients and is correlated with the 3-year survival rate. Factors influencing *HOXA6* expression include the number of tumor lesions, differentiation degree, and metastasis. Detecting *HOXA6* can help elucidate the mechanisms of tumor development and progression. Additionally, it can aid in assessing disease severity and guide clinical diagnosis of liver cancer. Therefore, clinical detection of *HOXA6* expression should be enhanced, and patient prognosis should be evaluated based on *HOXA6* expression levels to adjust treatment strategies accordingly.

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Disclosure statement

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Roles of Mutant *TP53* Gene in Cancer Development and Progression

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Abstract: *TP53* is a tumor suppressor gene that is mutated in most cancer types and has been extensively studied in cancer research. p53 plays a critical role in regulating the expression of target genes and is involved in key processes such as apoptosis, cell cycle regulation, and genomic stability, earning it the title “guardian of the genome.” Numerous studies have demonstrated p53’s influence on and regulation of autophagy, ferroptosis, the tumor microenvironment, and cell metabolism, all of which contribute to tumor suppression. Alterations in p53, specifically mutant p53 (mutp53), not only impair its tumor-suppressing functions but also enhance oncogenic characteristics. Recent data indicate that mutp53 is strongly associated with poor prognosis and advanced cancers, making it an ideal target for the development of novel cancer therapies. This review summarizes the post-translational modifications of p53, the mechanisms of mutp53 accumulation, and its gain-of-function, based on previous findings. Additionally, this review discusses its impact on metabolic homeostasis, ferroptosis, genomic instability, the tumor microenvironment, and cancer stem cells, and highlights recent advancements in mutp53 research.

Keywords: p53; Cancer; Mutant p53 (mutp53); Progression; Treatment

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1. Introduction

The “guardian of the genome” protein, known as the p53 tumor suppressor, is encoded by the *TP53* gene. Its primary biological role is the preservation of cellular DNA integrity. In addition, *TP53* is involved in cell differentiation, aging, and development. The p53 protein acts as a transcription factor, influencing numerous biological processes depending on the type of cellular stress signals it receives ^[1]. Oncogene activation, DNA damage, and replication stress are recognized stress signals that activate p53. In response to these stimuli, p53 undergoes post-translational modifications that promote the transcription of genes involved in specific cellular responses based on the type of stress, ultimately determining the fate of the cell ^[2].

Wild-type p53 (wtp53) protein binds to specific DNA response elements, leading to the expression of genes that prevent the onset and spread of cancer. Under normal conditions, the p53 signaling pathway is activated when cells encounter various stress signals. This activation allows the cells to engage in transcriptional

programs such as cell cycle arrest, DNA repair, senescence, and apoptosis, which inhibit tumor growth ^[3]. Inactivation of the *TP53* gene frequently results from loss-of-function mutations or negative regulation of wtp53 proteins in most, but not all, human malignancies ^[4]. The inactivation of *TP53* promotes cell survival, invasion, and proliferation, accelerating cancer progression. In more than 75% of *TP53* gene mutations, wtp53 functions are lost. Mutant p53 (mutp53) proteins may act as dominant negatives to wtp53 activity or acquire new tumorigenic traits that negate the tumor-suppressive effects of wtp53. p53 regulates the cell cycle, apoptosis, DNA repair, and senescence in response to hypoxia, oncogene activation, DNA damage, and nutrient deprivation ^[5].

The p53 transcription factor consists of six domains: the transactivation domain, the proline-rich domain, the DNA-binding domain, the nuclear localization signal domain, the oligomerization domain, and the C-terminal domain. The transactivation domain recruits transcriptional co-activators to enhance RNA transcription, while negative regulators can inhibit its activity ^[6]. The proline-rich domain supports transcription, and p300/CBP can bind to it to boost p53's transcriptional activity. The DNA-binding domain stabilizes the protein's structure, and single-point mutations in this domain can result in the loss of all p53 functions. The nuclear localization signal domain and oligomerization domain are essential for nuclear localization and transcriptional activity. The C-terminal domain acts as a negative autoregulatory domain by inhibiting DNA binding, which can be modulated by post-translational modifications ^[7].

Genomic instability is a key hallmark of human cancers and is largely driven by the gain-of-function activity of mutant p53. In breast cancer specimens, aberrant copy numbers are associated with mutant p53 and can lead to centrosome deviations, resulting in centrosome multiplication ^[8]. Furthermore, mutant p53 can bind to chromatin-regulated genes and promote histone methylation and acetylation, both of which contribute to genetic instability and cancer progression ^[9]. Mutant p53 complexes with p63 and p73 reduce their ability to control tumor growth and prevent apoptosis. Additionally, other proteins or transcription factors, such as disabled homolog 2-interacting protein (DAB2IP), poly(ADP-ribose) polymerase (PARP), nuclear transcription factor-Y (NF-Y), and sterol regulatory element binding proteins (SREBPs), collaborate with mutant p53 to promote breast cancer development via activation of the mevalonate pathway. Evidence suggests that different types of tumors have distinct *TP53* mutational spectra ^[10].

In a study of tissue samples from 10,000 cancer patients, *TP53* mutations were found in 42% of cases. The mutation frequency varies across cancer types, with small-cell lung cancer showing a mutation frequency of 89.02% and colorectal cancer at 72.69% ^[11]. However, cancers such as thyroid, cervical, and bone cancer exhibit significantly lower frequencies of *TP53* mutations. G to T transversions are commonly observed in lung and liver cancers, while CpG dinucleotide hotspot transitions are prevalent in leukemia, brain tumors, and colorectal cancer. Base pair mutations are frequently seen in esophageal cancer. Even within the same organ, different tumor subtypes can show variations in *TP53* mutation patterns. For example, an analysis of *TP53* mutations in 572 breast tumors found that truncating mutations were more common in basal breast cancers, while missense mutations, specifically A to G transitions, predominated in luminal breast cancers ^[12].

Additionally, there is a correlation between external risk factors and the *TP53* mutational spectrum in tumors. For instance, aggressive squamous cell carcinomas of the skin undergo CC to TT double base transitions when exposed to UV light, while smokers experience more G to T transversions in lung cancer compared to non-smokers. In primary hepatocellular carcinoma, aflatoxin B1 induces G to T transversions in codon 249 of *TP53*. Interestingly, *TP53* mutations are associated with poor prognoses in malignant tumors ^[13].

2. Role of microRNAs in influencing p53

It has been documented that several microRNAs (miRNAs) either directly or indirectly inhibit p53 expression and that p53, in turn, regulates the expression of miRNAs. For miRNAs to distinguish between mutant and wtp53, they must directly target the specific altered region of the mutant *p53* mRNA. So far, no miRNAs unique to mutant p53 have been shown to suppress wtp53. In specific cases, miRNAs that target important positive regulators of mutant p53 may reduce the expression of mutant p53. In line with this, artificial siRNA and shRNA oligonucleotides have been developed with the specific goal of targeting mutant p53 and reducing tumor growth *in vivo*.

Beyond its direct regulation of gene expression through interaction with the p53 response element (RE) of genes, p53 also regulates gene products by transcriptionally controlling miRNAs. It has been demonstrated that miRNAs activated by p53 play roles in the regulation of protein expression related to cell cycle progression, senescence, apoptosis, metastasis, angiogenesis, cellular stemness, and metabolic processes such as glycolysis^[14,15].

p53 has been found to regulate the transcription of several miRNAs, including the *miR-34* family, *miR-145*, *miR-107*, *miR-192*, and *miR-215*. The *miR-34* family (*miR-34a-c*), which reduces the expression of proteins associated with cell cycle progression and activation of cell growth and survival, as well as immune checkpoints like Cyclin E2, cyclin-dependent kinase 4 (CDK4), CDK6, BCL-2, and programmed death-ligand 1 (PDL-1), was the first group of miRNAs identified as being stimulated by p53. Other miRNAs, such as *miR-145* and *miR-107*, which regulate the oncogene *c-Myc* and hypoxia-inducing factor-1 beta (*HIF-1β*), respectively, are involved in oncogene repression and the response to hypoxia and angiogenesis^[16]. Through regulating CDK6 and RB transcriptional corepressor-like 2 (RBL2) expression, *miR-107* also plays a role in controlling the G1-S cell cycle transition.

Following genotoxic stress, p53 upregulates *miR-192* and *miR-215*, which in turn regulate the expression of molecules involved in cell cycle progression at the G1 and G2-M checkpoints, including RAD51, TOP1, MCM3, RB1, CDC7, MCM10, and MCM6^[17,18]. In addition to miRNA regulation of cell cycle genes, *miR-205* controls cell cycle progression by downregulating E2F1 and metastatic activity by targeting laminin subunit gamma-1 (LAMC1), which is associated with cell adhesion and migration. Other p53-regulated miRNAs implicated in epithelial-mesenchymal transition (EMT)-mediated metastasis include the *miR-34* family, which inhibits zinc finger E-box binding homeobox 1/2 (ZEB1/2) expression, and *miR-34*, which inhibits Snail1.

p53 also regulates its own stability and efficiency by activating miRNAs that target Mdm2 and Mdmx, such as *miR-192*, *miR-194*, *miR-215*, *miR-143*, *miR-145*, and *miR-34a*^[19].

3. p53 family members

The transcription factors p73 and p63 are encoded by the *TP73* and *TP63* genes, respectively, which are homologous to *TP53*. The DNA-binding domain is the most highly conserved motif among members of the p53 family, while the oligomerization domain and transactivation (TA) domains are the least similar. Due to these similarities, p73 and p63 can bind to conventional p53 REs, oligomerize, and transactivate p53 target genes^[20]. As a result, p73 and p63 also play roles in anti-tumor mechanisms, such as apoptosis and cell division regulation. However, due to their differences, p73 and p63 can be involved in distinct biological activities compared to p53. For instance, a variant of the TA domain prevents MDM2 from regulating p73^[21].

Moreover, while p53-null mice exhibit normal development but a wide range of tumor malignancies, p73- and p63-null mice survive but show developmental abnormalities. This suggests that p73 and p63 may have a stronger role in regulating cell differentiation compared to their tumor-suppressive functions, at least in the

early stages of development. Unlike p53, the tumor-suppressive properties of p73 and p63 are not lost due to mutation or allelic loss. Instead, the presence of two promoters results in the production of proteins that may either be pro-tumorigenic (ΔN , lacking the TA domain) or tumor-suppressive (TA). Indeed, the ΔN isoforms are expressed in several cancers, and alternative mRNA splicing processes produce distinct protein isoforms ^[22].

In *TP73*, there are 7 ΔN isoforms (ΔN - α , ΔN - β , ΔN - γ , ΔN - δ , ΔN - ϵ , ΔN - ξ , ΔN - η) and 7 TA isoforms (α , β , γ , δ , ϵ , ξ , η). In *TP63*, there are 5 ΔN isoforms (ΔN - α , ΔN - β , ΔN - γ , ΔN - δ , ΔN - ϵ) and 5 TA isoforms (α , β , γ , δ , ϵ). Although TA- α isoforms are more structurally similar to p53 and correspond with p63's transcriptional activity, TA- α is a more potent transcriptional activator and inducer of apoptosis in p73. The sterile α motif (SAM) is a critical distinction in the structures of p73, p63, and p53, as it allows p73 and p63 to interact with proteins involved in physiological processes ^[23].

Both p73 and p63 are activated in response to cellular stressors such as hypoxia and DNA damage. Similar to p53, their function is regulated by post-translational modifications such as ubiquitination, acetylation, and phosphorylation. Once activated, p63 and p73 can form both homo-tetramers and hetero-tetramers. Although some studies have shown that p73 can bind to p53, leading to the activation of Puma and Bax, others have found that neither p73 nor p63 forms hetero-tetramers with wtp53 ^[24]. This discrepancy may depend on the type of stress signal and the phosphorylation status of p53.

In certain models, p53 is required for apoptosis following DNA damage, and this process involves p73 and p63. P73 can transactivate p53 target genes such as *Puma*, *Noxa*, *RAD17*, and *p21* ^[25]. Similarly, p63 can upregulate p53 target genes such as *GADD45*, *PIG3*, *p21*, and *Bax*. Both p73 and p63 also regulate genes specific to their function that are not shared by p53. However, little is known about the unique gene regulation of TAp73 and TAp63 isoforms and their role in carcinogenesis. It is understood that each isoform has different functions in gene expression regulation and may be expressed differently in various tissues.

While p63 is generally expressed at low levels in both normal and malignant tissues, its high expression is primarily restricted to the female germline. Notably, the loss of the tumor-suppressive function of TA isoforms is associated with the production of ΔN isoforms. Although TA isoforms do not interact with wtp53, they can interact with mutant p53, thereby inhibiting the tumor-suppressive properties of TAp73 and TAp63 ^[26].

The potential cellular processes and key roles of the p53 family in cancer development, progression, and therapeutic options are summarized in **Table 1**.

Most tumor cells carry mutations in the *TP53* gene. According to genome sequencing of various human cancer cells, *TP53* mutations are present in 42% of cases. The DNA-binding domain (DBD) is the most frequently mutated region in *TP53*, and missense mutations, involving a single amino acid substitution, are the most common type of mutation. p53 mutants are classified into two major categories: structural mutations and DNA contact surface mutations. Structural mutants (e.g., R175H, R249S, G245S, and Y220C) exhibit reduced protein thermostability, leading to improper protein folding at physiological temperatures and the inability to bind DNA. Of these, R175H and C176Y mutations specifically affect the protein's affinity for zinc ions. DNA contact surface mutants (e.g., R273H/C, R248W) occur within the core DNA-binding region, where alterations prevent the protein from binding to DNA ^[27].

The most common mutation sites in *TP53*—R175, G245, R249, R282, R248, and R273—are collectively referred to as “hot spot” variants. These mutations not only bind to wtp53 to exert dominant-negative (DN) effects but also have the potential to acquire gain-of-function (GOF) properties, converting them into oncogenic proteins. Consequently, *TP53* differs from many “classical” oncogenes, which are typically inactivated by truncating or nonsense mutations that result in a non-functional, shortened protein ^[28].

Table 1. Critical roles of p53 in cancer development and treatment

Role of p53	Cancer development	Cancer progression	Treatment approaches
Tumor suppressor	p53 prevents the accumulation of DNA damage by inducing cell cycle arrest or apoptosis. Mutations in p53 lead to genomic instability and uncontrolled cell growth.	It inactivates pro-apoptotic pathways, promotes angiogenesis, and contributes to metastasis. Mutant p53 can also gain oncogenic functions.	The restoration of wild-type p53 function, inhibition of mutant p53 activity, and targeting p53-related pathways.
Cell cycle regulation	p53 pauses the cell cycle at the G1 checkpoint to allow DNA repair. Loss of p53 leads to uncontrolled cell division and tumor formation.	p53 disrupts cell cycle checkpoints, allowing for rapid cell proliferation and tumor growth.	Targeting cell cycle checkpoints and inducing apoptosis in p53-deficient cells.
Apoptosis	Induces programmed cell death to eliminate damaged cells. Loss of p53 leads to the accumulation of damaged cells and increased cancer risk.	p53 prevents apoptosis, allowing tumor cells to survive and proliferate.	Induction of apoptosis in cancer cells through various mechanisms.
DNA repair	Activates DNA repair pathways to maintain genomic stability. Loss of p53 impairs DNA repair, leading to genetic mutations and cancer development.	Contributes to genomic instability and accumulation of cancer-causing mutations.	Enhancing DNA repair mechanisms or targeting DNA damage response pathways.
Angiogenesis	p53 inhibits blood vessel formation to prevent tumor growth. Loss of p53 promotes angiogenesis, supporting tumor growth and metastasis.	p53 stimulates angiogenesis, providing nutrients and oxygen to the tumor.	Anti-angiogenic therapies to inhibit blood vessel formation.
Immune response	p53 regulates the immune response to cancer cells. Loss of p53 impairs immune surveillance, allowing tumor growth.	Suppresses immune response, creating an immunosuppressive tumor microenvironment.	Immunotherapy to enhance the immune response against cancer cells.

4. Post-translational modifications in mutant p53

Mutant p53 is also capable of undergoing post-translational modifications, although the resulting biological effects differ from those of wtp53. Interestingly, regions of wtp53 frequently modified post-translationally are also often mutated across various cancer types. This is partly due to the presence of common hotspot mutations in p53 that are not modified post-translationally. However, there are regions in all p53 domains that undergo post-translational modifications and have been identified as altered in human tumors. Post-translational modifications of mutant p53 do not affect its ability to bind to specific DNA sequences or perform tumorigenesis-related functions. While wtp53 binds to its respective RE, mutant p53 lacks a defined DNA sequence to which it binds. Instead, mutant p53 interferes with the transcriptional programs of other proteins, such as transcription factors ^[29].

Mutant p53 has been found to exhibit phosphorylation, ubiquitination, acetylation, and methylation. Compared to wtp53, the effects of post-translational modifications on mutant p53 are less well understood. For example, research has shown a link between the carcinogenic activity of the R175H mutant and its hyperphosphorylation at Ser392. However, this seems to be type-dependent, as hyperphosphorylation is absent in certain breast cancer cell lines. Mutant p53 interacts with cofactors and transcription factors via other phosphorylation sites, such as Ser15, which provides it with a GOF advantage. Similarly, hyperacetylation of mutant p53 at Lys373 and Lys382 promotes its localization to the nucleus.

Moreover, the effects of cellular stress signaling, such as glucose deprivation, on mutant p53 differ from those on wtp53 ^[30]. Under these stress conditions, mutant p53 undergoes acetylation, leading to metabolic reprogramming that enhances its survival. In contrast to wtp53, which is heavily ubiquitinated to regulate protein levels during stress response resolution, mutant p53 is rarely ubiquitinated. This is primarily due to

the lack of a negative feedback loop and the absence of *MDM2* gene transactivation. As a result, mutant p53 becomes highly stable in various tumor types. However, certain mutant p53 variants can interact with MDM2 and be targeted for degradation, in addition to other ubiquitin ligases known to regulate wtp53 stabilization ^[31].

5. Mutant p53 mechanisms of destabilization

The ubiquitin-proteasome system (UPS) is responsible for the degradation of mutant p53, similar to wtp53. Mutant p53 has been found to be ubiquitinated by several E3 ubiquitin ligases. MDM2 can target certain mutant p53 variants for proteasomal destruction and/or ubiquitination. However, the rate of mutant p53 degradation is not always directly correlated with its ubiquitination status. For example, hyper-ubiquitinated mutant p53 remains stable and may aggregate primarily in the cytoplasm rather than the nucleus, although this varies depending on the type of mutant p53. The primary reason for the increased stability of mutant p53 is its inability to transcriptionally regulate the target genes of wtp53, which disrupts the negative feedback loop mediated by MDM2 ^[32]. It is possible that additional E3 ligases may ubiquitinate mutant p53 because the interaction between mutant p53 and MDM2 depends on the MDM2 RING domain rather than its E3 ligase activity. Research has shown that while ARF-BP1 is unaffected, E3 ligases Cop1 and CHIP are involved. Mutant p53 can evade proper protein surveillance by binding to heat-shock proteins (Hsp), preventing degradation caused by its unfolded state. In this scenario, mutant p53 binds to Hsp90, protecting it from CHIP E3 ligases and MDM2. Notably, Hsp90 is overexpressed in various cancers, which may contribute to the stability and GOF properties of mutant p53 ^[33].

Autophagy represents a potentially unique mechanism for regulating mutant p53 protein levels. Macroautophagy, or simply autophagy, is the process of intracellular degradation that occurs in response to cellular damage or the need to recycle components to balance energy expenditure and maintain cellular homeostasis. Vesicles are generated in an organized manner to enclose targeted cellular elements for autophagy-mediated degradation. These vesicles eventually fuse with lysosomes, where their contents are broken down ^[34,35]. Mutant p53 can be degraded by the autophagy machinery in response to physiological stressors such as glucose deprivation, and this process depends on the presence of deacetylated p53. Both autophagy and chaperone-mediated autophagy (CMA), which does not rely on vesicle formation, can be used to degrade mutant p53. The 70 kDa heat-shock cognate protein (Hsc70) mediates the selective autophagy mechanism known as CMA. Lysosome-associated membrane protein type 2A (Lamp-2A) directs substrate proteins associated with Hsc70 to the lysosome and facilitates their internalization into the lysosomal compartment. Under metabolic stress, hypoxia, and non-proliferative cell conditions, aggregated mutant p53 is polyubiquitinated at K63 by CHIP and interacts with Hsc70 and Lamp-2A, leading to lysosomal degradation ^[23].

6. Mutp53 accumulation in cancer

Elevated levels of mutp53 expression in tumor cells are necessary for its gain-of-function effects. However, the exact mechanisms of mutp53 accumulation in cancers remain unclear. Post-translational modifications are central to the regulation of p53 and are involved in numerous cellular signaling processes. Wtp53 acts as a transcriptional regulator specific to DNA sequences and becomes active in response to various stress stimuli. Its activity can be modulated by post-translational modifications, including phosphorylation, acetylation, and ubiquitination, which also affect mutp53 similarly to wtp53. Studies have shown that phosphorylation at sites such as Ser15, Thr81, and Ser392 can influence mutp53. For instance, phosphorylation of mutp53 at Ser15/Ser37 by DNA-PK enhances its stability and GOF in ovarian cancer. Conversely, in prostate cancer, nuclear

factor kappa-B (NF-κB) restriction leads to phosphorylation of mutp53 at Ser15, restoring its DNA-binding ability. Additionally, mutp53 can be modified through acetylation^[36]. Transformation/transcription domain associated protein (TRRAP), a component of several histone acetyltransferase complexes, is overexpressed, increasing mutp53 levels, while TRRAP silencing reduces mutp53 accumulation in lymphomas and colon cancers. Along with phosphorylation and acetylation, ubiquitination also plays a role in modulating mutp53. Normally, MDM2 regulates wtp53 at low levels by targeting it for proteasomal degradation. However, mutp53 does not effectively activate MDM2, which impairs MDM2's negative regulatory function. Nonetheless, one study found that the p53 R172H mutant is stabilized by MDM2 deletion^[37]. Mutp53 can be ubiquitinated and degraded by additional E3 ubiquitin ligases, including CHIP, COP1, and Pirh2. Co-chaperone and chaperone proteins like BAG5, Hsp90, and Hsp70 are also associated with the accumulation of mutp53 in human malignancies. BAG5 protects mutp53 from ubiquitin-mediated degradation by MDM2 and CHIP, while Hsp90 and Hsp70 help stabilize mutp53 through interactions with its DNA-binding domain^[38].

7. The p53 pathway

p53 is a transcription factor that is distributed in the nucleus and cytoplasm, binds specifically to DNA, and activates a variety of genes. Under normal conditions, cellular p53 protein levels are kept very low due to strict regulation by its negative regulators, MDM2 and MDMX, which promote p53 degradation through ubiquitination. In response to internal and external stresses such as DNA damage, hypoxia, starvation, and cancer cell risk, p53 ubiquitination is inhibited. This results in a significant increase in intracellular p53 protein levels^[39]. Post-translational modifications, including phosphorylation, acetylation, and methylation, enhance and maintain elevated levels of p53. Stabilized p53 binds to its target DNA, forms tetramers in the nucleus, and regulates gene transcription, thereby influencing downstream signaling pathways. As a well-studied tumor suppressor gene, p53 transcriptionally activates multiple genes involved in apoptosis and cell cycle regulation in response to cellular stress, thereby halting cellular processes and preventing the division of cells with damaged or mutated DNA. In addition to these classical functions, p53 also regulates several “non-classical” pathways, such as autophagy, metabolic balance, ferroptosis, stem cell differentiation, and the tumor microenvironment, as reported in various studies^[40].

7.1. Metabolic homeostasis

For tumor cells to grow rapidly and continuously, they require substantial amounts of biological energy and raw materials. According to the Warburg effect, tumor cells use glucose differently from normal cells, characterized by increased lactate production and heightened glycolysis. By regulating the glycolytic pathway, p53 acts as a tumor suppressor by maintaining cellular metabolic balance. p53 can transcriptionally regulate genes involved in oxidative phosphorylation, such as *SCO2*, and genes that inhibit glycolysis, like *TIGAR*^[41]. Additionally, to suppress the pentose phosphate pathway in tumor cells, p53 binds to G6PDH, the rate-limiting enzyme of the pathway. p53 also inhibits glucose uptake and glycolysis by reducing the production and translocation of glucose transporter proteins, including GLUT1 and GLUT4. In a dynamic sense, glycolysis and gluconeogenesis can be considered opposing mechanisms, with p53 suppressing glycolysis and promoting gluconeogenesis. Since the Warburg effect and glycolysis are crucial for tumor cell growth and metastasis, p53's suppression of glycolysis typically inhibits cancer development^[42].

Cancer cells can initiate different metabolic processes depending on environmental conditions. Mutant p53 stimulates the Warburg effect and enhances tumor metabolism by promoting the translocation of GLUT1

to the plasma membrane. Mutant p53 also enhances mitochondrial efficiency and promotes cancer metastasis by binding to and activating PGC-1 α , a key regulator of oxidative phosphorylation. This suggests that cancer cells with mutant p53 may exhibit greater metabolic plasticity, helping them adapt to stressful conditions and increasing their capacity for growth and metastasis. Tumor cells require lipids to proliferate and expand, and p53 promotes lipolysis, thereby inhibiting tumor growth. Cholesterol and nonsteroidal isoprenoid synthesis is regulated through the mevalonate pathway, in which SREBP2 plays a key transcriptional role. p53 prevents SREBP2 activation by transcriptionally activating the *ABCA1* cholesterol transporter gene and downregulating *USP19* and *SOAT1* to inhibit cholesterol esterification. Furthermore, p53 promotes fatty acid oxidation by upregulating *CPT1C*, *MCD*, and *PANK1* expression^[43].

Ammonia is a common byproduct of cellular metabolism. Cancer cells generate large amounts of ammonia during amino acid metabolism, which can serve as a nitrogen source for tumor formation. p53 regulates ammonia levels in cancer cells through the urea cycle. By inhibiting the expression of three key enzymes in the urea cycle—*CPS1*, *OTC*, and *ARG1*—p53 controls ammonia levels and, in turn, suppresses tumor growth. In addition to other metabolic signaling pathways, p53 plays a role in regulating tumor cell metabolism. The overproduction of reactive oxygen species (ROS) in tumor cells has a dual effect: it promotes tumor growth while also triggering ROS-dependent destruction pathways that lead to tumor cell elimination. p53 regulates ROS in two ways^[44]. As an upstream signal, ROS activates p53 to either promote or inhibit tumor growth, depending on the context. p53 then transcribes antioxidant genes, such as manganese superoxide dismutase and *GPX1*. Additionally, ROS can act as a downstream regulator of p53 to induce apoptosis and ferroptosis, leading to tumor cell death. p53 also influences both oxidative phosphorylation and the tricarboxylic acid cycle, mediating cancer cell death and regulating redox processes. Furthermore, p53 controls the metabolism of lipids, amino acids, and nucleotides^[45].

7.2. Mutp53 exerts gain-of-function

Different p53 mutations confer GOF in various ways. First, for mutp53 to function, it must interact with transcription factors (TFs). Wtp53 binds to DNA RE and recruits TFs, RNA polymerase II (to initiate transcription at open promoters), histone acetyltransferases (HATs) like p300, and chromatin-remodeling complexes (CRCs) like SNF and SWI, which adhere to acetylated histones. However, mutp53 is unable to bind to p53 DNA RE. Instead, it exerts its GOF by alternative mechanisms, often promoting cancer. For instance, mutp53 regulates the transcription of target genes by interacting with various TFs and cofactors, including NF-Y, p73, NRF2, and Ets-1^[46]. Mutp53 binds to NF-Y in response to DNA damage, recruiting p300 to acetylate histones, which leads to the overexpression of cell cycle genes and fosters tumor growth. Mutp53 can also bind to specific DNA structures, such as matrix attachment sites, regulating transcription in specific contexts. Additionally, mutp53 interacts with other proteins, modifying or inhibiting their functions. In colorectal and pancreatic cancers, mutp53 antagonizes p63/p73-mediated tumor suppression via the Notch1 signaling pathway. Notably, the gain-of-function activity of mutp53 is also influenced by its cellular localization. While mutp53 is typically found in the nucleus, some mutations cause it to localize to the cytoplasm. For example, a study found that p53 E258K, R273H, and R273L mutants localized to the cytoplasm and inhibited autophagy in colon cancer, while p53 P151H and R282W mutants remained in the nucleus and did not exhibit this effect^[47,48].

7.3. Genetic instability

Genomic instability is considered a hallmark of human cancers. As the “guardian of the genome,” wtp53 plays a critical role in maintaining genomic stability, whereas mutp53 can promote genomic instability. It has

been shown that mutp53 drives chromosomal instability and cell proliferation. For instance, in osteosarcoma, mutp53 interacts with topoisomerase I to induce gene amplification ^[49]. In pre-tumor thymocytes, mutp53 causes inter-chromosomal translocations. In lung cancer, mutp53 promotes the formation of DNA replication origins and stabilizes replication forks, leading to micronuclei formation and the propagation of cells with abnormal genomes. Additionally, mutp53 prevents the MRE11-RAD50-NBS1 complex from binding to DNA damage sites, thereby inactivating ATM and promoting genetic instability. In lung and breast cancer, mutp53 inhibits BRCA1 and RAD17 expression, contributing to genomic instability and impairing DNA damage repair. Intriguingly, cell-in-cell structures, a feature observed in many solid tumors, are facilitated by mutp53. In lung adenocarcinomas, mutp53 drives the formation of these structures through the engulfment of live cells, leading to abnormal mitosis, while wtp53 promotes the breakdown of these cells. Therefore, the interaction between mutp53 and genomic instability is crucial to cancer development ^[50,51].

7.4. Role of ferroptosis

Ferroptosis, an iron-dependent form of cell death, has been identified as a distinct mechanism for inhibiting tumor growth. Notably, the regulation of ferroptosis involves p53 in a complex but crucial manner. While most studies provide evidence supporting p53's role in promoting ferroptosis, p53 can also inhibit ferroptosis under certain conditions. In lung cancer, wtp53 induces ferroptosis by suppressing the expression of SLC7A11, which reduces cystine uptake. This reduction in cystine absorption diminishes cellular antioxidant capacity and GPX4 activity. Besides inhibiting SLC7A11, wtp53 also decreases the level of H2Bub1 by promoting the nuclear translocation of the deubiquitinase USP7 ^[52,53]. Additionally, wtp53-induced ALOX12 expression, via SLC7A11 reduction, triggers ALOX12-dependent ferroptosis. Mutant p53 inhibits SLC7A11 expression in lung and esophageal cancers by interacting with NRF2, a transcription factor known for its antioxidant role, which increases ROS accumulation and induces ferroptosis. For instance, researchers created acetylation-deficient p53 3KR mutant mice by replacing lysine residues at positions 117, 161, and 162 of p53 with arginine. These mice did not regulate the cell cycle or apoptosis like wtp53, but they suppressed SLC7A11 expression and induced ferroptosis. Ectopic SLC7A11 expression in tumors carrying mutp53 increases resistance to ferroptosis-inducing drugs, indicating that mutp53 suppression of SLC7A11 sensitizes cancer cells to ferroptosis ^[54].

The integration of ferroptosis with genomic instability could significantly accelerate senescence. In the context of *XRCC4* deletion, a gene involved in DNA double-strand break repair, p53 3KR mice exhibited senescence-like symptoms, and p53-mediated ferroptosis was markedly increased in their testes. However, the creation of p53 4KR mutant mice (K98R + 3KR) revealed that these mice not only failed to suppress tumor growth but also could not inhibit SLC7A11 expression or induce ferroptosis ^[55]. Tumors emerged earlier in p53 4KR mice compared to p53 3KR mice. Moreover, wtp53 interacts with SLC25A28 and translocates to mitochondria in hepatic stellate cells through its interaction with BRD7, resulting in an abnormal accumulation of redox-active iron and promoting ferroptosis. Conversely, the p53 S392A mutant reduces BRD7's binding to p53, preventing p53's mitochondrial translocation and delaying the onset of ferroptosis. In lung cancer, wtp53 regulates the level of the long noncoding RNA (lncRNA) LINC00336 by inhibiting the expression of ELAVL1, which in turn decreases the expression of cystathionine- β -synthase (CBS) and promotes ferroptosis. Wtp53 also induces ferroptosis by regulating the expression of SAT1, GLS2, and PTGS2 ^[56].

Remarkably, wtp53 can also prevent the initiation of ferroptosis. For example, in lung cancer, wtp53 may delay ferroptosis by promoting the expression of iPLA2 β at low-stress levels, though this effect diminishes under high-stress conditions. On the other hand, p53 mutants R175H, R273H, and R248W are unable to rapidly induce iPLA2 β expression. In colorectal cancer, wtp53 inhibits ferroptosis by preventing DPP4 function in a

transcription-independent manner. In fibrosarcoma, wtp53 delays ferroptosis in response to cystine deprivation by regulating CDKN1A expression. Additionally, wtp53 may inhibit ferroptosis caused by cystine depletion by upregulating Parkin expression and reducing ROS levels. These findings suggest that p53 can regulate ferroptosis, which has significant implications for cancer therapy ^[57].

7.5. Role of tumor microenvironment

There is increasing evidence that mutp53 may regulate the tumor microenvironment. Solid tumors are often characterized by tumor-associated macrophages (TAMs). Wtp53 promotes M1 macrophage polarization and creates an anti-tumor environment, which inhibits tumor growth. Interestingly, in colon cancer, mutp53 produces exosomes containing miR-1246 that specifically target nearby macrophages, resulting in miR-1246-dependent reprogramming into an M2 state, which supports tumor progression ^[58]. Mutp53 may also promote tumor neo-angiogenesis. In non-small cell lung cancer (NSCLC), mutp53 activates the ID4 protein, which encourages the expression of pro-angiogenic factors such as IL8 and GRO- α . When mutp53 is reduced, ID4 expression is also diminished. In leukemia, mutp53 stimulates the production of VEGF, fostering a microenvironment conducive to cell proliferation. Additionally, tumors are often characterized by chronic inflammation. Mutp53 exacerbates inflammation by promoting TNF-induced NF- κ B activation in breast cancer. In colon adenocarcinoma, mutp53 inhibits the expression of sIL-1Ra, leading to a pro-inflammatory tumor microenvironment that can increase tumor aggressiveness ^[59,60].

7.6. Cancer stem cells

Mutp53 has also been found to play a role in the acquisition of cancer stem cell (CSC) phenotypes. CSCs are characterized by their ability to generate a variety of tumor cells, which is crucial for cancer development and metastasis. Wtp53 typically acts as a barrier to CSC development and suppresses the expression of CSC-associated markers. In contrast, mutp53 enhances the expression of CSC markers such as CD44, Lgr5, and ALDH, and promotes the expansion of CSC subpopulations, thereby encouraging colorectal cancer progression. In glioblastoma and breast cancer, mutp53 overexpression not only increases the expression of CSC markers but also drives CSC proliferation ^[61]. Additionally, in colorectal cancer, the p53 R273H mutation regulates the expression of lncRNAs, such as lnc273-31 and lnc273-34, which promote CSC self-renewal and tumor growth. Mutp53 also regulates miRNAs to enhance cancer stemness. For example, in basal-like breast cancer, mutp53 increases cancer stemness by modulating the miR-200c-PCK2 axis. In lung adenocarcinoma, mutp53 regulates the miR-324-5p-CUEDC2-NF- κ B pathway to promote cancer stemness. These findings suggest that mutp53 plays a critical role in regulating cancer stemness, offering a potential new approach to targeting tumors ^[62].

7.7. The effect of erastin on p53 and its outlook in cancer treatment

Ferroptosis can be induced through two primary mechanisms. The first involves the cysteine-glutamate transporter, which is affected by glutamate, erastin, and sulfasalazine. The second pathway involves RSL3 and DP17, which directly inhibit glutathione peroxidase (GPX) to trigger ferroptosis. Erastin differs from other ferroptosis inducers in that it has a multi-targeted stimulating action that is efficient, fast, and long-lasting. In other words, erastin does not act through a single pathway. One of its functions is to influence the voltage-dependent anion channel (VDAC), an ion channel located in the outer mitochondrial membrane. VDAC regulates ion and molecular exchange between the cytoplasm and the mitochondria. When medications alter VDAC's accessibility, this leads to the generation of ROS, disruptions in mitochondrial metabolism, and ultimately oxidative cell death. As a tubulin antagonist, erastin can open the VDAC channel, altering the

permeability of the outer mitochondrial membrane ^[63].

VDAC opening results in three biological effects: increased ROS production, reduced glycolysis, and enhanced mitochondrial metabolism. Since many cancer cells exhibit suppressed glycolysis and mitochondrial metabolism, VDAC opening caused by drugs and subsequent ROS generation can target and destroy these cells. One of erastin's advantages as a VDAC-tubulin antagonist is its selective toxicity to cancer cells, as non-proliferating cells lack the high levels of free tubulin characteristic of tumor cells. Thus, by regulating metabolism, erastin offers potential as a novel anti-cancer strategy. Importantly, erastin can activate p53, enhancing ferroptosis. Wild-type p53 induces ferroptosis by inhibiting the function of the XC system ^[64].

Research has shown that treating A549 lung cancer cells with erastin significantly affects p53 transcription factors and increases ROS levels. The findings suggest that p53 activation, dependent on ROS generated by erastin, triggers the downstream p53 cascade. In acetylation-deficient p533KR mutant cells, even though wtp53 no longer induces apoptosis, these cells still block SLC7A11 transcription. Human cancers commonly overexpress SLC7A11, and ROS-induced ferroptosis inhibits its function. By suppressing SLC7A11, a key antiporter in the cysteine/glutamate system, p53 blocks cysteine uptake and triggers ferroptosis in cells. The results revealed that cell death was minimal ($\leq 10\%$) in p53-deficient cells, but highly significant ($> 90\%$) in p533KR mutant cells treated with erastin. However, erastin treatment significantly reduced cell death (20%) in p533KR mutant cells with high SLC7A11 activity ^[65].

A different type of mutp53, p534KR98, loses its ability to regulate SLC7A11 transcription. When treated with erastin, this mutant model showed a dramatic decrease in tumor-suppressive activity and led to cell elimination. These findings suggest that erastin-induced p53 activation may be crucial for inhibiting tumor growth by suppressing SLC7A11 transcription and promoting ferroptosis. It is important to note that while some cells may undergo ferroptosis due to erastin-induced p53 activation, this approach could reduce the side effects of chemotherapy by selectively targeting cancer cells for destruction while sparing normal cells. This leaves a promising area for future research ^[66].

8. Targeting wtp53 tumors through MDM2 and MDM4 inhibitors

The most common therapeutic strategy targeting p53 in cancers that still retain wtp53 is to prevent its breakdown. The most extensively studied mechanism for p53 degradation is ubiquitylation, mediated by the E3 ubiquitin ligase MDM2, which leads to p53 degradation by proteases. MDM2 overexpression is observed in various types of cancer, particularly in tumors that retain wtp53. Since MDM2-mediated ubiquitylation and degradation rely on direct interaction with p53, researchers are developing small molecules that inhibit the MDM2-p53 interaction to stabilize p53 and restore its function ^[67].

Nutlins, a group of cis-imidazolines, were the first of these inhibitors discovered through a chemical library screen. In cancer cells with wtp53, nutlins activated p53, while no such activation occurred in cells with mutp53. RG7112, a derivative of nutlin, was the first MDM2 inhibitor to enter clinical trials. RG7112 activated wtp53 in patients with refractory or relapsed acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). The effects included stabilization of the p53 protein and increased expression of several p53 target genes, such as *CDKN1A* (encoding p21, a cyclin-dependent kinase inhibitor) and *BBC3* (encoding PUMA, a pro-apoptotic protein). Encouragingly, most of these patients showed anti-leukemic activity. A subset of patients without *TP53* mutations also showed clinical responses, suggesting that RG7112 might have p53-independent effects, such as inhibiting hypoxia-inducible factor 1 α to suppress angiogenesis. However, RG7112 required high doses for efficacy, leading to adverse effects such as gastrointestinal discomfort and reduced platelet

production. High concentrations of RG7112 were also associated with neutropenia and thrombocytopenia in liposarcoma patients. Progenitor cells in the gastrointestinal tract and bone marrow may be particularly sensitive to excessive p53 activity, possibly due to high *TP53* mRNA expression, typically coupled with rapid p53 protein turnover. Moreover, RG7112 was shown to cause thrombocytopenia by impairing the ability of megakaryocytes to produce platelets ^[68].

Idasanutlin (RG7388), a third-generation derivative, replaced RG7112. Several clinical trials are currently evaluating the safety and efficacy of idasanutlin in various cancers, although results remain preliminary. In a phase III trial involving patients with relapsed or refractory AML, idasanutlin combined with cytarabine did not meet the primary endpoint of improved overall survival (OS) or complete responses, although the overall response rate was higher. Similarly, in a phase I trial, idasanutlin showed promising results in patients with polycythemia vera, but in a later phase II trial, it was frequently discontinued due to hematological and gastrointestinal toxicity. The presence of wtp53 in normal tissues, where p53 overexpression is not well tolerated, presents a significant challenge to the clinical use of nutlin derivatives, despite their solid scientific rationale and promising anticancer activity in early-phase trials ^[69].

Other compounds that inhibit MDM2-p53 binding have been developed or are in development in addition to nutlin derivatives. For instance, in preclinical models of AML, the orally bioavailable MDM2 inhibitor APG-115 exhibited potent antitumor activity and enhanced the radiosensitivity of gastric cancer xenografts. APG-115 is currently being tested in clinical trials as a monotherapy and in combination with immune checkpoint inhibitors or chemotherapy. Another oral MDM2 inhibitor, AMG 232, has shown the ability to stabilize wtp53 and induce tumor regression in osteosarcoma cells. In head-to-head comparisons, AMG 232 demonstrated greater activity than other MDM2 inhibitors, such as idasanutlin ^[70]. When combined with cytotoxic chemotherapy, AMG 232 showed more pronounced antitumor activity than when used alone. Now called KRT-232, AMG 232 has been evaluated in over 10 clinical trials, including a phase III trial for myelofibrosis after JNK inhibitor withdrawal. Based on its promising results, AMG 232 received fast-track designation from the FDA and has been tested in prior phase trials for other cancers. Additional MDM2 inhibitors, such as milademetan and siremadlin, are also under investigation (NCT03634228, NCT04116541). Interestingly, both drugs showed greater efficacy when administered intermittently at high doses rather than continuously for two weeks in preclinical and phase I trials ^[71].

While MDM2 inhibition holds promise, it is unclear whether newer MDM2 inhibitors will cause less damage to healthy tissues. Since p53 is present in almost all normal tissues, especially in proliferative regions, it is not a cancer-specific target. Therefore, it may be more feasible to combine smaller, well-tolerated doses of an MDM2 inhibitor with a cancer-specific therapy or develop a method for selectively delivering the inhibitor to tumor cells, rather than aiming to create an MDM2 inhibitor without any adverse effects. MDM4, a protein related to MDM2, is also a critical negative regulator of p53 ^[72]. Unlike MDM2, MDM4 lacks inherent E3 ubiquitin ligase activity but can directly bind to p53 and inhibit its transcriptional activity while supporting MDM2's E3 ligase function. MDM4 is overexpressed in many cancers, making it an attractive therapeutic target. Notably, wtp53 is commonly retained in hematologic malignancies such as AML and myelofibrosis, often in conjunction with increased MDM2 or MDM4 expression. MDM4 is highly expressed in leukemic stem cells, unlike MDM2, making MDM4 inhibitors particularly promising for leukemia treatment ^[73].

Stapled peptides have emerged as a novel alternative to small-molecule drugs in recent years. Hydrocarbon stapling techniques have been used to develop a stapled peptide (SAH-p53-8) that disrupts MDM2 and MDM4 interactions with p53. However, subsequent *in vitro* studies suggested that SAH-p53-8 might be cytotoxic independently of p53, raising concerns about its therapeutic potential. Later, ALRN-6924 and other bispecific

stapled peptides targeting both MDM2 and MDM4 were developed. ALRN-6924 demonstrated potent activity in p53-mutant cells and strong efficacy against several wtp53 breast cancer cell lines^[74]. Similar wtp53 selectivity was observed in AML cell lines, outperforming idasanutlin, though these effects were abolished with p53 knockdown. The initial phase I trial of ALRN-6924 showed antitumor activity in solid tumors and lymphoma, with mild side effects. Recently, crystal structure analysis of the MDM4-nutlin 3a complex revealed additional intermolecular interactions that could enhance the binding affinity of nutlin 3a for MDM4. This insight may enable the development of more potent dual MDM2/MDM4 inhibitors that could suppress the growth of lung and colorectal cancer cell lines. More dual inhibitors targeting both MDM2 and MDM4 are expected to emerge^[75].

9. Challenges and perspectives

Developing drugs that target p53 presents numerous challenges. Two primary issues are the lack of binding pockets and the absence of a well-established process for protein reactivation. While the activity of many proteins can be largely inhibited by blocking their active sites with small molecules, it remains unclear how drug binding can restore a protein's function. Additionally, treating p53-related issues is complicated by factors such as *TP53* deletions, off-target effects, and potentially harmful side effects from p53 overexpression in healthy tissues. The full structures of p53 and its binding partners in association with various DNA targets are not yet fully understood. This lack of structural information limits structure-based drug development for certain p53 mutants that are difficult to produce or whose architectures are not readily available. However, recent advancements in artificial intelligence and cryo-electron microscopy offer hope. With these technological developments, there is reason to believe that p53 structural research will progress, providing a stronger foundation for the development of p53-targeting drugs.

Several additional considerations must be addressed in p53-targeted therapy. First, not all *TP53* mutations are alike, and they vary widely. This diversity makes it unlikely that a single drug could be effective against all *TP53* mutations, suggesting that different p53 mutants may require different therapies. Second, cancer treatment may need more than just p53-targeted therapies. Combination treatments could offer a synthetic lethal approach, such as the simultaneous inhibition of the p53-BCL-2 and MDM2-p53 pathways. Third, new therapeutic strategies might include targeting p53 mRNA, disordered structural domains, mutant protein degradation, or even genome editing using CRISPR-Cas9. Gene editing technologies, like CRISPR-Cas9, are already being explored as cancer therapies. With further scientific progress, CRISPR-Cas9 may provide effective solutions for addressing *TP53* mutations in cancer treatment.

Despite many years of unsuccessful attempts to develop drugs targeting p53, recent progress offers renewed hope. Once considered an undruggable gene, p53 is now more accessible thanks to technological advancements, much like other previously “undruggable” targets such as KRAS. Given the high frequency of *TP53* mutations in human cancers, there is good reason to believe that drugs targeting p53 will continue to advance and may lead to a breakthrough in cancer treatment.

Author contributions

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Disclosure statement

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The Role of MSCT in Evaluating Tumor Size, Density, Immunohistochemical Classification, and Pathological Risk in GIST Patients

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Abstract: *Objective:* To investigate the role of multilayer spiral CT (MSCT) in evaluating patients with gastrointestinal stromal tumors (GIST), particularly its utility in determining tumor size, immunohistochemical classification, and pathological risk. *Methods:* A retrospective analysis was conducted on 22 GIST patients, confirmed by surgical pathology between January 2019 and December 2023. All patients underwent MSCT examination prior to surgery. Tumor size, density, and immunohistochemical classification from the MSCT results were compared with the postoperative pathological findings. Additionally, the ability of MSCT to predict GIST risk grade was evaluated in combination with immunohistochemical analysis results. *Results:* No significant differences were found between the preoperative MSCT findings and postoperative pathological results in terms of tumor size, density, or immunohistochemical classification in GIST patients. MSCT also enhanced the ability to predict GIST risk grades. *Conclusion:* MSCT demonstrates significant clinical value in the diagnosis and risk assessment of GIST, aiding in the prediction of the tumor's biological behavior and patients' treatment responses.

Keywords: Gastrointestinal stromal tumor; Multilayer spiral CT; Tumor size; Immunohistochemistry; Pathological risk

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1. Introduction

Gastrointestinal stromal tumor (GIST) is the most common tumor of gastrointestinal stromal origin ^[1]. Unlike adenomas and carcinomas, which originate from the epithelial cells of the digestive tract, GIST typically presents with no specific symptoms or only mild gastrointestinal discomfort. However, some patients may experience symptoms such as gastrointestinal bleeding or obstruction. With advances in imaging technology, particularly the application of multilayer spiral CT (MSCT), the diagnostic accuracy of GIST has significantly improved. Early and accurate diagnosis is essential for effective treatment planning and prognosis assessment. The purpose of this study is to evaluate the value of MSCT in determining GIST tumor size, density, immunohistochemical classification, and pathological risk.

2. Materials and methods

2.1. Study subjects

Twenty-two patients with GIST, confirmed by surgical pathology in Zhanjiang Central People's Hospital between 2019 and 2023, were selected for retrospective analysis. All patients underwent an MSCT scan before surgery. The detailed image analysis provided by MSCT showed that it could not only accurately measure tumor size and density but also offer important information to determine the malignant potential of the tumors ^[1].

2.2. MSCT scan

A Toshiba 64-row spiral CT machine was used for scanning. Scanning parameters included a 5 mm layer thickness, no spacing, 120 kV, and automatic mAs regulation, with image reconstruction using a soft tissue algorithm. Enhanced scans were performed using nonionic contrast agents, with a total volume of 1.5 mL/kg body weight and a flow rate of 3.0 mL/s.

2.3. Image analysis

Image analysis was independently conducted by two radiologists, each with more than ten years of experience. The analysis included tumor location, maximum diameter, morphology, boundaries, density, and enhancement features. Tumor location was categorized as stomach, jejunum, ileum, or abdominal cavity. Tumor morphology was classified as irregular, mass, nodule, round, or cystic. Size was based on the long axis, and boundaries were noted as either clear or unclear. Growth patterns were categorized as luminal, extraluminal, or mixed. Tumor enhancement was classified as mild (< 20 HU), moderate (20–40 HU), or severe (> 40 HU) ^[1].

2.4. Immunohistochemistry and pathological risk assessment

Immunohistochemical staining for CD117 and DOG-1 was performed on postoperative pathological specimens, and evaluated according to Fletcher's risk classification criteria ^[2].

2.5. Observation indicators

A retrospective analysis of tumor location, diameter, morphology, boundaries, density, and enhancement characteristics was conducted using MSCT imaging, and these findings were compared with pathological results obtained after surgery. The study also compared tumor size, density, and immunohistochemical classification, assessing the predictive ability of MSCT for GIST risk classification.

3. Results

Among the 22 patients with GIST, 3 tumors originated in the stomach (**Figure 1 left**), 14 in the small intestine (including the jejunum and ileum) (**Figure 1 middle**), and 5 in the abdominal cavity (including 1 patient with a recurrent intraperitoneal tumor, as shown in **Figure 1 right**).



Figure 1. MSCT scans of 22 patients with GIST showed the stomach (**left**), small intestine (**middle**), and abdominal cavity (**right**).

Table 1 shows the tumor size of the 22 patients, as measured by MSCT and described pathologically.

Table 1. Comparison of tumor size

No.	Mass size (MSCT)	Mass size (pathology description)
1	98 mm × 65 mm × 66 mm	10 cm × 6 cm × 6 cm
2	44 mm × 25 mm × 26 mm	4 cm × 2 cm × 2 cm
3	50 mm × 36 mm	5.3 cm × 3.5 cm × 3.0 cm
4	100 mm × 56 mm	11 cm × 5 cm × 4 cm
5	89 mm × 126 mm	13 cm × 10 cm × 8 cm
6	20 mm × 15 mm × 18 mm	2 cm × 1 cm
7	92 mm × 15 mm × 65 mm	9 cm × 6 cm × 5 cm
8	31 mm × 23 mm	3.0 cm × 2.5 cm × 2.0 cm
9	44 mm × 37 mm	diameter 4.5 cm
10	53 mm × 75 mm × 59 mm	7 cm × 5 cm × 5 cm
11	75 mm × 43 mm × 55 mm	7.5 cm × 5.0 cm × 3.0 cm
12	122 mm × 118 mm × 53 mm	22 cm × 12 cm × 5 cm
13	70 mm × 58 mm × 32 mm	8 cm × 7 cm × 7 cm
14	63 mm × 35 mm	6.0 cm × 4.0 cm × 3.5 cm
15	75 mm × 65 mm × 72 mm	7cm × 6 cm × 7 cm
16	103 mm × 90 mm × 80 mm	11 cm × 9 cm × 7 cm
17	53 mm × 43 mm × 42 mm	5.5 cm × 4.5 cm × 4.0 cm
18	26 mm × 39 mm × 39 mm	4.0 cm × 3.0 cm × 2.5 cm
19	72 mm × 52 mm × 80 mm	7 cm × 6 cm × 5 cm
20	65 mm × 43 mm × 50 mm	6.5 cm × 5.0 cm × 4.0 cm
21	62 mm × 54 mm × 28 mm	6 cm × 5 cm × 2 cm
22	62 mm × 50 mm	diameter 6.8 cm

As shown in **Table 1**, the tumor sizes measured by MSCT were highly consistent with those of the surgical specimens. After analysis, no significant difference was found between the MSCT measurements and the postoperative pathology results ($P > 0.05$), as shown in **Figure 2**.

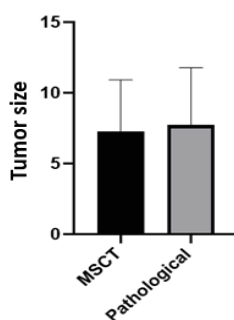


Figure 2. Comparison of tumor size based on MSCT and pathological analysis

Table 2 compares tumor density based on MSCT descriptions and risk classifications based on pathological findings.

Table 2. Patient’s tumor density and risk classifications based on MSCT and pathological descriptions

No.	Tumor density based on MSCT descriptions (1 as no uniform reinforcement; 2 as uniform reinforcement)	Risk classifications based on pathological descriptions (1 as low risk; 2 as high risk; 3 as extremely high risk)
1	1	2
2	1	2
3	1	2
4	1	2
5	1	2
6	2	1
7	1	3
8	2	1
9	1	2
10	1	2
11	1	2
12	1	2
13	1	2
14	1	2
15	1	2
16	1	2
17	2	1
18	1	2
19	1	2
20	1	3
21	1	2
22	1	3

As shown in **Table 2**, the MSCT descriptions indicate that uniform tumor enhancement corresponds to low risk in the pathological results, while non-uniform enhancement correlates with high risk. After analysis, the tumor density differed significantly between low-risk and high-risk (including extremely high-risk) GISTs ($P < 0.05$), as shown in **Figure 3**.

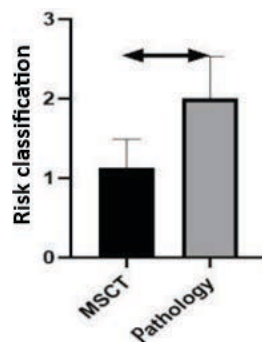


Figure 3. Relationship between tumor density and risk classification based on MSCT and pathological analysis

Table 3 compares MSCT examination results of tumor density with immunohistochemical classifications (CD117 and DOG-1 expression).

Table 3. Comparison of MSCT examination results of tumor density with immunohistochemical classifications

No.	Tumor density based on MSCT descriptions (1 as no uniform reinforcement; 2 as uniform reinforcement)	CD117 (1 as positive; 2 as negative)	DOG-1 (1 as positive; 2 as negative)
1	1	1	1
2	1	1	1
3	1	1	1
4	1	1	1
5	1	1	1
6	2	1	1
7	1	1	1
8	2	1	1
9	1	1	1
10	1	1	1
11	1	1	1
12	1	1	1
13	1	1	1
14	1	1	1
15	1	1	1
16	1	1	1
17	2	1	1
18	1	1	1
19	1	1	1
20	1	1	1
21	1	1	1
22	1	1	1

As shown in **Table 3**, both CD117 and DOG-1 were positive. Upon analysis, there was no significant difference between MSCT-detected uniform enhancement and CD117 or DOG-1 expression ($P > 0.05$), as shown in **Figure 4**.

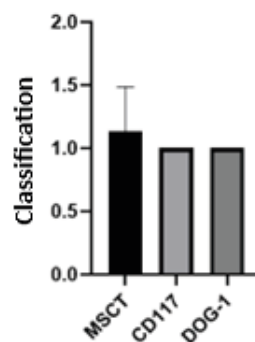


Figure 4. Comparison of tumor density classification with immunohistochemical classifications

Table 4 presents the pathological risk assessment for each patient.

Table 4. Pathological risk assessment for each patient

No.	Mass size (MSCT)	MSCT description (1 as clear; 2 as opaque)	Tumor density based on MSCT descriptions (1 as no uniform reinforcement; 2 as uniform reinforcement)	Risk classifications based on pathological descriptions (1 as low risk; 2 as high risk; 3 as extremely high risk)	CD117 (1 as positive; 2 as negative)	DOG-1 (1 as positive; 2 as negative)
1	98 mm × 65 mm × 66 mm	2	1	2	1	1
2	44 mm × 25 mm × 26 mm	2	1	2	1	1
3	50 mm × 36 mm	2	1	2	1	1
4	100 mm × 56 mm	2	1	2	1	1
5	89 mm × 126 mm	2	1	2	1	1
6	20 mm × 15 mm × 18 mm	1	2	1	1	1
7	92 mm × 15 mm × 65 mm	2	1	3	1	1
8	31 mm × 23 mm	1	2	1	1	1
9	44 mm × 37 mm	2	1	2	1	1
10	53 mm × 75 mm × 59 mm	2	1	2	1	1
11	75 mm × 43 mm × 55 mm	2	1	2	1	1
12	122 mm × 118 mm × 53 mm	2	1	2	1	1
13	70 mm × 58 mm × 32 mm	2	1	2	1	1
14	63 mm × 35 mm	2	1	2	1	1
15	75 mm × 65 mm × 72 mm	2	1	2	1	1
16	103 mm × 90 mm × 80 mm	2	1	2	1	1
17	53 mm × 43 mm × 42 mm	1	2	1	1	1
18	26 mm × 39 mm × 39 mm	2	1	2	1	1
19	72 mm × 52 mm × 80 mm	2	1	2	1	1
20	65 mm × 43 mm × 50 mm	2	1	3	1	1
21	62 mm × 54 mm × 28 mm	2	1	2	1	1
22	62 mm × 50 mm	2	1	3	1	1

As shown in **Figure 5**, the multiple characteristics of MSCT, such as tumor size, boundary, and density, as well as immunohistochemistry results, were significantly associated with high-risk GISTs after analysis ($P < 0.05$).

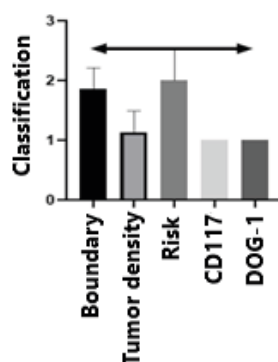


Figure 5. Classification of MSCT characteristics, risk, and immunohistochemistry results

4. Discussion

GIST is the most common stromal-derived tumor of the digestive tract. Imaging plays a crucial role in the diagnosis, treatment, and follow-up of GIST. Recently, MSCT has become an indispensable imaging method for GIST patients due to its rapid speed, high resolution, and powerful postprocessing capabilities. As a non-invasive method, MSCT provides images with high temporal and spatial resolution^[3], allowing for a particularly clear visualization of GIST^[4]. GIST typically appears as a well-demarcated, soft tissue density mass in MSCT scans. Smaller GISTs usually show uniform enhancement, while larger tumors may exhibit heterogeneous enhancement due to hemorrhage or necrosis. Intravenous contrast injection enables the observation of tumor vascular supply and blood flow dynamics, which aids in evaluating the tumor's malignancy and resectability. In particular, MSCT has demonstrated high application value in both diagnosing and assessing the risk of GIST. Through precise tumor size measurement and densitometric analysis, MSCT helps predict the biological behavior of GIST and the patient's treatment response. Moreover, combining MSCT characteristics with immunohistochemical results can further improve the accuracy of GIST risk assessment.

5. Conclusion

This study confirms that the MSCT examination provides a comprehensive and accurate assessment of GIST, including tumor size, density, immunohistochemical classification, and pathological risk. This information is critical for clinical decision-making, treatment planning, and prognosis evaluation. With its advantages of being non-invasive, rapid, and offering high-resolution imaging, MSCT plays a pivotal role in the diagnosis, preoperative evaluation, treatment monitoring, and follow-up of GIST patients^[5]. As technology continues to advance, MSCT will remain crucial in GIST management, offering patients more accurate diagnostic information and optimized treatment strategies. Future studies may further explore the combined use of MSCT with other advanced imaging technologies to enhance the diagnosis and management of GIST.

Disclosure statement

The authors declare no conflict of interest.

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Corrigendum: EGFR Mutation and FHIT Methylation: Inverse Relationship in Patients with Lung Adenocarcinoma and Tuberculosis

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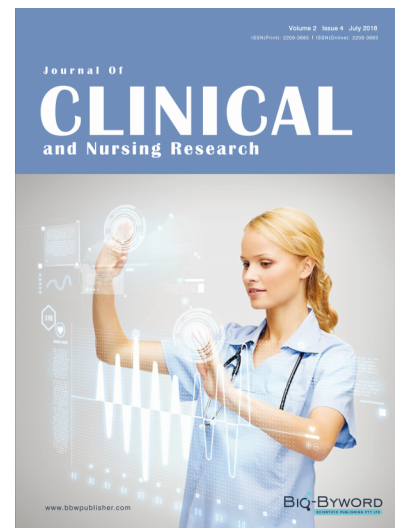
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