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Advances in Biomarkers for Multiple Myeloma

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Abstract: Multiple myeloma (MM) is the second most common malignancy in hematology. MM is characterized by the malignant proliferation of plasma cells in the bone marrow, accompanied by the secretion of monoclonal immunoglobulin, mainly occurring in the elderly. The clinical manifestations of MM include renal dysfunction, bone destruction, infection, anemia, hemorrhage, hypercalcemia, and hyperviscosity syndrome. The recent discovery of biomarkers related to the diagnosis or prognosis of MM provides an important basis for the diagnosis and treatment of MM. This paper reviews the research progress of biomarkers expressed in tissues and peripheral blood at home and abroad.

Keywords: Multiple myeloma; Markers; miRNA

Online publication: January 12, 2023

1. Introduction

Multiple myeloma (MM) is a plasma cell malignancy, accounting for about 10% of all hematological malignancies, with a higher incidence in male than in female [1], and mainly occurring in the elderly population. It poses serious threats to the health of the population. However, the cause of MM remains unknown.

MM is characterized by the accumulation of malignant plasma cells in bone marrow and the production of high levels of immunoglobulin (Ig). MM can be divided into IgG, IgA, IgM, IgD, IgE, light chain, non-secretory, double, or polyclonal according to the type of M protein produced and its detection in blood and urine. The most common types are IgG kappa and IgA kappa [2]. Some of the common clinical manifestations of MM include increased blood calcium (C), kidney damage (R), anemia (A), bone damage (B), and other CRAB symptoms. The presence of these symptoms signifies an active or symptomatic myeloma (AMM). The onset of MM can be divided into three stages, with a slow progress from monoclonal gammopathy of undetermined significance (MGUS) to smoldering multiple myeloma (SMM), and then to AMM. In China, there are 27,800 newly diagnosed MM patients every year. MGUS and SMM progress toward AMM at a rate of 1% and 2% per year, respectively [3]. Although there are many traditional biomarkers used in the experimental diagnosis of MM, their sensitivity and specificity are low. Hence, it has always been a challenge to make an early diagnosis and carry out early intervention for MM. The survival and prognosis of MM are still not ideal although its treatment methods are constantly being innovated. With the development of research, there are new biomarkers that have shown potential in predicting the disease progression of early MM, which is beneficial for its early diagnosis and subsequent targeted therapy. In this paper, we review several potential biomarkers related to the early experimental diagnosis of MM that have been discovered in recent years to provide a theoretical basis for improving the clinical diagnosis rate of MM.

2. Biomarkers expressed in serum

2.1. Circulatory tumor cells (CTCs)

Circulatory tumor cells (CTCs) are tumor cells that shed from the primary tumor or metastasis and subsequently enter the circulation [4]. CTCs are present in every stage of MM. CTC load can be used to evaluate the efficacy of treatment and the prognosis of patients. It has been shown that patients with high CTC load after treatment tend to have poorer prognosis [5]. Studies have shown good consistency between CTCs in MM and tumor cells at the primary site; hence, the number and genetic characteristics of CTCs can be used as stratified indicators of MM risk, and the characteristics of primary tumor cells can be reflected by studying CTCs when evaluating the efficacy and biological characteristics of tumor cells during treatment [4].

2.2. Circulating tumor deoxyribonucleic acid (ctDNA)

ctDNA is a characteristic tumor biomarker. It is a DNA fragment from the tumor genome that carries certain characteristics (including mutation, deletion, insertion, rearrangement, abnormal copy number, methylation, *etc*.) in the circulatory system and is mainly derived from necrotic or apoptotic tumor cells, circulating tumor cells, and exosomes secreted by tumor cells [6]. Due to the high degree of consistency between certain corresponding tumor tissues and plasma samples, especially in metastatic breast cancer, colorectal cancer, and non-small cell lung cancer, ctDNA can be used for noninvasive gene mutation analysis.

2.3. Micro-ribonucleic acid (miRNA)

2.3.1. Micro-ribonucleic acid (miRNA)

miRNA is a short (about 22 nucleotides long) non-coding RNA fragment with the ability to regulate gene expression at the post-transcriptional level [7]. It is involved in the occurrence and development of tumors. Abnormal expression of miRNA has been observed in various conditions, including hematologic malignancies. Common changes in gene expression have been observed in tumor cells' miRNA, which may be caused by deletion, translocation, or amplification. These mechanisms lead to changes in target gene expression. Depending on the genes they affect, miRNAs can act as carcinogenic or tumor suppressor genes. Therefore, miRNAs can be used as promising biomarkers for cancer detection and prognosis [8]. The most well-recognized miRNAs are *miR-15a* and *miR-16*. They are located at chromosome *13q14* and have similar sequences. They perform tumor suppressive functions and are involved in the regulation of cell proliferation, differentiation, and apoptosis. The decreased expression of *miR-15a* and *miR-16* in MM promotes proliferation by increasing the expression of calcineurin binding protein 1 (CABIN1) [9].

The results of a study showed that *miR-15a-5p*, which belongs to cluster *15a/16* located on chromosome *13q14*, is upregulated in MM. Three miRNAs *miR-134-5p*, *miR-107*, and *miR15a-5p* have also been found to be upregulated in MM and MGUS. These three miRNAs may be used as potential diagnostic markers. Combining *miR-107* and *miR-15a-5p* with hemoglobin can help distinguish MM from MUGS, thereby enabling early treatment and thus improving prognosis [10]. The role of miRNA as a biomarker for detecting the transition from asymptomatic to symptomatic MM is critical in clinical settings. However, the current available data are still too tentative to be statistically significant. Hence, this should be the focus of follow-up research.

2.3.2. Exosomal micro-ribonucleic acid

Currently, bone marrow biopsy and tissue biopsy are the main diagnostic methods for MM in clinical settings. However, these procedures may cause pain to patients. Since miRNAs can be stably contained in exosomes, their composition is less complex than that of serum, and samples can be obtained in a non-invasive way, serum exosomal miRNAs can be used as an ideal, non-invasive, and reliable tumor marker

[11]. Studies have shown that serum exosomes contain high abundance of specific miRNAs with good stability and have the potential to be used as new non-invasive molecular markers for disease diagnosis and prognosis [12]. Exosomes are vesicles that oscillate between 50 nm and 100 nm in size and can be released by various cells. They contribute to the pathogenesis and progression of MM. Exosomes contain proteins, cytokines, lipids, microRNAs, long non-coding RNAs, and circRNAs that regulate interactions between MM plasma cells. Through exosomes, mesenchymal stem cells confer chemotherapy resistance to MM cells, while myeloma cells promote angiogenesis, influence immune responses, and cause bone damage. They can affect the prognosis of MM patients [13]. A growing body of evidence has shown that exosomes, isolated from the peripheral blood of patients with MM, may be used as biomarkers to predict the progression of MM. In a study by Zhang *et al*. [14], an overlap of 10 miRNAs with the greatest variation was observed. The upregulation of *miR-513a-5p*, *miR-20b-3p*, and *let-7d-3p* and the downregulation of *miR-16-5p*, *miR-15a-5p*, *miR-20a-5p*, *miR-17-5p*, *miR-125b-5p*, *miR-19a-3p*, and *miR-21-5p* were involved in bortezomib resistance in MM patients. These 10 miRNAs are considered to be a potential predictive group for drug resistance in MM patients. Such predictive panels are important for selecting the most appropriate treatment for patients. Therefore, more extensive and in-depth studies are needed to prove the use of exosomal miRNAs as novel markers in the diagnosis, survival, and prognosis assessment of MM patients.

2.4. Multicolor flow cytometry (MFC) immunophenotype

The Chinese Guidelines for Diagnosis and Treatment of Multiple Myeloma (revised in 2020) have recommended the use of antibodies labeled with more than four colors when MFC is performed for MM examination; the antibodies should include CD38, CD138, CD56, CD19, CD45, CD20, kappa light chain, and lambda light chain. Conditions can be added to other antibodies, such as CD27, CD28, CD81, CD117, CD200, *etc*.

2.4.1. CD38

CD38 is a type II transmembrane glycoprotein consisting of a 45kD single chain with three distinct domains, namely intracellular (20 amino acids), transmembrane (23 amino acids), and extracellular (257 amino acids) structures. It is expressed in early differentiated CD34+ stem cells and mature immune cells, including T cells, B cells, granulocytes, and natural killer (NK) cells, but not in quiescent immune cells. However, the expression of CD38 is not limited to immune cells (including mature cells and precursor cells); instead, it is also expressed in solid tissues, such as brain, eye, prostate, intestine, pancreas, muscle, bone, and kidney [15]. CD38 is a transmembrane glycoprotein that mainly plays the role of cell membrane receptor and extracellular enzyme in the human body. It is highly expressed on the surface of myeloma cells but continuously expressed on the surface of lymphocytes, myeloid cells, red blood cells, and other cells at low levels [16]. CD38 is highly specific and highly expressed in plasma cells of patients with MM compared to normal lymphocytes and bone marrow cells [17]. Based on this characteristic, CD38 is considered to be a promising target in the treatment of MM.

2.4.2. CD56

CD56 is a nerve cell adhesion molecule that can mediate mutual adhesion between myeloma cells and stroma. The expression of CD56 molecule can be detected in most myeloma cells of MM patients. The immunophenotype CD56 is a cell adhesion molecule involved in the homing of MM cells. MM cells with low CD56 expression have a higher capacity for proliferation and metastasis [18]. Therefore, low CD56 expression may be involved in the extramedullary lesions and extramedullary recurrence of MM patients. Patients with MM have a high incidence of extramedullary lesions, some of which are occult and

underestimated due to the limitation of examination. However, there is a lack of effective detection indices for extramedullary lesions in clinical work. A negative expression of immunophenotype CD56 has been found to be associated with the occurrence of extramedullary lesions and extramedullary recurrence. Therefore, CD56 may be a potential index that can predict the occurrence of extramedullary lesions and the extramedullary recurrence of MM. The detection of the expression of immunophenotype CD56 may aid early clinical detection of extramedullary lesions or the identification of patients who may suffer from extramedullary lesions [19].

2.4.3. CD45

CD45 is a single chain transmembrane glycoprotein, a common expression antigen of human leukocytes, and a receptor tyrosine protein phosphatase, which is expressed on the surface of various hematopoietic cells. CD45 is a prerequisite for B cell activation and a key molecule in cell membrane signal transduction. CD45 is widely found on the surface of leukocytes. In normal hematopoietic cells, only red blood cells and platelets do not express CD45. In B-cell acute lymphoblastic leukemia and acute megakaryoblastic leukemia, abnormal naive cells may or may not express CD45 on the surface. A study has found a correlation between the expression of CD45 in tumor plasma cells of patients with extramedullary recurrence of MM and the prognosis of these patients, irrespective of other prognostic factors. Therapeutic strategies need to be established for patients with extramedullary recurrence and CD45-MM cells to ameliorate adverse outcomes [20].

3. Biomarkers expressed in tissues

3.1. Programmed death factor 1 (PD-1)

As a negative costimulatory molecule, PD-1 is mainly expressed in activated and/or depleted T cells, B cells, NK cells, and antigen-presenting cells. Programmed death-ligand 1 (PD-L1) is expressed in various solid tumors and immune cell subsets [21], while PD-L2 is mainly expressed in activated dendritic cells, macrophages, and mast cells [22]. The binding of PD-1 to PD-L1/2 in MM bone marrow microenvironment can lead to immune escape, migration, and proliferation of tumor cells. MM patients often have immune dysfunction, which may be related to the interaction between T lymphocytes expressing PD-1 and PD-L1/2 and the tumor cells. Blocking this pathway may counteract the proliferation potential and drug resistance of myeloma cells [23].

3.2. Adiponectin (APN)

APN is a cytokine that is mainly secreted by adipocytes. After binding to its receptor, it regulates cell survival, apoptosis, and metastasis through a series of signaling pathways. It plays an antitumor role in a variety of tumors [24]. There is an increasing number of evidence supporting the role of obesity in MM etiology, which may be related to decreased serum adiponectin levels in obesity. Studies have confirmed that MM patients have significantly lower serum adiponectin levels compared with normal controls. The decrease in adiponectin levels may be related to the progression of MGUS to MM [25].

4. Discussion

The pathological process of MM is complex, and it remains a challenge to diagnose MM in the early stage. A comprehensive study of the dynamic changes in gene and protein expressions in the bone marrow microenvironment; CTCs, miRNAs, MFC immunophenotypes, and ctDNAs in peripheral blood; and other potential biomarkers is of great significance to the early detection and treatment of patients with MM. In addition, the detection of miRNAs in peripheral blood and CTCs, MFC immunophenotypes, and ctDNAs in blood biopsies can help clinicians assess the disease status and diagnose MM early without invasive bone

marrow tests. These emerging biomarkers have shown potential in the diagnosis and prognosis of MM. However, the standardization of these biomarkers still requires continuous testing and validation before they can be applied in clinical trials of MM in the future.

Disclosure statement

The authors declare no conflict of interest.

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Therapeutic Effect of Integrated Traditional Chinese and Western Medicine on Anal Pruritus After Anorectal Surgery

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Abstract: *Objective:* To explore the therapeutic effect of integrated traditional Chinese and western medicine on anal pruritus after anorectal surgery. *Methods:* Ninety-eight patients with anal pruritus after anorectal surgery in our hospital were selected as the research subjects. They were divided into two groups, the control group (50 cases) and the study group (48 cases), according to the treatment plan. The control group was under a simple western medicine treatment plan, while the study group was under an integrated traditional Chinese and western medicine treatment plan. The overall efficacy, severity of anal pruritus, time to eliminate clinical symptoms, and anxiety and depression scores of the two groups of patients under different treatment plans were compared. *Results:* After two weeks of treatment, the total effective rate of the study group was 95.83%, which was significantly higher than that of the control group (82.00%, $P < 0.05$). After 7 and 14 days of treatment, the anal pruritus scores improved significantly in both the groups, but the study group was superior to the control group, with statistical difference ($P < 0.01$). The time of disappearance of skin itching and skin damage in the study group was shorter than that in the control group. After 14 days of treatment, the anxiety and depression scores of both groups were lower than those after 7 days of treatment; however, there was statistical difference between the two groups ($P < 0.01$). *Conclusion:* In the clinical treatment of anorectal postoperative diseases, such as anal pruritus, the combination of traditional Chinese and western medicine can significantly improve the symptoms of pruritus, shorten the time of disappearance of clinical symptoms, improve depression and anxiety, and create a positive clinical application value in promoting the rehabilitation of patients and improving their quality of life.

Keywords: Integrated traditional Chinese and western medicine; Anorectal surgery; Anal pruritus

Online publication: January 12, 2023

1. Introduction

Anal pruritus is a common complication after anorectal surgery, which not only brings pain to patients, but also affects their normal life. Studies have shown that about 70% of patients with long-term anal itching symptoms will have anal dysfunction, and other diseases will develop with improper treatment [1]. At present, there are many ways to treat anal pruritus, including traditional conservative treatment [2] + surgical resection [3] + external treatment of Chinese medicine and modern scientific and technological means, but the outcome is not ideal [4,5]. In recent years, with the advancements in medical research and the integration

of traditional Chinese and western medicine treatment methods and advanced diagnosis and treatment technologies, the combination of traditional Chinese and western medicine treatment has become the main means to address the issues of anal pain and perianal itching [6,7]. In order to explore the therapeutic effect of the combination of traditional Chinese and western medicine on anal pruritus after anorectal surgery, 98 patients with anal pruritus after anorectal surgery were studied. Some of the patients were under a simple western medicine treatment plan, while others were under a combination of traditional Chinese and western medicine treatment plan. The overall efficacy, severity of anal pruritus, time of disappearance of clinical symptoms, and anxiety and depression scores of the two groups of patients under different treatment plans were compared.

2. Data and methods

2.1. General information

Patients with anal pruritus after anorectal surgery who were admitted to Ankang Traditional Chinese Medicine Hospital from October 2021 to September 2022 were selected as the research subjects. They were divided into two groups, the control group and the study group, according to the treatment plan. Among them, there were 50 cases in the control group, including 29 male and 21 female patients, age ranging from 19 to 65, with an average age of 32.5 ± 4.8 years; there were 48 cases in the study group, age ranging from 21 to 67, with an average age of 31.8 ± 5.2 years. The general data of the patients in both the groups showed $P > 0.05$. The study was approved by the hospital ethics committee for implementation, and the patients and their families had given informed consent.

2.2. Diagnostic criteria

2.2.1. Western medicine

Referring to the Diagnosis and Treatment of Anorectal Diseases in China, the diagnostic criteria for anal pruritus are as follows: (1) a persistent and stubborn history of perianal itching (mild itching, as if insects crawling under the skin; severe itching, severe and intolerable, which may cause insomnia, irritability, and other symptoms); (2) in the early stage of the disease, only slight flushing or no obvious abnormalities observed across the anal skin; with disease progression, new and old scratches, scabs, local skin lesions, wrinkles, and other skin injuries are observed around the anus; in the extended course of the disease, local grayish white skin changes are observed around the anus.

2.2.2. Traditional Chinese medicine

Referring to the Guidelines for Diagnosis and Treatment of Common Diseases in the Anorectal Department of Traditional Chinese Medicine and the Therapeutic Effect Criteria for Diagnosis of Diseases and Syndromes of Traditional Chinese Medicine, the diagnostic criteria of anal pruritus with damp heat block syndrome [8] are as follows: presence of one main symptom (local itching around the anus, with varying degrees of dampness, exudation, and ulceration and severe itching pain after stimulation of anal pruritus) and 2 or more secondary symptoms (flushing, irritability, bitter taste in the mouth, dry throat, and sticky stools) with red tongue, yellow and greasy coating, and stringy pulse.

2.3. Inclusion and exclusion criteria

2.3.1. Inclusion criteria

(1) Those who met the aforementioned diagnostic criteria for anal pruritus (damp heat block type) in traditional Chinese medicine and western medicine; (2) age ranging from 19 to 67 years old; (3) those who were not under any other treatment regimens that may affect the research results during the study; (4) those who understood and signed the informed consent form voluntarily.

2.3.2. Exclusion criteria

(1) Patients with perianal skin pruritus caused by hemorrhoids, anal fistula, anal fissure, perianal abscess, perianal eczema, parasites, and other anal diseases; (2) patients with perianal skin itching caused by drug and food allergy; (3) elderly or infirm and mentally ill patients who could not cooperate; (4) female patients who were menstruating, in pregnancy, or lactating; (5) those allergic to the drug of study or unwilling to participate in the study.

2.4. Treatment methods

The control group was treated with western medicine alone. First, the patients first instructed to pay attention to personal hygiene at all times and wash regularly to avoid contamination around the anus and perineum; the patients were prohibited to scratch with their hands and to eat spicy or stimulating food. Second, triamcinolone acetonide econazole cream (Pevisone, 15 g/piece, provided by XI'an Janssen Pharmaceutical Co., Ltd., National Drug Approval No. H20000454) was prescribed to the patients for external use; the drug was evenly applied to the itching area at the anus and the surrounding area, gently rubbing for 1 min, once in the morning and another in the evening, over two courses, with each course lasting seven days; the dosage of ointment or the frequency of medication was reduced if the symptoms were alleviated.

The study group was treated with a combination of traditional Chinese and western medicine. In addition to the cream, the patients were treated with traditional Chinese medicine fumigation and hip bath. In order to boil 800 mL of Xiaoyang decoction, 20 g of *Sophora flavescens*, 20 g of rhubarb, 15 g of *Kochia scoparia*, 10 g of *Cnidium monnieri*, 10 g of *Phellodendron chinense*, 10 g of *Angelica dahurica*, 10 g of black plum, and 10 g of argyi leaf were used. Placing 400 mL of the decoction into a basin each time and adding 1,000 mL of boiled water, fumigation of the anus with traditional Chinese medicine was done; the patients were requested to sit in a sitz bath, soaking their anal region in the medicinal solution for 10 min, when the temperature drops to a comfortable, warm temperature (36°C–40°C), and then wipe dry with a clean, soft cloth.

2.5. Observations

(1) Overall efficacy

According to the Guiding Principles for Clinical Research of New Chinese Medicines (trial) and the Diagnostic Efficacy Standards of TCM Anorectal Diseases and Syndrome, the overall efficacy of the two groups after two courses of treatment was analyzed. Based on the efficacy index, the patients were divided into four categories: cured, markedly effective, effective, and ineffective. Total effective rate = (cured + markedly effective + effective)/total number of people × 10%.

(2) Severity of anal pruritus

According to the 2012 Guidelines for Diagnosis and Treatment of Common Diseases in Anorectal Department of Traditional Chinese Medicine, the severity of anal pruritus was scored for the two groups of patients before and after treatment. The points were given as follows: (1) 0, no anal pruritus; 2, mild anal pruritus, requiring no treatment; 4, moderate anal pruritus, requiring local medication; 6, severe anal itching, without relief after local medication. The clinical effects of different treatment schemes were observed.

(3) Time of disappearance of clinical symptoms

The time of disappearance of skin itching and skin damage between the two groups was compared.

(4) Anxiety and depression

An assessment for anxiety and depression was carried out in combination with the clinical evaluation criteria, and the improvements in anxiety and depression as well as other adverse psychology in the two groups at different treatment stages were compared [9].

2.6. Statistical analysis

SPSS 22.0 was used to process the research data, and the counting data were expressed in percentage (%) by chi-squared (χ^2) test, while the measurement data were expressed in mean ± standard deviation ($\bar{x} \pm s$). After t-test, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Comparison of the overall efficacy between the two groups

After two weeks of treatment, the overall efficacy of both the control group and the study group improved. The total effective rate of the study group was 95.83%, which was significantly better than that of the control group (82.00%, $P < 0.05$), as shown in **Table 1**.

Table 1. Comparison of the overall efficacy of the two groups after 2 weeks of treatment (n/%)

Group	n	Cured	Markedly effective	Effective	Ineffective	Overall efficacy
Control group	50	6 (12.00)	21 (42.00)	14 (28.00)	9 (18.00)	41 (82.00)
Study group	48	8 (16.67)	22 (45.83)	16 (33.33)	2 (4.17)	46 (95.83)
χ^2						4.7030
p						0.0301

3.2. Comparison of the severity of anal pruritus between the two groups before and after treatment

Before treatment, the anal pruritus scores of the control group and the study group were 5.88 ± 2.24 and 6.02 ± 2.09, respectively, with no comparable difference ($P > 0.05$). However, after 7 and 14 days of treatment, the anal pruritus scores of the control group were 4.32 ± 1.78 and 2.28 ± 1.07, respectively, while the anal pruritus scores of the study group were 3.58 ± 1.42 and 1.26 ± 1.04, respectively, indicating that the symptom improved significantly in both groups of patients. The improvement in symptoms after 14 days of treatment was better than that after 7 days of treatment. The comparison between the two groups showed that the study group was superior to the control group, with statistical difference ($P < 0.01$), as shown in **Table 2**.

Table 2. Comparison of anal pruritus severity between the two groups before and after treatment ($\bar{x} \pm s$, points)

Group	n	Before treatment	After treatment			
			7 d	14 d	t	*P*
Control group	50	5.88 ± 2.24	4.32 ± 1.78	2.28 ± 1.07	6.9456	0.0000
Study group	48	6.02 ± 2.09	3.58 ± 1.42	1.26 ± 1.04	9.1320	0.0000
t		0.3196	2.2692	4.7826		
P		0.7500	0.0255	0.0000		

3.3. Comparison of time of disappearance of clinical symptoms between the two groups

By comparing the time of disappearance of skin itching and skin damage between the two groups, it was found that the duration was significantly shorter in the study group compared to the control group ($P < 0.05$). See **Table 3** for details.

Table 3. Comparison of time of disappearance of clinical symptoms between the two groups ($\bar{x} \pm s$, h)

Group	n	Skin itching	Skin damage
Control group	50	16.55 ± 7.52	48.52 ± 8.46
Study group	48	10.46 ± 4.23	25.66 ± 6.54
t		4.9133	14.9225
P		0.0000	0.0000

3.4. Comparison of anxiety and depression scores (HADS) between the two groups

Before treatment, there was no significant difference in the anxiety and depression scores between the two groups ($P > 0.05$). However, after 7 and 14 days of treatment, the anxiety and depression scores of the patients in the control group were 4.32 ± 1.78 and 2.28 ± 1.07, respectively, while the anxiety and depression scores of the patients in the study group were 3.58 ± 1.42 and 1.26 ± 1.04, respectively, indicating that anxiety and depression as well as other negative emotions significantly improved in both the groups. After 14 days of treatment, the anxiety and depression scores of both the groups were lower than those after 7 days of treatment. The comparison between the two groups showed that the study group was superior to the control group, with statistical difference ($P < 0.01$), as shown in **Table 4**.

Table 4. Comparison of anxiety and depression scores between the two groups before and after treatment ($\bar{x} \pm s$, points)

Group	n	Before treatment	After treatment			
			7 d	14 d	t	*P*
Control group	50	12.48 ± 3.92	9.41 ± 3.42	2.37 ± 1.94	12.6606	0.0000
Study group	48	12.25 ± 3.07	8.02 ± 3.31	1.65 ± 0.89	12.8758	0.0000
t		0.3225	2.0432	2.3449		
P		0.7478	0.0438	0.0211		

4. Discussion

Anal pruritus is a common complication after anorectal surgery and a major problem that often puzzles patients and their families. Patients usually have difficulty defecating or experience anal itching after defecation. Anal pruritus after anorectal resection affects not only the quality of life of patients, but also their psychological aspect [10]. The main causes of anal pruritus are as follows: (1) wound infection caused by improper operation during surgery; (2) the body's resistance against infection reduces after surgery; (3) non-compliance or untimely consumption of oral medications after surgery; (4) not keeping the anal area clean after surgery; (5) damage of certain nerve fibers as a result of the surgery; (6) excessive friction and scratching of the skin around the anus during surgery, thus causing itching; (7) local skin inflammation or fibrous tissue hyperplasia; (8) adhesion between anorectal sphincter and multiple wounds from surgery. In order to treat anal pruritus after anorectal surgery, western medicine, traditional Chinese medicine, and a combination of both can be adopted.

In western medicine, local application of drugs is mainly used. According to the severity of pruritus, three grades of antipruritic agents can be used accordingly: A, B, and C. For patients with mild symptoms, Grade A antipruritic agents can be used. These agents can generally relieve the symptoms. For those with severe symptoms but no more than 3 days, Grade B antipruritic agents should be used first, followed by Grade C antipruritic agents if the symptoms persist. Common antipruritic agents include chlorpheniramine, promethazine, loratadine, terbutaline, Pevisone, and desonide, but they should be used under the guidance of a doctor [11].

There are three main therapeutic methods in traditional Chinese medicine: drug therapy, acupuncture and moxibustion therapy, and traditional Chinese medicine fumigation therapy. (1) Drug therapy. Anal pruritus is often caused by accumulation of damp and heat, invasion of external evils, and chronic anal eczema, blocked by the evils of cold and dampness, and the loss of qi and blood from nourishing. The itching site is often accompanied by skin rash, swelling, and raised temperature. At times, there may be even pustules around the itching area. Therefore, the most direct and effective treatment method is to use Chinese medicine for internal adjustment and local external use. (2) Acupuncture and moxibustion therapy. Traditional Chinese medicine treatment for anal pruritus after anorectal surgery is mainly based on the TCM theory and meridian theory. Acupuncture and moxibustion therapy can warm yang and dredge fu organs, dissipate phlegm and disperse knots, as well as dredge collaterals and relieve pain when treating external diseases. It can also stimulate the body's healthy qi, help the healthy qi, improve the patient's physique, and enhance the disease resistance. This condition should be treated according to its characteristics based on syndrome differentiation and treated with syndrome addition and subtraction. (3) Traditional Chinese medicine fumigation therapy. Traditional Chinese medicine fumigation therapy is commonly used by applying it on the affected part to remove dampness and relieve itching and pain. Common methods include acupoint injection, moxibustion, *etc*. This therapy is individualized and mainly aims at the treatment of anal pruritus based on syndrome differentiation, which would not only relieve the pain of patients, but also improve their postoperative physique, thus improving their quality of life and reducing the recurrence rate [6].

The combination of traditional Chinese medicine and western medicine clears away heat and damp, dispels wind, and relieves itching. In this study, a traditional Chinese medicine decoction was prepared with Kushen, rhubarb, *Phellodendron*, wormwood, and other traditional Chinese herbs that have the effect of heat clearing, detoxification, as well as swelling and dampness elimination. Fumigating and washing the skin of patients locally can promote skin absorption and relieve anal pruritus. In combination with western medicine treatment using antibacterial drugs, antipruritic ointment is applied externally, so as to achieve the effect of contraction and promote the healing of skin injury and intestinal balance. Triamcinolone acetonide, a western medicine, is also applied externally. It is a glucocorticoid, which has anti-inflammatory, antipruritic, and anti-allergic effects. Therefore, it is effective for a variety of skin inflammatory conditions caused by bacteria and fungi.

In this study, the overall efficacy, severity of anal itching, time of disappearance of clinical symptoms, and anxiety and depression scores of the two groups of patients under the two treatment plans (pure western medicine treatment and integrated traditional and western medicine treatment) were compared. It was found that both treatment plans had positive effects on relieving pruritus and promoting the prognosis of patients. However, all the outcome indicators of the study group were better than those of the control group. After two weeks of treatment, the total effective rate of the study group was 95.83%, which was significantly better than that of the control group (82.00%, $P < 0.05$). Before treatment, the anal pruritus scores of the control group and the study group were 5.88 ± 2.24 and 6.02 ± 2.09, respectively, with no comparable difference ($P > 0.05$); after 7 and 14 days of treatment, the anal pruritus scores of the patients in the control group were 4.32 ± 1.78 and 2.28 ± 1.07, respectively, while the anal pruritus scores of the patients in the

study group were 3.58 ± 1.42 and 1.26 ± 1.04, respectively, indicating that the symptom significantly improved in both the groups. The improvement of anal pruritus after 14 days of treatment was better than that after 7 days of treatment. The comparison between groups showed that the study group was superior to the control group, with statistical difference ($P < 0.01$). The time of disappearance of skin itching and skin damage in the study group was shorter than that in the control group, with statistically significant difference ($P < 0.05$). Before treatment, there was no significant difference in the anxiety and depression scores between the two groups ($P > 0.05$); however, after 7 and 14 days of treatment, the anxiety and depression scores of the patients in the control group were 4.32 ± 1.78 and 2.28 ± 1.07, respectively, while the anxiety and depression scores of the patients in the study group were 3.58 ± 1.42 and 1.26 ± 1.04, respectively, indicating that the anxiety and depression as well as other negative emotions in both the groups had significantly improved. After 14 days of treatment, the anxiety and depression scores of the patients were lower than those after 7 days of treatment. The comparison between the two groups showed that the study group was superior to the control group, with statistical difference ($P < 0.01$).

In conclusion, the use of a combination of traditional Chinese medicine and western medicine for patients with anal pruritus after anorectal surgery can significantly relieve their pruritic symptoms, promote the healing of skin injuries, and support their rehabilitation; thus, it can be widely used in clinical practice.

Disclosure statement

The authors declare no conflict of interest.

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Bioinformatics Analysis and Experimental Verification of Prognostic and Biological Significance of Autophagy-Related Long Non-Coding RNAs in Gastric Carcinoma

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Abstract: *Background:* Long non-coding RNAs (lncRNAs) play a vital role in autophagy modulation and tumor progression. However, the key lncRNAs and their functions in gastric cancer (GC) remain largely unknown. *Methods:* A bioinformatic analysis of GC patients' gene expression profiling data from the Cancer Genome Atlas database was performed to identify autophagy-related lncRNAs that are associated with predictive risk. Through Cox regression and Lasso regression analyses, the autophagy-related lncRNAs that are associated with prognosis were identified, and a novel prognostic model for GC was established. The model was then used to evaluate the clinical features and predictive risk of individuals with GC. By using two datasets, GSE 62254 (n = 300) and GSE 15459 (n = 192), from Gene Expression Omnibus, its effectiveness was verified. Gene set enrichment analysis according to hallmark and Kyoto Encyclopedia of Genes and Genomes were used to determine the possible biological roles of these lncRNAs. Furthermore, the HOXD antisense growth-associated long non-coding RNA (HAGLR) mechanism in GC was discovered through *in vitro* and *in vivo* experiments. *Results:* Six lncRNAs associated with autophagy in GC were identified, and a new prognostic risk model based on these lncRNAs was established. The six-lncRNA signature was significantly associated with adverse clinicopathological features and found to be an independent GC prognostic factor. The model was proven to be effective and robust by GSE62254 and GSE15459. According to gene set enrichment analysis, the six lncRNAs appeared to be tightly linked to autophagy-related and cancer-related mechanisms. HAGLR was also found to promote tumor growth by enhancing autophagy signaling in GC. *Conclusion:* A novel prognostic model integrating HAGLR that can effectively evaluate and predict the prognostic risk of GC patients was established. The results indicated that HAGLR promotes gastric cancer progression by enhancing autophagy and is anticipated to be a potential new target for the treatment of gastric cancer.

Keywords: Gastric cancer; Autophagy; Long non-coding RNA; Prognostic risk; HAGLR

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1. Introduction

Gastric cancer (GC) is one of the most life-threatening malignant tumors worldwide [1]. Although there has been tremendous progress in the management of this disease, the overall 5-year survival rate of individuals with GC is still low, as it is often diagnosed at an advanced stage [2]. Therefore, the treatment of GC is greatly challenged.

Long non-coding RNAs (lncRNAs) are RNAs with a length of 200 nucleotides or more with limited ability for protein-coding [3]. However, they tend to drive the formation and growth of malignant tumors by providing signals of malignant transformation through precise regulation of their own transcription [4]. Furthermore, lncRNAs dictate the autophagy role in tumors [5-7] and influence the degree of autophagy in various stages of cancer progression [8], thereby regulating tumor growth. Therefore, lncRNAs are known to be strictly associated with tumor progression [9-12]. In recent years, they have been widely utilized as prognostic and diagnostic markers of various cancers [13].

In this investigation, six autophagy-related lncRNAs associated with GC prognosis was identified to establish a novel prognostic model, which can facilitate early prognostic risk stratification and individualized treatment regimens for GC patients. The tumor-enhancing role of the lncRNAs and the HOXD antisense growth-associated long non-coding RNA (HAGLR) mechanism in GC were also explored and verified through experiments, thus presenting a new potential target for GC treatment.

2. Materials and methods

2.1. Flowchart of the study and data source

Supplementary Figure 1A shows the detailed workflow of the development and validation of the new predictive model of six autophagy-related lncRNAs in GC. The transcriptome and medical data of the training set, containing 375 gastric cancer specimens, were acquired from The Cancer Genome Atlas (TCGA) database. RNA-sequencing (RNA-Seq) information was downloaded in FPKM format and normalized to log2(FPKM+1). The corresponding clinical data of the patients included age, gender, overall TNM stage, individual TNM stage, tumor grade, overall survival (OS) status, and time. The gene expression profiles and medical data of 300 subjects with GC and 192 patients with GC obtained from Gene Expression Omnibus (GEO) datasets (GSE 62254 and GSE 15459) were used as verification data.

2.2. Autophagy-related long non-coding RNAs screening

Geno biotypes were annotated with genomic information from Homo sapiens (version GRCh38.99), and 14,081 and 1,105 lncRNAs were identified from TCGA and GEO datasets. Then, 222 and 328 genes related to autophagy were acquired from the Human Autophagy Database (HADb) and the Gene Ontology (GO) gene set (GOBP_REGULATION_OF_AUTOPHAGY, M10281) of Molecular Signatures Database (MSigDB), respectively. After removing duplicated genes, 495 genes related to autophagy were included for further analysis. By using $|R2| > 0.3$ and $P < 0.01$ as selection principles, Pearson correlation analysis between autophagy-related genes and lncRNAs was conducted to identify the lncRNAs related to autophagy in the TCGA dataset.

2.3. Competing endogenous (ce)RNA network construction

The miRcode database (http://www.mircode.org/) was used to predict microRNA (miRNA) and autophagy-related lncRNA relationship pairs. Three databases, TargetScan (http://www.targetscan.org/), miRTarBas (http://mirtarbase.cuhk.edu.cn/), and miRDB (http://mirdb.org/), were employed to obtain the experimentally validated interaction between these miRNAs and 495 autophagy-related messenger RNAs (mRNAs). Then, a ceRNA network was created using the lncRNAs, miRNAs, and mRNAs obtained by the above methods. Cytoscape 3.8.0 was used to visualize this network.

2.4. Prognostic risk model validation and construction

The overlapping genes from 1,105 lncRNAs obtained from the GEO datasets and cell autophagy-related lncRNAs identified in the TCGA dataset were employed for further analysis to establish a predictive risk model. First, univariate Cox regression analysis was performed to determine if the training group prognosis was substantially influenced by autophagy-related lncRNAs. Then, lncRNAs that overlapped with other lncRNAs were eliminated by Lasso regression analysis using the R package glmnet. Third, multivariate Cox regression analysis was performed to obtain a minimal gene set for prognostic risk prediction. The following formula was used to compute the prognostic risk score: risk score = coef1*expr1 + coef2*expr2 +…+ coefn*exprn. Coefn and exprn represent the coefficient and gene n expression value. The samples were distributed into low- and high-risk groups based on the median risk score as the critical value. Then, the difference in survival between low- and high-risk groups was assessed using Kaplan-Meier survival analysis.

In order to further analyze the relationship between the risk score and clinicopathological factors, age, sex, overall TNM stage, individual TNM stage, tumor grade, and risk score were all included in the multivariate and univariate Cox regression analyses to determine if risk score is an independent prognostic factor in individuals with GC. A multi-indicator receiver operating characteristic (ROC) curve was employed to evaluate the risk scoring precision in the prognosis of individuals with GC. The assessment of this risk scoring model's stability was performed in a similar manner using the GEO datasets as a validation dataset.

2.5. Gene set enrichment analysis

The functional difference between the low- and high-risk groups was estimated by gene set enrichment analysis. In this study, GSEA was employed to identify the potential function enriched in the high-risk group that most likely leads to a poor prognosis.

2.6. Cell culture and transfection

Normal gastric epithelial cell line GES-1 and GC cell lines AGS and SGC-7901 were obtained from the Cell Bank of the Chinese Science Academy. The cells were kept in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 1% penicillin-streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS; Gibco) at 37°C under 5% carbon dioxide (CO_2). Negative control small interfering RNA (si-NC), small interfering RNA against HAGLR (si-HAGLR)#1, and si-HAGLR#2 were designed and created in GenePharma Inc. and transfected into cells following the manufacturer's protocol. **Supplementary Table S1** shows the si-NC, si-HAGLR#1, and si-HAGLR#2 sequences.

2.7. RNA extraction and quantitative real-time polymerase chain reaction

TRIzol reagent was used to extract the total RNA from cells and tissues (Thermo Fisher, USA). PrimeScript™ reverse transcription (RT) kit (Takara, Kyoto, Japan) was used to transcribe complementary DNA reversibly, and TB Green Premix Ex Taq™ II kit (Takara, Kyoto, Japan) was employed to determine the expression quantity through quantitative real-time PCR (qPCR) following the manufacturer's protocol. β-actin was utilized as an internal reference. **Supplementary Table S1** shows the primer sequences utilized for qPCR by means of $2^{-\Delta\Delta CT}$ to analyze relative gene expression.

2.8. Cell proliferation assay

AGS cells (10^3 cells/well) were plated into 96-well plates. The well was injected with 10 μL Cell Counting Kit-8 (CCK-8) assay (Beyotime) and incubated for 4 h at 37°C. The absorbance was detected at 450 nm to

determine cell growth. For the colony formation assay, 2×10^3 AGS cells were supplemented to six-well plates. The cells were incubated the following day in conditioned media, which was changed every three days. Cell colonies were fixed with 4% polyformaldehyde and stained with 0.1% crystal violet on the tenth day. The quantity of cell colonies was then calculated.

2.9. Transwell assay

A cell migration test was performed on 24-well transwell cell culture chambers with 8-μm diameter holes (Corning, USA); 200 μL serum-free medium suspension containing 2×10^5 AGS cells was introduced to the superior chambers, while 750 μL complete culture medium was injected into the inferior chambers. After one day, the remaining superior chamber cells were eliminated, and the inferior chamber cells were stained with 0.1% crystal violet and fixed with 4% paraformaldehyde. The cells were counted after at least five randomized microscopic fields were captured on camera (at 100× magnification).

2.10. Western blot

Western blot was performed as described in previous research [14]. Secondary antibodies (1:2000; Jackson ImmunoResearch), β-actin (1:10000; Abcam), and primary antibodies against microtubule-associated protein 1A/1B-light chain 3 (LC3B, 1:1000; Proteintech) were used.

2.11. Immunofluorescence assay

The inoculated cells were seeded onto a glass coverslip in a 24-well plate. After that, the cells were permeabilized for 15 min with 0.5% Triton X-100 at room temperature and then fixed for 30 min with 4% paraformaldehyde. Primary anti-LC3B antibodies (1:300; Invitrogen) were incubated on cells before adding Goat Anti-Rabbit Alexa Fluor 488 (1:400; Jackson ImmunoResearch). The BX53 Fluorescence Microscope (Olympus) was used to capture images, while the nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI).

2.12. Mice tumor models

The Institutional Animal Care and Use Committee of Tongji University (LL-2021-SCI-005) authorized all animal investigations conducted following the National Institutes of Health (NIH) Strategies for the Care and Use of Laboratory Animals. Thirty female nude mice (BALB/c, 6weeks, 18–20 g) were obtained from Shanghai Jiesijie Lab Animals Co., Ltd. for two experiments. In the first experiment, 15 mice were assigned to two groups (7 mice in the si-NC group and 8 mice in the si-HAGLR group). The cell suspension (1×10^7 cells/100 μL) was injected subcutaneously in the mid-dorsal region of the mice. All mice were observed, and the observations were recorded once every 3 days. The following formula was used to estimate the cancer volume: volume = (length × width^2)/2. The nude mice were killed after 21 days, and the cancers were photographed and quantified. Western blot analysis was performed to determine if LC3B was expressed in the malignant tissues of nude mice. The experiment was repeated using the same number of mice in each group as that in the first experiment.

2.13. Statistical analysis

Data analysis was performed, and plots were generated using R program version 4.0.0. One-way analysis of variance (ANOVA) was carried out for multiple comparisons, while Student’s t-test was employed for two-group comparisons. Data were expressed in mean ± standard error of mean (SEM). A *P*-value smaller than 0.05 was identified to be statistically significant.

3. Results

3.1. Autophagy-related competing endogenous RNA network

After performing Pearson correlation analysis between lncRNAs and autophagy-related genes in the TCGA dataset, 843 lncRNAs were recognized as autophagy-related lncRNAs using $|R^2| > 0.3$ and $P < 0.01$ as the threshold. Thirty-four autophagy-related lncRNA and miRNA relationship pairs were obtained through miRcode. Then, TargetScan, miRTarBas, and miRDB were utilized to predict the relationship between autophagy-related genes and miRNA. An autophagy-related ceRNA network was obtained, which included 33 lncRNAs, 29 miRNAs, and 75 mRNAs (**Supplementary Figure 1B**).

3.2. Building a predictive risk model based on autophagy-related lncRNAs

A total of 136 autophagy-related lncRNAs were filtered after the overlap between the 843 autophagy-related lncRNAs in the TCGA dataset and the 1,105 GEO lncRNAs. Based on the TCGA dataset, 136 autophagy-related lncRNAs were associated with the survival data of GC patients, and 10 autophagy-related lncRNAs were found to be associated with prognosis by univariate Cox regression analysis (**Figure 1A**). Two autophagy-related lncRNAs were eliminated by Lasso Cox regression analysis (**Figure 1B–C**). Multivariate Cox regression analysis showed that six lncRNAs with prognostic significance, namely LINC01023, LINC00963, HAGLR, MIR100HG, LINC01315, and LINC00857, were associated with autophagy (**Figure 1D**). The following formula was used to estimate the risk score for individuals with GC: risk score = (0.379944 × LINC01023) + (-0.32266 × LINC00963) + (0.316604 × HAGLR) + (0.158644 × MIR100HG) + (-0.56624 × LINC01315) + (-0.19173 × LINC00857). GC patients were distinguished as high-risk (n = 175) and low-risk (n = 175) groups based on the median risk score. Kaplan-Meier survival curve revealed that GC patients with elevated risk score showed significantly shorter OS (median OS: 383 days versus 526 days; $P < 0.001$; **Figure 1E**). GC patients were ranked based on their risk scores (**Figure 1F**), and the scatter dot plot (**Figure 1G**) showed that patients with higher risk scores had shorter survival. The heatmap showed significant difference in lncRNA levels related to 6 prognostic signals between low- and high-risk individuals with GC (**Figure 1H**). Furthermore, there were significant group differences in the expression of all six autophagy-related lncRNAs (**Supplementary Figure 1C–H**; high- versus low-risk scores; $P < 0.05$).

3.3. Correlation between autophagy-related long non-coding RNA predictive risk score and clinicopathological factors

From the association between autophagy-related lncRNA predictive risk score and age, gender, tumor grade, overall TNM stage, and individual TNM stage, the outcomes indicated that there was no significant difference in the risk scores between age > 60 and ≤ 60 as well as between men and women (**Supplementary Figure 1I–J**). However, the risk score was higher in stage II–IV than in stage I ($P < 0.05$) and in G3 than in G1–2 ($P < 0.001$; **Supplementary Figure 1K–L**). These outcomes indicate that risk score may be associated with GC progression.

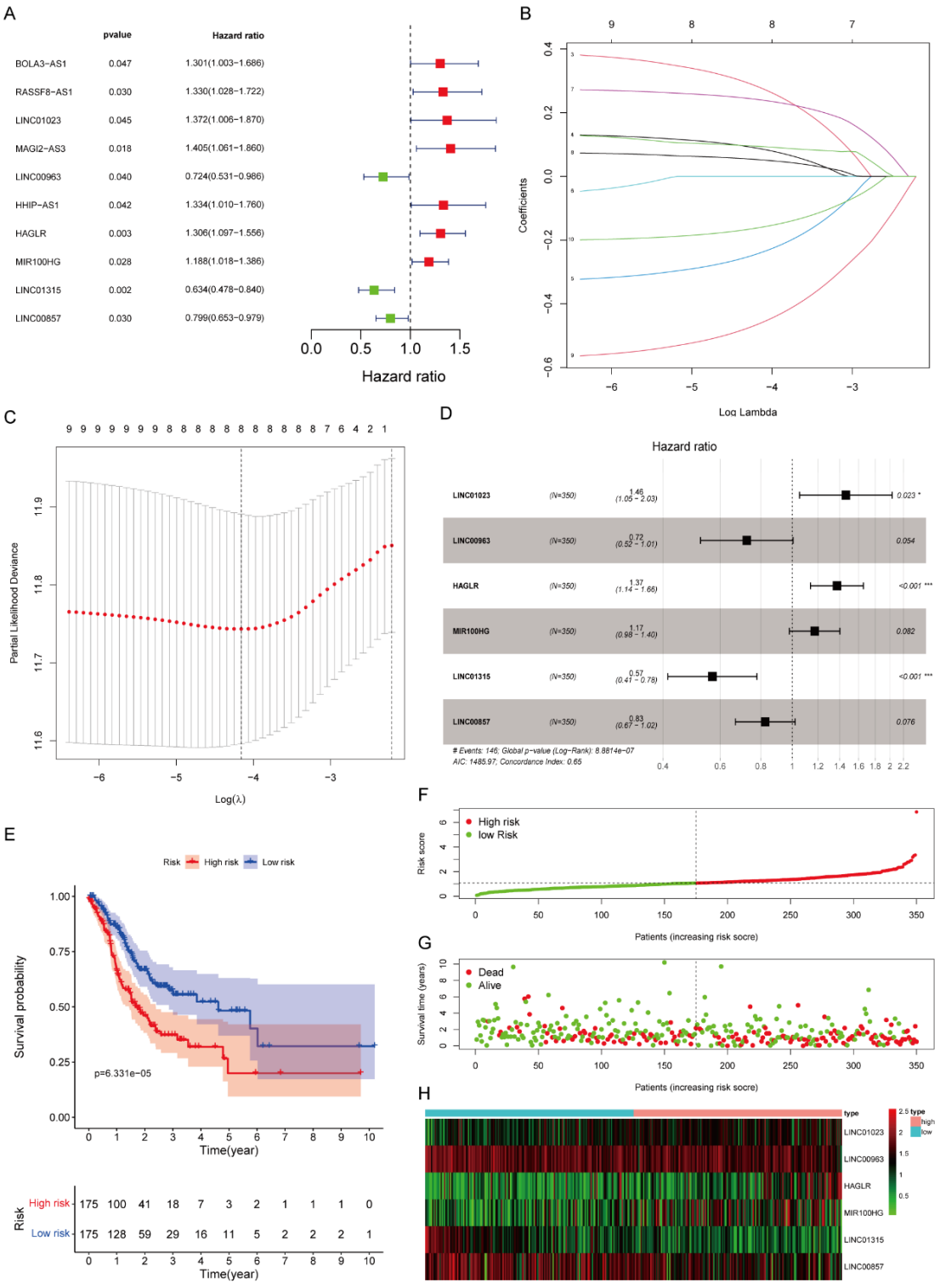

Figure 1. (A) Forest plot of hazard ratios showing 10 autophagy-related long non-coding (lnc)RNAs associated with overall survival in gastric cancer (GC). (B) Lasso coefficient profiles of prognostic lncRNAs. (C) Lasso coefficient values and vertical dashed lines at the best log (lambda) value. (D) Six prognostic autophagy-related lncRNAs in GC. (E) Kaplan-Meier survival analysis of high- and low-risk groups based on the risk model for GC patients in The Cancer Genome Atlas database. (F) Distribution of risk scores for each patient. (G) Survival status of GC patients. (H) Expression heatmap of six autophagy-related lncRNAs.

3.4. The six-autophagy-related lncRNAs risk score is an independent prognostic factor in GC

Univariate Cox regression analysis was performed to prove that the six incRNAs are independent prognostic factors. The analysis showed that many factors, including age and sex, were associated with OS (**Figure 2A**). Multivariate analysis showed that autophagy-related lncRNA predictive risk score and age were significantly linked to OS (**Figure 2B**). According to the ROC curve, the area under the curve (AUC) of the autophagy-related lncRNA prognostic risk score was 0.705 (**Figure 2C**). On the basis of these findings, the autophagy-related lncRNA predictive risk score is an independent prognostic factor for individuals with GC.

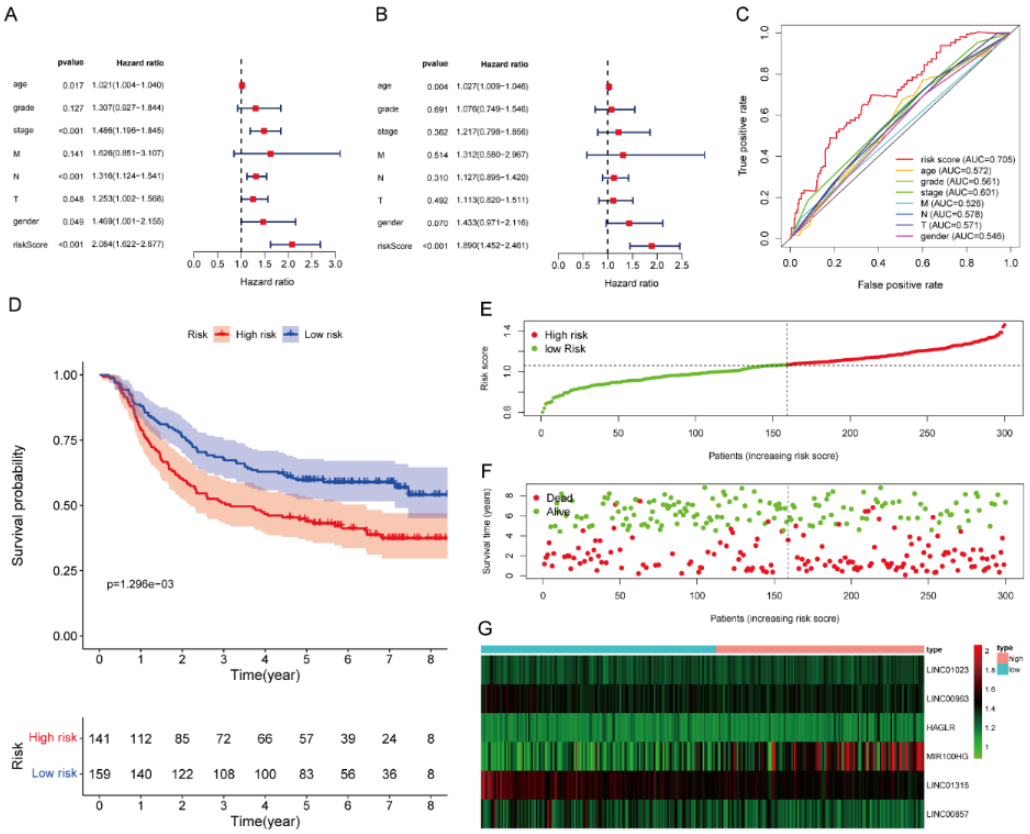

Figure 2. (A) Univariate Cox regression analysis for risk score, age, gender, tumor grade, overall TNM stage, and individual TNM stage. (B) Forest plot for multivariate Cox regression analysis showing that risk score and age were independent prognostic factors. (C) Multivariate receiver operating characteristic curve analysis showed predictive accuracy of the prognostic signature. (D) Kaplan-Meier survival analysis of high- and low-risk groups based on the risk model for gastric cancer (GC) patients in the Gene Expression Omnibus dataset (GSE62254). (E) Distribution of risk scores for each patient. (F) Survival status of GC patients. (G) Expression heatmap of six autophagy-related long non-coding RNAs.

3.5. Prognostic risk model validation in the Gene Expression Omnibus datasets

Two independent validation datasets (GSE62254 and GSE15459) from the GEO database were employed to evaluate the validity of the prognostic risk model. First, the risk score for GC patients was estimated depending on the six lncRNAs expression in the GEO datasets. The patients in the two GEO cohorts were distributed into high- and low-risk groups based on the TCGA cohort median risk score. According to Kaplan-Meier survival curve analysis, GC patients with high-risk scores had significantly shorter OS than those with low-risk scores (**Figure 2D** and **Supplementary Figure 2A**). The survival status, risk score, and lncRNAs expression pattern distributions of the high- and low-risk groups in the validation sets are shown in **Figure 2E–G** and **Supplementary Figure 2B–D**. These outcomes showed consistent trends in those

observed in the TCGA dataset. Furthermore, the ROC curve indicated that the AUC values of the autophagy-related lncRNA prognostic risk model were 0.615 and 0.598 (**Supplementary Figure 2E–F**). Overall, the above outcomes indicate that this established prognostic risk model of six autophagy-related lncRNAs can provide reliable prognostic risk prediction for GC patients.

3.6. Gene set enrichment analysis

GSEA was performed for both low- and high-risk groups of the lncRNA prognostic risk model. The analysis showed that 40 mechanisms were significantly enriched in the high-risk group (**Supplementary Table 2**). These mechanisms include many important tumor-related signaling pathways, such as interleukin

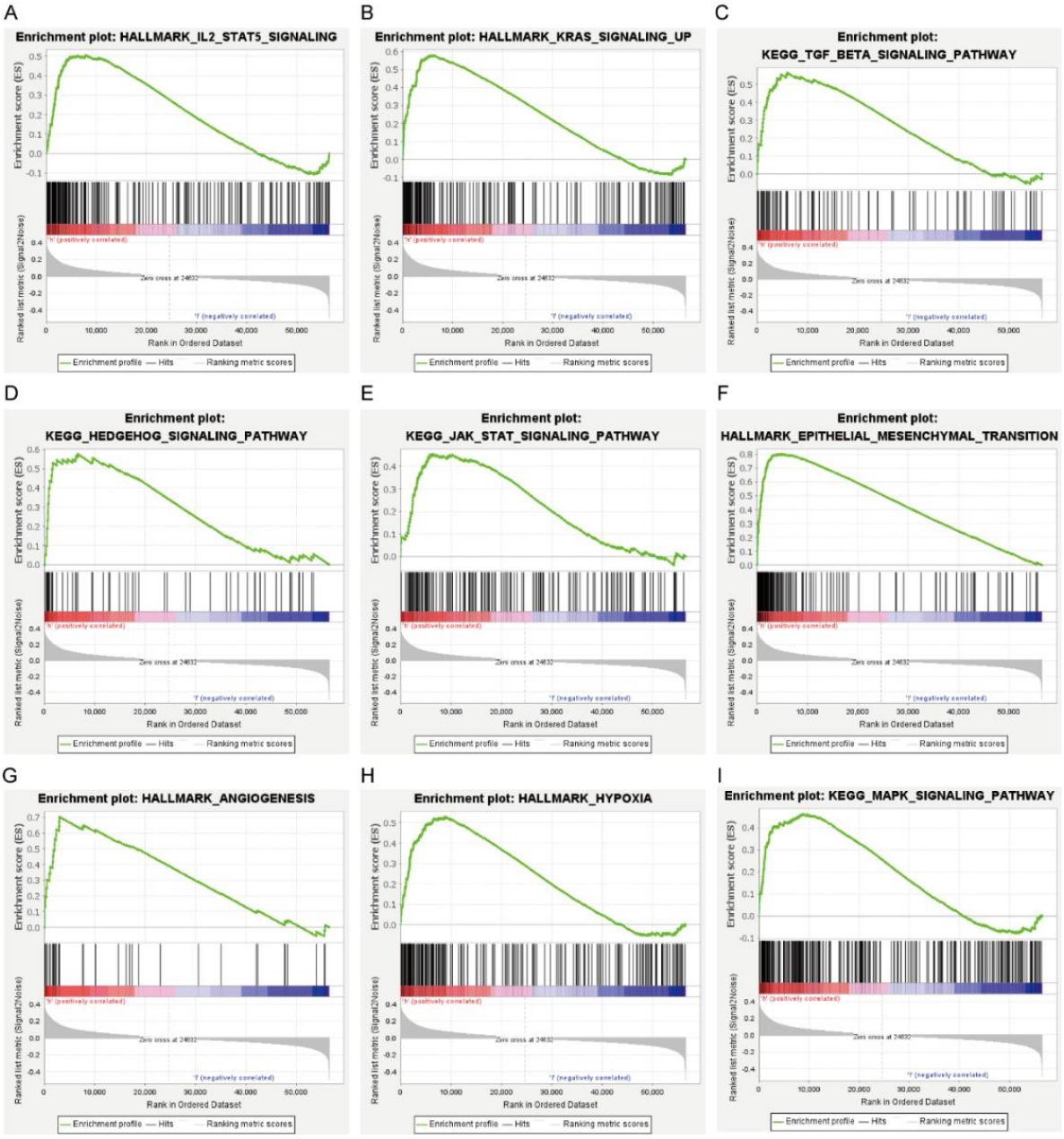

Figure 3. (A–E) Gene set enrichment analysis indicated significant enrichment of multiple cancer-related pathways in the high-risk group based on The Cancer Genome Atlas database. (F–G) Two major pathways associated with tumor progression and metastasis were significantly enriched in the high-risk group. (H–I) Autophagy signaling pathways that were significantly enriched in the high-risk group (determined by gene set enrichment analysis).

2 (IL2)/signal transducer and activator of transcription 5 (STAT5), Kirsten rat sarcoma viral oncogene homolog (KRAS), transforming growth factor beta (TGF-β), Hedgehog, and Janus kinase (JAK)/STAT signaling pathways (**Figure 3A–E**), as well as epithelial-mesenchymal transition and angiogenesis pathways, both of which are involved in cancer invasion and metastasis (**Figure 3F–G**). Moreover, hypoxia and mitogen-activated protein kinase (MAPK) signaling pathways that are closely associated with autophagy were observed in the high-risk group (**Figure 3H–I**). The above outcomes indicate that these autophagy-related lncRNAs are closely associated with tumor formation and progression.

3.7. HAGLR promotes gastric cancer cell proliferation and migration

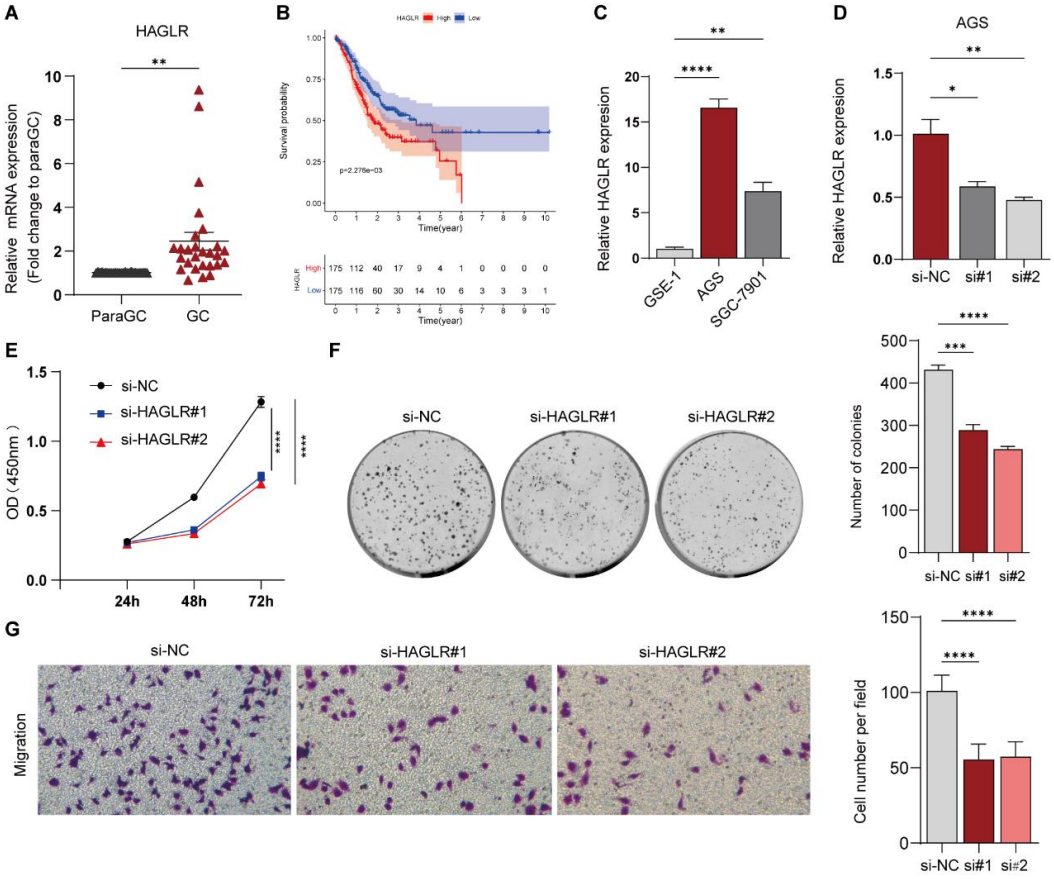

Figure 4. (A) qPCR analysis of HAGLR messenger RNA expression levels in human paired GC tissues and adjacent tissues. (B) Kaplan-Meier survival analysis of GC patients from TCGA database showing HAGLR as a significant prognostic risk factor for patients with GC ($P < 0.01$). (C) The expression levels of HAGLR in GSE-1, AGS, and SGC-7901 were assessed by qPCR. (D) The efficiency of MSR1 knockdown was confirmed by qPCR. (E–F) The proliferation ability of AGS cells after HAGLR knockdown was measured by (E) CCK-8 and (F) colony formation assays. (G) The effect of HAGLR knockdown on AGS cells migration was measured by transwell migration. Representative images showing the results of the assays. Magnification: 100× (left panel). Histogram showing the number of migration cells (right panel). Data are displayed as mean ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$; Paired Student's t-test (A) for two-group comparisons, and one-way ANOVA (C–D and F–G) or two-way ANOVA (E) with Tukey's method for multiple comparisons. Abbreviations: ANOVA, analysis of variance; CCK-8, Cell Counting Kit-8; GC, gastric cancer; HAGLR, HOXD antisense growth-associated long non-coding RNA; qPCR, quantitative polymerase chain reaction; SEM, standard error of mean; si-NC, negative control small interfering RNA; si-HAGLR, small interfering RNA against HAGLR; TCGA, The Cancer Genome Atlas.

From the six lncRNAs identified, the HAGLR with the largest AUC value was selected for further research (**Supplementary Figure 2G**). The qPCR results of 27 pairs of GC and nearby healthy tissues revealed significantly increased HAGLR expression in GC tissues (**Figure 4A**); furthermore, patients with increased HAGLR expression had shorter survival time (**Figure 4B**), suggesting that HAGLR, as an oncogene, may be involved in GC development and thus affect the prognosis of patients. In addition, the elevated HAGLR expression in AGS cells was found to be significantly more than that in GSE-1 cells by qPCR detection (**Figure 4C**). In AGS cells, HAGLR expression was suppressed by siRNA HAGLR transfection (**Figure 4D**). Then, CCK-8 and colony formation assays were used to identify HAGLR impact on GC cells' growth ability; it was found that HAGLR knockout effectively inhibited AGS cells' growth ability (**Figure 4E–F**). Besides, AGS cells' migratory capability was also impaired following HAGLR knockdown, as shown in **Figure 4G**. These results suggest that HAGLR contributes to tumor proliferation and migration.

3.8. HAGLR promotes gastric cancer cell growth by enhancing autophagy *in vitro*

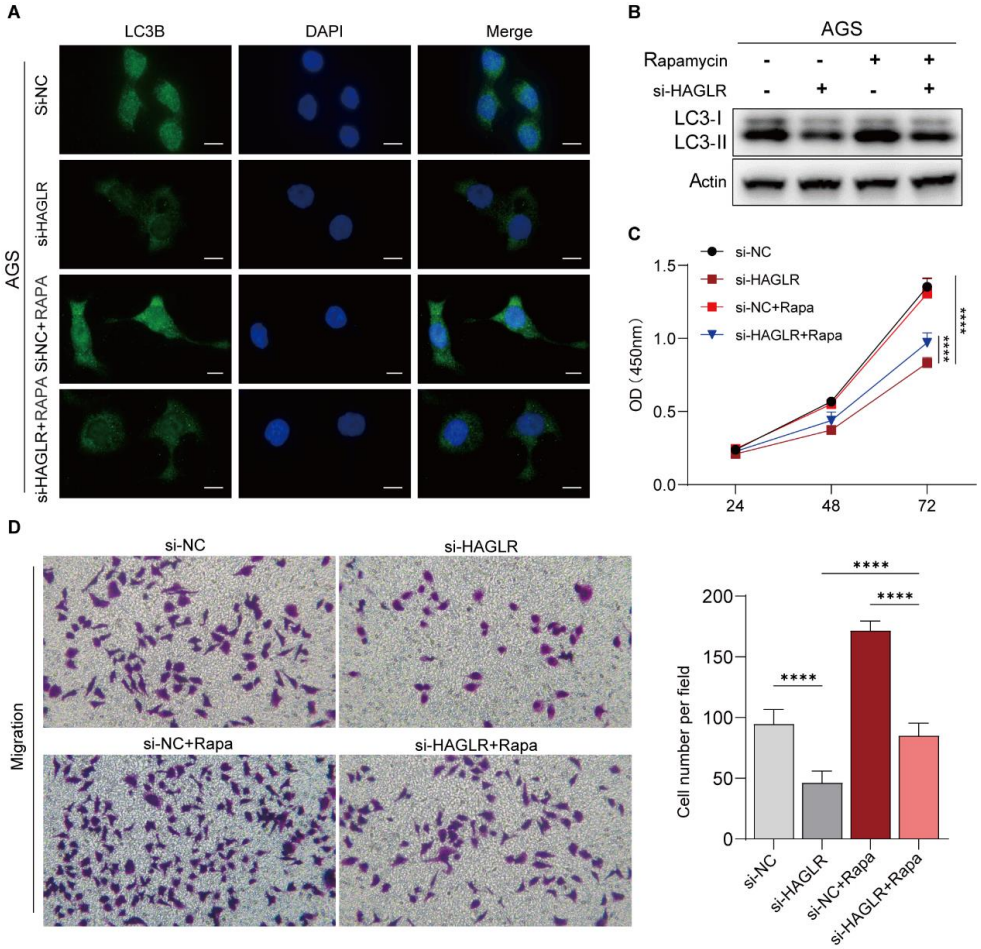

Figure 5. (A) HAGLR-knockdown AGS cells were treated with 1 μM rapamycin over 6 h for the rescue assay, and autophagy-related protein LC3B expression was detected by immunofluorescence staining (scale bar: 10 μm). (B) Western blot analysis of LC3B expression in rescue assay. (C) The proliferative capacity of AGS cells in the rescue assay was detected by CCK-8. (D) Cell migration was evaluated in the rescue assay by transwell migration assay. Magnification: 100× (left panel). Histogram showing the number of migration cells (right panel). Abbreviations: CCK-8, Cell Counting Kit-8; DAPI, 4',6-diamidino-2-phenylindole; HAGLR, HOXD antisense growth-associated long non-coding RNA; RAPA/Rapa, rapamycin; si-NC, negative control small interfering RNA; si-HAGLR, small interfering RNA against HAGLR.

In order to further investigate the potential influence of HAGLR regulatory pathway on GC progression, immunofluorescence staining and western blot were performed. It was discovered that when HAGLR was knocked out, the aggregation of LC3B protein in AGS cells was significantly suppressed (**Figure 5B**) and LC3B protein expression was significantly reduced, indicating an inhibition of autophagy flux. However, the inhibition of LC3B protein aggregation and expression caused by HAGLR silencing was rescued when AGS cells were treated with rapamycin to increase their autophagic flux (**Figure 5A–B**). Interestingly, rapamycin also compensated for the diminished cell proliferation (**Figure 5C**) and migration (**Figure 5D**) ability caused by HAGLR silencing. Overall, HAGLR can promote the growth of GC cells by enhancing autophagy.

3.9. HAGLR promotes gastric cancer progression by enhancing autophagy *in vivo*

In order to verify the above findings, a mice tumor model was established by subcutaneous introduction of AGS cells. It was found that HAGLR silencing significantly inhibited local tumor formation (**Figure 6B**); the subcutaneous cancers in the HAGLR knockdown group were smaller and lighter (**Figures 6A** and **6C**). Furthermore, the silencing of HAGLR suppressed LC3B expression in tumor tissues (**Figure 6D**). These outcomes indicate that HAGLR may be an important regulator of autophagy signaling that promotes GC development and a potential target in GC treatment.

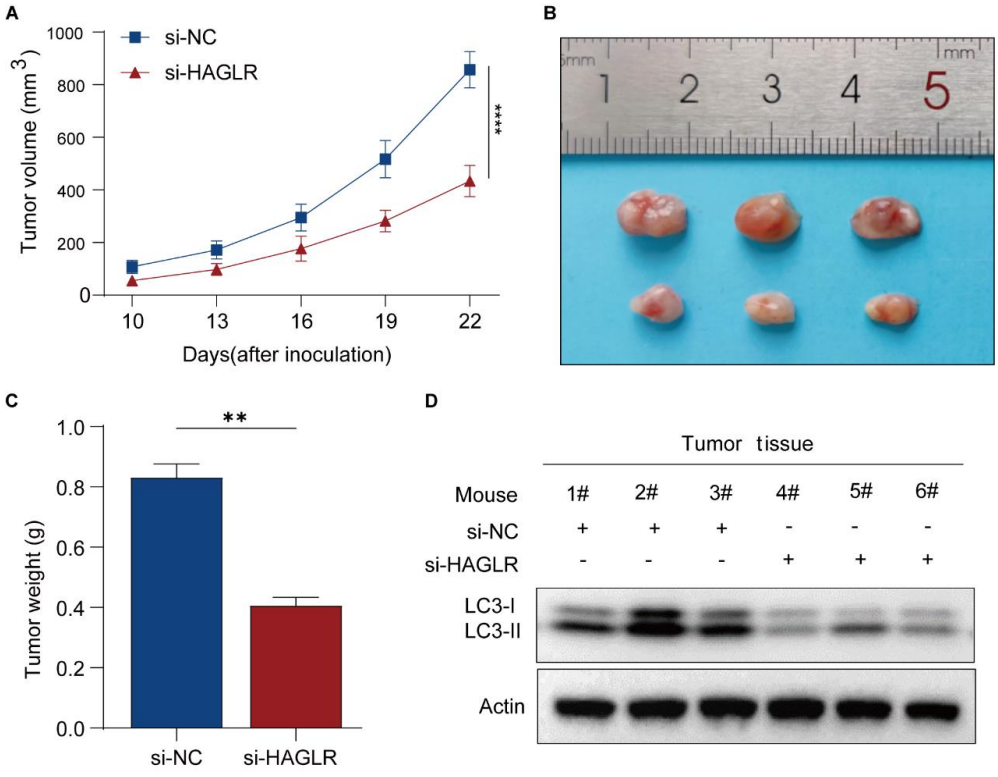

Figure 6. (A) Subcutaneous tumor volumes in the si-NC and si-HAGLR groups determined at the indicated time points. N = 6–8 animals per group. (B) Representative image of subcutaneous tumors at day 22. (C) Weight of the isolated tumor tissues. (D) Western blot analysis of the expression of autophagy-related protein LC3B in tumor tissues of the two groups of mice. Abbreviations: HAGLR, HOXD antisense growth-associated long non-coding RNA; si-NC, negative control small interfering RNA; si-HAGLR, small interfering RNA against HAGLR.

4. Discussion

Investigations have revealed that lncRNAs are related to autophagy modulation in tumors and can regulate autophagy via various mechanisms [8,15]. The most typical example is the equivalence of lncRNAs to

molecular sponges in modulating the expression of autophagy-related genes by absorbing autophagy-related miRNAs [9-11, 16]. Moreover, most autophagy-related lncRNAs influence tumor formation and progression [12]. Therefore, autophagy-related lncRNAs are very promising targets for cancer treatment and prognostic evaluation. In recent years, investigations on the predictive value of autophagy-related lncRNAs in different malignancies have been carried out.

Jiang *et al*. have built a predictive model of 16 autophagy-related lncRNAs, which can accurately predict the prognosis of lung cancer patients [13]. A study has identified six autophagy-related lncRNAs to build a model of risk score, which has been proven to be effective in differentiating between high and low-risk colorectal tumor patients as well as in predicting their OS [17]. In endometrial cancer, a predictive signature of five autophagy-related lncRNAs has also been developed. Compared with other traditional clinical indicators, this signature has been proven to be more efficient as an independent prognostic factor for endometrial cancer [18]. Although advances have been made in this field, the prognostic significance of autophagy-related lncRNAs in GC has yet to be investigated.

In the present study, autophagy-related genes were obtained from HADb and MSigDB, while the RNA sequence and medical data of GC patients were acquired from the TCGA database. Pearson correlation analysis was used to evaluate the association between autophagy-related genes and lncRNAs to identify autophagy-related lncRNAs. An autophagy-related ceRNA network was established based on these autophagy-related lncRNAs using four databases (miRcode, miRDB, miRTarBas, and TargetScan). Thereafter, univariate, multivariate, and lasso Cox analyses were performed to screen six autophagy-related lncRNAs (LINC01023, LINC00963, HAGLR, MIR100HG, LINC01315, and LINC00857), and a novel model of risk score that can precisely predict OS in individuals with GC was thus established. According to the model, the OS of GC patients in the high-risk group was significantly shorter. Moreover, in comparison to traditional medical prognostic factors, this risk scoring model has better predictive performance and can be assumed as an independent predictive factor for GC prognosis. This study validated this model to be effective and robust in predicting GC prognosis through two independent GEO datasets.

Among the six autophagy-related lncRNAs in the risk scoring model, LINC00857, MIR100HG, and LINC00963 have been established to be upregulated in GC patients with the worse prognosis; they have also been established as independent prognostic biomarkers of GC [19-21]. The other three lncRNAs (LINC01023, LINC01315, and HAGLR) are novel lncRNAs that have been identified in GC. Yu *et al*. have found that LINC01023 has an oncogenic function in glioma through IGF1R/AKT pathway activation and can be an applicable treatment target [22]. LINC01315, on the other hand, can facilitate the development and invasion of papillary thyroid carcinoma cells and colorectal cancer cells by sponging miR-497-5p and miR-205-3p, respectively [23, 24]. According to reports, HAGLR, which promotes colon tumor growth through the miR-185-5p/CDK4/CDK6 axis, is a potential target for colon cancer [25]. These studies demonstrate that these lncRNAs are very important in cancer progression; however, the biological processes involved remain unclear. In order to determine the underlying pathway of these lncRNAs in cancers, GSEA analysis was performed in the present study; it was found that mechanisms related to malignancy and autophagy were substantially enriched in the high-risk group. Interestingly, most of the cancer-related pathways enriched are closely associated with autophagy. For example, TGF-β signaling can regulate autophagy [26], and oncogenic KRAS can prompt NIX-mediated mitophagy to promote pancreatic malignancy [14]. The six autophagy-related lncRNAs identified in the present study are closely associated with GC formation and progression; they might influence GC progression by regulating autophagy, thus providing a reliable basis for future molecular mechanism research. A growing body of evidence has demonstrated that HAGLR possesses tumor-promoting ability; for instance, LncRNA-HAGLR can promote the development of triple-negative breast cancer via Wingless-type MMTV integration site family, member 2 (WNT2) regulation by sponging miR-335-3p [27] and colon cancer by sponging miR-185-5p and

triggering CDK4 and CDK6 [25]. The present study showed that individuals with GC and high HAGLR expression had shorter survival time, thus suggesting that HAGLR may have the ability to promote malignant GC development. Nevertheless, to date, whether HAGLR can regulate autophagy in CG and influence its malignant progression remains unclear. In the present study, *in vitro* experiments demonstrated that HAGLR promoted tumor cell growth and migration by enhancing autophagic flux in GC cells, while *in vivo* experiments demonstrated that HAGLR regulated autophagy-related pathways and promoted subcutaneous tumor growth in mice, thus further validating the *in vitro* results. These findings suggest that HAGLR can promote GC progression by enhancing autophagy.

5. Conclusion

A novel predictive signature integrating HAGLR was established in the present study. This predictive signature may contribute to individualized treatment and the follow-up of patients with GC. *In vitro* and *in vivo* experiments confirmed that HAGLR can promote GC progression by enhancing autophagy, thus suggesting this lncRNA as a potential novel target in GC treatment.

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Disclosure statement

The author confirms that there were no financial or commercial relations that could be considered as having possible conflict of interest.

Author Contributions

This is a single-authored paper. The author confirms sole responsibility for the following: conceptualization, data curation, methodology, writing of original draft and revision, as well as visualization, including figure preparation.

Availability of data

In this investigation, publicly accessible datasets were examined. This information is available here: https://portal.gdc.cancer.gov/ and https://www.ncbi.nlm.nih.gov/gds/.

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Advances in the Restoration and Fixation of Lateral Femoral Wall Fracture

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Abstract: Hip fractures, especially intertrochanteric fractures, are more common with aging. After decades of progress, it is a general consensus to carry out internal fixation for this group of patients. However, the recent focus is on unstable intertrochanteric fractures to ensure better prognosis and prevent internal fixation failure. The lateral femoral wall, as a novel concept, is often disregarded. Many scholars have recognized that the lateral wall of the proximal femoral plays a crucial role in the stability of internal fixation for intertrochanteric fractures. In this paper, the historical evolution, definition, clinical significance, injury classification, choice of internal fixation, and possible prognosis of lateral femoral wall fracture are reviewed in order to provide clinicians strong evidence of treatment strategies.

Keywords: Intertrochanteric fracture; Lateral femoral wall; Hip fracture; Surgery

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1. Introduction

Intertrochanteric fracture occurs at the base of the femoral neck between the greater and lesser trochanters [1]. It frequently occurs in the elderly, in which the most frequent and severe fracture among all fractures is caused by osteoporosis, resulting in disability, poor prognosis, and even death [2]. With the continuous improvement of living standards, although the age-standardized incidence rate of intertrochanteric fractures in many countries is declining, the incidence rate is skyrocketing with aging [3]. However, the treatment of intertrochanteric fracture has seen a shift from non-operative conservative treatment to surgical treatment in view of its high mortality and complications. Surgical intervention has become the preferred treatment for intertrochanteric fractures [4]. In order to obtain better outcomes and prevent internal fixation failures, the research focus has shifted to unstable intertrochanteric fractures. In the early days, intertrochanteric fractures were often fixed with extramedullary fixation with a long force arm, which provides support through the posterior medial bone fragment containing the femoral calcar and the lesser trochanter. Therefore, it was believed that the key factor determining the stability of internal fixation was the integrity of the internal structure, especially that of the posterior medial bone fragment, including the lesser trochanter and the femoral calcar [4,5]. With the introduction of internal fixation devices, intramedullary fixation has gradually replaced extramedullary fixation, which was the mainstream treatment for intertrochanteric fractures. The placement of the main nail of the intramedullary nail system in the proximal

femoral medullary cavity shifts the center of gravity inward, shortening the force of the arm and reducing the internal fixation stress as well as the failure rate to a certain extent. However, compared with external fixation, intramedullary fixation has higher requirements for the stability of external structures.

For intertrochanteric fractures with lateral wall fracture, the failure rate of intramedullary pin fixation remains high. Research has shown that the integrity of the lateral wall is also a key factor that constitutes the stability of intertrochanteric fractures, which necessitates surgeons to pay close attention to when considering the use of intramedullary nails [6-8].

2. Insights into the lateral wall of the proximal femur

The lateral wall of the proximal femur, which is positioned at the proximal femur, rises upward from the femur to the lateral femoral bone cortex. Although research has long focused on the lateral wall of the proximal femur, it has recently gained popularity in academic circles. The proper assessment of this distinctive, unique anatomical feature remains a contention.

In 1991, Ritter *et al*. [9] discovered that inserting a blade through the lateral femoral cortex into the neck of the femur with an angled plate in the treatment of intertrochanteric fractures frequently caused iatrogenic greater trochanter fracture. However, the concept of "lateral femoral cortex" still received little attention at that time. In 1996, Parker [10] investigated failure instances of dynamic hip screws (DHS) in treating femoral intertrochanteric fractures and discovered that medial femoral shaft sliding might greatly increase the operation failure rate; additionally, he felt that the bone cortex outside the proximal femur could prevent femoral shaft sliding and improve internal fixation stability. However, he was not responsible for introducing the concept of the lateral wall. Instead, Gotfried [11] was the one who proposed the concept of the lateral wall in 2004. He was credited for establishing the concept of the lateral wall, a concept that is now generally accepted. He suggested that sliding compression screws should be inserted into the femoral head via the lateral cortex of the proximal femur. The lateral wall of the proximal femur, described as the extension of the femoral stem to the proximal end, provides vital support. Gotfried initially abstracted the notion of lateral femoral wall fracture from complicated unstable fracture types, attracting attention from researchers and a series of follow-up investigations. However, he merely conceived the lateral wall as a surgical concept for the use of sliding compression screws (such as DHS); he did not provide specific descriptions of the starting or ending point of the lateral wall. In 2007, Palm *et al*. [12] proposed that the lateral femoral wall extends from the distal femoral cortex to the lateral muscle crest. In 2014, Haq *et al*. [13] defined the lateral wall as follows: if a tangent line is drawn on a hip X-ray film along the upper and lower cortices of the femoral neck, the region produced by the junction of the two tangent lines with the lateral aspect of the femur is called the lateral wall; the tangent lines form the lateral wall with the lateral side of the femur. Many experts have deliberated the definition of the lateral wall. According to Zhang *et al*. [14], the lateral wall anatomically refers to the proximal lateral cortex of the femur far from the lateral femoral muscle crest, *i.e*., the lateral cortex of the femur above the plane of the lesser trochanter; the line of the lower boundary of the lateral wall to the midpoint level of the lesser trochanter [15]. It has been recommended that the lateral wall shall be defined as a 2-cm distance from the lateral femoral crest to the lower margin of the lesser trochanter [16]. These definitions of the lateral wall are all based on hip X-ray films. In 2018, Zhou *et al*. [17] proposed the extent of the lateral wall based on three-dimensional (3D) computed tomography (CT) reconstruction of hip fractures; the upper boundary of the lateral wall is the lateral femoral muscle crest, while the lower boundary is the intersection of the tangent line of the bone cortex at the lower edge of the femoral neck with the lateral femoral cortex; the area between the upper and lower boundaries is the lateral wall. The advantage of this classification is that the cortical bone is below the lateral femoral muscle crest, and it is thus more straightforward to recognize clinically. In contrast, fractures below the lower boundary are clinically classed as femoral subtrochanteric fractures. Using the

CT volumetric rendering technique, the anterior and posterior borders of the lateral wall have been identified.

3. Clinical significance of the lateral wall

Currently, most femoral intertrochanteric fracture fixation procedures include tension screws, helical blades, or other internal fixation devices that are inserted into the femoral head and neck via the lateral wall of the proximal femur. An intact lateral wall ensures fixation stability, provides lateral support to the femoral head and neck at the proximal end of the fracture, as well as limits the sliding of the femoral head and neck along the axis of the tension screw so that the bone fragments are in close contact, thus promoting healing. When the bone fragments are forced against one other, they can resist the inward movement of the femoral shaft, preventing the collapse and rotation of bone fragments [14]. When the lateral wall fails, the femoral neck fracture fragment slips laterally while the femoral stem moves medially, undermining the entire internal fixation mechanism and consequently resulting in screw withdrawal and internal fixation failure. The lateral wall can offer three-point external action points for the tension screw or helical blade and minimize lever stress at the interface between the medial femoral head and the middle intramedullary nail, preventing the screw from cutting out and the main nail from bending and fracturing [11,14]. Furthermore, when the lateral wall is shattered, the tension screw or helical blade may be pushed from the broken line of the bone, dispersing the bone fragments with unsatisfactory reduction and an increased risk of internal fixation failure. Abram *et al*. [16] presented a three-point stable proximal femur structure: tip apex distance (TAD). The integrity of the lateral wall and the entrance point of the major nail are essential factors in determining the effectiveness of treatment, followed by TAD. Hsu *et al*. [17] evaluated the criteria for predicting the stability of internal fixation for fractures, in which lateral wall thickness was found to be the essential element among several parameters, including the patient's age, fracture type, gender, lateral wall thickness, and maximum distance.

The idea of the lateral femoral wall has made people aware that there are five sections in the anatomical structure of the proximal femur: femoral head and neck, femoral stem, greater trochanter, lesser trochanter, and lateral wall [14].

4. Classification of intertrochanteric fractures in relation to lateral wall fractures

Intertrochanteric fractures are common fractures whose treatment has evolved through time with the progress of knowledge and advances of internal fixation devices. There are various classifications for intertrochanteric fractures depending on age, and they are constantly evolving. The idea of classification has shifted from emphasizing on the stability of the posterior medial bone mass to the integrity of the lateral wall. However, there are only a few classifications for intertrochanteric fractures involving the lateral wall as the research on lateral femoral wall fracture started relatively late. Jensen *et al*. [18] hypothesized that the involvement of both greater and lesser trochanters is related to the stability of intertrochanteric fractures. As a result, the original Evans classification has been modified, and intertrochanteric fractures are classified into five categories. Type III represents a three-part fracture with separation of the great trochanter, while Type V represents a four-part fracture with separation of the greater and lesser trochanters. Although the classification involves lateral wall fractures, the concept of the lateral wall was not introduced at the time and Jensen did not describe lateral wall fractures in his classification.

According to the Arbeitsgemeinschaft für Osteosynthesefragen/Orthopaedic Trauma Association (AO/OTA) intertrochanteric fracture classification [19] (**Figure 1**) in 1990, Palm *et al*. [12] proposed three categories of intertrochanteric fractures depending on the integrity of the lateral wall of the proximal femur. This classification has been recognized by several scholars [13,20,21].

(1) Intact lateral wall (type A1): 31A1.1, 31A1.2, 31A1.3, and 31A2.1. These fractures are not prone to intraoperative lateral wall fractures, and the prognosis is good.
(2) Weak lateral wall (type A2): 31A2.2 and 31A2.3. These two types of fractures are prone to intraoperative lateral wall fractures, and the prognosis is relatively poor.
(3) Lateral wall fracture (type A3): 31A3.1, 31A3.2, and 31A3.3. These three types of posterolateral wall fractures are preoperative fractures, and the term posterolateral wall was not used in the original classification.

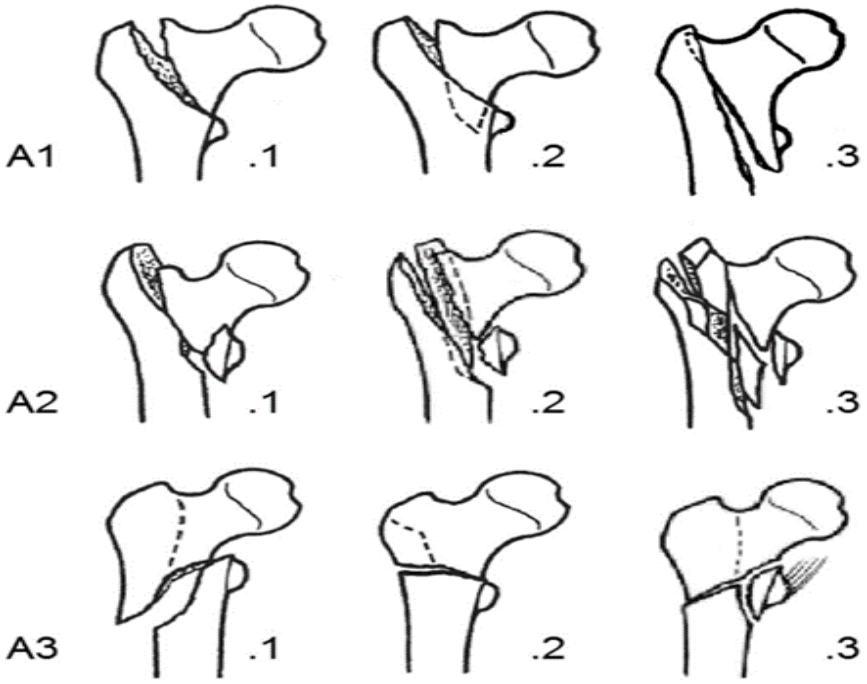

Figure 1. Schematic diagram of the 1990 version of the AO/OTA intertrochanteric fracture classification

Gu *et al*. [22] proposed a classification of intertrochanteric fractures based on the involvement of the lateral wall and the posterior medial bone mass. According to Gu *et al*., type I is a simple comminuted fracture of the lateral wall, involving the screw entry point of the spiral blade, but the medial support basically exists after reduction. Type II is considered when there is lateral wall splitting along with femoral calcar fracture. The bone condition is acceptable and can stabilize the spiral blade, but the inner side lacks support. Type III represents a comminuted fracture of the lateral wall and subtrochanteric femur, involving the screw entry point of the spiral blade on the lateral wall after fracture reduction, but the inner side also lacks support. Different treatment plans are proposed according to this typology.

Zhang *et al*. [23] proposed a 3D CT reconstruction-based subtype of lateral femoral wall fractures: type A, intact lateral wall; type B, partial lateral wall fracture; and type C, complete lateral wall fracture. However, no detailed criteria have been put forward for differentiation between subgroups.

In 2018, the AO/OTA classification was revised [24]. For type A3 fractures, the 2018 classification is consistent with that of the 1990 classification, but for type A1 and A2 fractures, the classification method in the 2018 classification is based on whether or not the lesser trochanter is intact and whether the lateral wall is intact or weak (also known as lateral wall danger) as the basis for differentiating between type A1 and type A2 fractures; it also provides a method of measuring lateral wall weakness [25]. The new AO/OTA classification affirms the role of the lateral wall in the stability of intertrochanteric fractures, reflecting the current situation in which more attention is paid to the importance of the lateral wall. The disadvantage is that the method of measuring lateral wall thickness is dependent on X-ray films, which have stringent

criteria over the patient's posture when the radiograph is taken. If the anterior and posterior X-ray films are not standardized, it may impair the accuracy of the measurement. In addition, it may be difficult to determine if there is a coronal fracture of the lateral wall based only on anterior and posterior X-ray films.

Zhang *et al*. [26] proposed a regional classification method based on the integrity of the lateral wall of the proximal femur and the presence of an independent fracture mass on the posterior medial side. It is a classification of the proximal femur by the Third Hospital of Peking Medical College. There are four types of proximal femoral fractures. Type I fractures are known as intertrochanteric fractures since the lateral femoral fracture line is located between the base of the femoral neck and the lateral pole of the greater trochanter. Type Il is an intertrochanteric fracture since the lateral femoral fracture line is located between the lateral pole of the greater trochanter and the corresponding lateral femoral cortex at the distal end of the lesser trochanter; additionally, the lateral wall is fractured. Type III is referred to as subtrochanteric fracture since the lateral femoral fracture line is located between the lateral femoral cortex corresponding to the distal end of the lesser trochanter and the lateral femoral cortex corresponding to 7.5 cm away from the lesser trochanter. Type IV is referred to as a complex fracture since the lateral femoral fracture line is located in the subtrochanteric region, and it is a complex fracture, *i.e*., type III + type I, type III + type II, and type III + type I + type II.

Each type is classified into two categories A and B based on whether or not there is an independent fragment on the posterior medial side. Subtype A represents the full posterior medial side, while subtype B represents an independent fragment on the posterior medial side. The classification system represents the clinical features of various types of fractures and guides the selection of internal fixation techniques. In order to minimize misunderstandings, the 2018 AO/OTA classification of hip fractures has been universally accepted.

5. Selection of internal fixation for lateral wall fracture

5.1. Dynamic hip screw

Since its inception, DHS has been regarded as a traditional approach for treating intertrochanteric fractures [27]. The advantages of DHS include easy surgery, short operating time, and sliding compression, which promotes fracture healing in stable intertrochanteric fractures. However, for unstable intertrochanteric fractures, particularly those with non-intact lateral wall, it is believed that the extent of soft tissue stripping is large during DHS, which would affect the blood supply at the broken end of the fracture, and the wrapping effect of soft tissue on the lateral wall fragment will be lost, making the lateral wall fracture more prone to displacement [28].

The triple drill bit used in pressing the nail head is relatively thick, which may increase the degree of crushing and displacement of lateral wall fractures. More crucially, lateral wall fractures are still being repaired with DHS, and the lag screw's sliding axis is consistent with the direction of the fracture line. Due to the loss of support and obstruction of the lateral wall, the proximal femoral head and neck fragment will slide excessively along the sliding axis of the lag screw, resulting in uncontrollable outward retreat, outward movement, and collapse as well as relative inward movement of the distal femoral shaft, leading to loss of fracture reduction, cutting out of the lag screw, or extraction and fracture [14]. Experts have thus agreed that traditional DHS is ineffective in the treatment of lateral intertrochanteric wall fractures [29].

5.2. Extramedullary nail plate system

The extramedullary nail plate system mainly consists of the early-stage inverted femoral less invasive stabilization system (LISS), discovered by Zhou *et al*. [30,31], and the late-stage proximal femoral locking plate (PFP) and percutaneous compression plate (PCP), specifically created for trochanteric fractures (PCCP). Inverted LISS fixes intertrochanteric fractures by utilizing a minimally invasive internal fixation

technology initially intended for distal femoral fractures. It employs the biological fixation concept of minimally invasive placement. Screws are used to fix the exterior wall bone fragment, resulting in robust fracture fixation, while minimizing injury to the bones and the surrounding soft tissues as well as providing an environment favorable for callus formation.

In PFP, four locking screws are placed in the lateral wall of the proximal femur, and a 6-mm incision is made [32]. When the plate is inserted percutaneously and PCCP is used, two lag screws are placed in the lateral wall of the proximal femur to fix the femoral head and neck. The surgical incision is about 25 cm [33-35]. The placement of screws can disperse the stress on the lateral wall, and the screws are easily held by the bone cortex of the lateral wall. At the same time, there is less soft tissue peeling over the lateral wall with the smaller incision. The protection of soft tissue over the lateral wall prevents the excessive displacement of the lateral wall bone fragment [36]. Both PFP and PCCP adhere to the concepts of minimally invasive placement and multiple stabilizing screws, which not only meet the needs of lateral wall stabilization, but also take into account of biological fixation and reliable stability; thus, they can be used in the treatment of lateral intertrochanteric wall fractures.

Han *et al*. [37] compared 52 cases of lateral femoral wall fractures treated with inverted LISS and Gamma intramedullary nails, respectively, and discovered that there was no statistical difference between the two groups in terms of fracture healing time, complications, hospital stay, and Harris hip score one year after surgery. In another study, PFP was used to treat 98 cases of intertrochanteric fractures, comprising 22 cases of stable type and 76 cases of unstable type [32]. After a year of follow-up, all fractures had healed without inversion or screw removal. Knob *et al*. [38] conducted a prospective controlled experiment, in which 108 cases of type 31A2 intertrochanteric fractures were treated differently, with PCCP and proximal femoral nail anti-rotation (PFNA). There was no statistical difference between the two groups in terms of internal fixation failure rate, hip joint function, or mortality; however, the lateral wall fracture rate in the PCCP group was 7%, compared to 30% in the PFNA group. PCCP was thought to provide improved lateral wall protection. After analyzing the clinical efficacy of 97 cases of A1 to A2.2 intertrochanteric fractures, Gotfried [34] concluded that the use of PCCP in the treatment of intertrochanteric fractures, particularly unstable ones, is interesting and can be used as another option for the treatment of such fractures.

5.3. Intramedullary nail system

In this period, it is widely assumed that intramedullary fixation for lateral femoral wall fractures will result in better prognoses [12,39,40]. The intramedullary nail technique has several advantages in the treatment of lateral wall fractures: (1) less soft tissue stripping, which allows soft tissue to protect the lateral wall, strengthen the wrapping effect, and prevent further displacement of lateral wall fractures [40]; (2) thin nail head, resulting in less bone damage to the posterolateral wall during drilling and nail placement [41]; and (3) the thick main rod of the intramedullary pin provides some lateral support to the femoral neck bone mass and prevents outward dislocation [41,42], *i.e*., a “metal lateral wall” [43].

Haq *et al*. [13] carried out a randomized controlled trial of 40 patients with type A2.2 to A3.3 intertrochanteric fractures, assigning them to different treatment groups: intramedullary nail system or DHS system. The intramedullary nail group considerably outperformed the DHS group in terms of operation duration, blood transfusion, fluoroscopy time, reoperation rate, and hip function score. In a study by Sadowski *et al*. [44], 39 cases of type A3 intertrochanteric fracture were randomized to receive different treatments: dynamic hip screw or intramedullary nail. Although there was no significant difference in hip function between the two groups after one year of follow-up, the intramedullary nail group performed considerably better than the dynamic hip screw group in terms of operation duration, blood transfusion, hospital stay, and reoperation rate. Several researchers have also demonstrated the effectiveness of intramedullary nail in treating lateral femoral wall fractures [45-47]. Recently, InterTan intramedullary needle

has shown promising results in the treatment of lateral femoral wall fractures [48,49].

However, the intramedullary nailing approach may not be a universal treatment for lateral femoral wall fractures. Ciufo *et al*. [50] retrospectively reviewed the data of 362 patients who had intramedullary nailing for intertrochanteric fractures, among which 6% had screw removal following surgery. According to the regression analysis, even with the intramedullary nail system, lateral wall fracture remained the most important risk factor for screw cut-out (OR = 8.0), outweighing unsatisfactory neck stem repositioning (OR = 4.3) and incomplete posterior medial bone mass (OR = 3.6). Although the intramedullary nail has mechanical benefits for lateral wall fractures, it cannot adequately fix the bulk of the lateral wall fracture, thus making early lateral wall stability improbable. As a result, using the intramedullary nail method may still require surgeons to protect and reconstruct the lateral wall as much as possible, achieving sufficient reduction and appropriately positioning the internal fixation.

5.4. Lateral wall reconstruction

It ought to be emphasized that lateral wall reconstruction refers to a surgical approach to dealing with lateral wall fracture fragments rather than a specific internal fixation procedure. In any sort of fracture, poor reduction is associated with internal fixation failure. In intertrochanteric fractures, an inadequate reduction of bone fragments in the lateral wall may result in delayed bone healing, reduced intertrochanteric fracture stability, and internal fixation failure. Lateral wall reduction and fixation can restore lateral wall support, enhance the stability of intertrochanteric fractures, and improve the prognosis. Gupta *et al*. [51] used DHS in combination with a trochanteric protection plate to treat 46 patients with lateral femoral wall fractures. It has been hypothesized that lateral wall restoration can minimize internal fixation failure rates and enhance postoperative hip function. The function of lateral wall reconstruction in the treatment of lateral wall fractures should be considered. Kulkarni *et al*. [52] divided 154 patients suffering from unstable intertrochanteric fracture into two groups. In the first group (lateral wall reconstruction group), lag screws and steel cable were employed to reconstruct the lateral wall, whereas intramedullary nail fixation was used in the second group (control group). The lateral wall reconstruction group outperformed the control group in terms of fracture non-healing rate, screw resection rate, and reoperation rate. Wang [53] used a combination locking plate for lateral wall reconstruction and discovered that lateral wall reconstruction, when compared to PFNA fixation alone, can minimize internal fixation failure rate, shorten fracture healing time, and improve hip function. Scholars, both at home and abroad, have attempted various methods to reconstruct the lateral wall (including but not limited to DHS combined with trochanter protection plate, lag screw, steel cable, intramedullary nail combined with locking plate, steel cable, single or combined application of proximal femoral plate, *etc*.), but there are still dissensions on the need for reconstruction in lateral wall fractures and how it should be done.

As of now, there is no unanimous conclusion as to whether all lateral wall fractures of the proximal femur require a one-stage lateral wall reconstruction. Kim *et al*. [54] discovered that some lateral wall fracture fragments reduce spontaneously without additional fixation and proposed that the recovery of vastus lateralis strength may form a point similar to a hinge at the lateral femoral muscle crest and exert a force similar to a door closing to reduce bone fragments. On this basis, it is arguable that if the displaced lateral wall fracture can be spontaneously reduced with only intramedullary nail fixation, then the lateral wall bone fragment can be repaired without further reconstruction. According to Wu *et al*. [55], for fractures with lateral wall displacement, it is necessary to pay attention to the posterior medial bone fragment of the lesser trochanter. Suppose the displacement is small and there is a possibility of healing, in that case, reconstructing the lateral wall is unnecessary, such as that seen in cases where the posterior medial bone mass is displaced or a continuing shift during follow-up. When there is a possibility of non-union, the lateral wall should be reconstructed.

6. Conclusion and prospects

The lateral femoral wall is represented by the upward continuation of the bone cortex from the femoral shaft to the lateral femur. Internal fixation devices are often placed into the femoral head and neck by surgeons. There is no one hypothesis that defines its upper and lower boundaries, anterior and posterior boundaries, thickness, and biomechanical strength. However, with the ongoing research on lateral wall, many physicians and researchers are beginning to comprehend the importance of the lateral wall and direct their attention to such unstable fractures. Through this, we can successfully avert future treatment failures. This may be an essential objective and point of reference for us to investigate in reference to the lateral wall. Furthermore, scientists have yet to develop a clearer understanding in the classification of lateral wall fractures. We conclude that there is an urgent need to develop a more clinical and unambiguous classification of lateral wall fractures in order to provide better treatment for lateral wall fractures.

Intramedullary nailing is still considered the gold standard for treating proximal femoral fractures. However, for proximal femoral fractures with lateral wall fractures, multicenter prospective cohort studies and appropriate biomechanical studies are still required. There are also significant dissensions on the need for lateral wall reconstruction and how it should be done. Surgeons should ensure personalized treatment when dealing with lateral wall fractures of the proximal femur, fully evaluate the pathology, carefully formulate management plans, protect the external wall during surgery, reduce and fix it as much as possible, and ensure close follow-up after the surgery to prevent complications and ensure satisfactory prognoses.

Disclosure statement

None of the authors has potential conflict of interest.

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Exploring the Protective Effect of the Ethanolic Extract of *Rosa laevigata* Michx. Fruit on Rats with Mesangial Proliferative Glomerulonephritis Based on NLRP3 Inflammasome Pathway

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Abstract: *Objective:* To investigate the effect of the ethanolic extract of *Rosa laevigata* Michx. fruit on rats with mesangial proliferative glomerulonephritis based on the NLRP3 inflammasome pathway. *Methods:* Thirty Wistar rats were divided into three groups, a blank control group, a diabetic nephropathy (DN) model group, and an ethanolic extract intervention group, according to the random number table method, with 10 rats in each group. One day before the experiment, basic feeding was initiated for all the rats; the changes in activity and weight of each group of rats were observed and recorded after 7 d, and a rat model of renal function injury was established after 1 d. *Results:* Compared with the control group, the model group had significantly higher kidney/body ratio, 24 h urine protein, serum creatinine (SCr), blood urea nitrogen (BUN), glomerular mesangial cell (GMC) count, and extracellular matrix (ECM) positive area ratio ($P < 0.05$); the same indicators were significantly lower in the intervention group than in the model group ($P < 0.05$). The NLRP3 inflammasome pathway in renal intrinsic cells was activated in the intervention group. The overactivation of NLRP3 inflammasome is known to promote interleukin (IL)-1β release, which was inhibited in the intervention group. *Conclusion:* The ethanolic extract of *Rosa laevigata* Michx. fruit has a protective effect on renal intrinsic cells and may be related to NLRP3 inflammasome pathway, suggesting that the fruit of *Rosa laevigata* Michx. has a potential role in protecting renal intrinsic cells from inflammatory damage. NLRP3 inflammasomes are involved in the development of various chronic inflammatory diseases, such as acute and chronic glomerulonephritis and renal fibrosis.

Keywords: Ethanolic extract of *Rosa laevigata* Michx. fruit; Glomerulonephritis; NLRP3

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1. Introduction

Glomerulonephritis is a common pathological type of chronic kidney disease, and its occurrence is associated with excessive activation of renal lamina propria cells. In clinical practice, NLRP3 inflammasomes have been recognized as a new immunomodulatory factor. *Rosa laevigata* Michx., of which its fruit is known as wild golden cherry, hawthorn seed, *etc*., is a plant of the Rosaceae family and is widely distributed across the country. In recent years, despite the numerous studies on the ethanolic extract of wild golden cherry, there very few studies on its mechanism of action. In China, about 20%–40% of patients

with mesangial proliferative glomerulonephritis developed end-stage renal disease. Timely monitoring and individualized treatment can effectively stop or delay the progression of this condition. Since the common feature of all mesangial proliferative glomerulonephritis is the proliferation of glomerular mesangial cells, which leads to functional kidney damage, biomarkers of these cells can be used to monitor the disease [1-8]. We used immunohistochemistry, reverse transcription polymerase chain reaction (RT-PCR), and Western blot to study the protective effect of the ethanolic extract of wild golden cherry on rats with mesangial proliferative glomerulonephritis and the role of NLRP3 inflammasome pathway.

2. Methods

2.1. Animal model and experiment

In this study, 30 Wistar rats were divided into three groups, a blank control group, a diabetic nephropathy (DN) model group, and an ethanolic extract intervention group, according to the random number table method, with 10 rats in each group. One day before the experiment, basic feeding was initiated for all the rats. After 7 d, changes in activity and weight of the rats in each group were observed and recorded, and a rat model of renal function injury was established after 1 d. In order to establish a rat model of kidney injury, the animals were anesthetized and fixed on a collagen membrane with 1% glutaraldehyde; the model group was given saline instead of urea, the control group was given a high-salt diet, and the intervention group was given sodium citrate instead of saline. After the experiment, the kidney specimens of each group were examined by immunohistochemistry. Immunohistochemical staining under light microscope was performed to observe the changes in glomerular pathology before and after the intervention. The kidney specimens were incubated with 5% carbon dioxide (CO_2) for 30 min, washed with phosphate-buffered saline (PBS) 5 times, and further incubated for another 10 min with new samples to ensure no contamination of samples. The kidney sections were fixed with 2% silver nitrate for 6 h and then placed into 10% neutral formalin solution for storage. The prepared specimens were subsequently incubated in 5% CO_2 for Western blot analysis on 96-well plates.

2.2. Observation indicators

(1) *Rosa laevigata* Michx. fruit extract: ethanolic extract was extracted from fresh *Rosa laevigata* Michx. fruit, and the content was 100%. (2) Urine: fresh Wistar rat urine was collected; bacterial culture was performed; and 100 μL of urine sample was taken for analysis after isolation, inactivation, and dilution. (3) Protein extraction: ultra-high temperature instantaneous rotating disk centrifugal method and ultra-high-performance liquid chromatography (UPLC) were used to isolate each group of proteins in rat serum and determine the content of each component in each group of samples (purity 100%), respectively. (4) Cytomorphological examination: rat glomeruli were stained by immunohistochemical staining to observe the morphological changes in the glomeruli. (5) Determination of urinary protein by enzyme standardization: samples were measured by A and B wavelength absorbance meter at 600 nm with enzyme label; according to the calculation of urine protein volume, albumin and total cholesterol in urine were determined by automatic colorimetry and radioimmunoassay (RIA), respectively. (6) Electron micrographs were taken by laser confocal microscopy to observe pathological changes in the glomeruli.

3. Results

3.1. Comparison of kidney/body ratio and 24 h urine protein level of each group

Compared to the control group, the model group had significantly higher kidney/body ratio and 24 h urine protein level ($P < 0.05$); compared with the model group, the intervention group had significantly lower kidney/body ratio and 24 h urine protein level ($P < 0.05$), as shown in **Table 1**.

Table 1. Comparison of kidney/body ratio and 24 h urine protein level of each group

Group	Kidney/body ratio	24 h urine protein (mg)
Control group	4.92 ± 0.32	2.20 ± 0.21
Model group	10.04 ± 0.49[a]	8.75 ± 0.37[a]
Intervention group	7.43 ± 0.56[b]	5.41 ± 0.46[b]

Data are given as mean ± SD, n = 10; [a]$P < 0.05$, compared with the control group; [b]$P < 0.05$, compared with the model group

3.2. Comparison of serum creatinine and blood urea nitrogen levels of each group

Compared with the control group, the model group had significantly higher SCr and BUN ($P < 0.05$); compared with the model group, the intervention group had significantly lower SCr and BUN levels ($P < 0.05$), as shown in **Table 2**.

Table 2. Comparison of serum creatinine (SCr) and blood urea nitrogen (BUN) of each group

Group	SCr (μmol/L)	BUN (μmol/L)
Control group	16.32 ± 3.96	8.96 ± 2.19
Model group	32.36 ± 4.49[a]	21.54 ± 3.65[a]
Intervention group	22.94 ± 5.99[b]	14.03 ± 4.96[b]

Data are given as mean ± SD, n = 10; [a]$P < 0.05$, compared with the control group; [b]$P < 0.05$, compared with the model group

3.3. Comparison of glomerular mesangial cell (GMC) count and extracellular matrix (ECM) positive area ratio of each group

Compared with the control group, the model group had significantly higher GMC count and ECM positive area ratio ($P < 0.05$); compared with the model group, the intervention group had significantly lower GMC count and ECM positive area ratio ($P < 0.05$), as shown in **Table 3**.

Table 3. Comparison of GMC count and ECM positive area ratio of each group

Group	GMC count (pcs)	ECM positive area ratio
Control group	41.36 ± 0.32	0.13 ± 0.21
Model group	72.39 ± 0.49[a]	0.25 ± 0.37[a]
Intervention group	47.63 ± 0.56[b]	0.16 ± 0.46[b]

Data are given as mean ± SD, n = 10; [a]$P < 0.05$, compared with the control group; [b]$P < 0.05$, compared with the model group

4. Discussion

4.1. Ethanolic extract of *Rosa laevigata* Michx. fruit

Rosa laevigata Michx. is a deciduous tree or shrub of the Rosaceae family. In recent years, research has found that it contains a variety of nutrients, bioactive substances, and chemical components. As a traditional Chinese herbal medicine for more than a millennium, wild golden cherry is mainly distributed in northeast, north, and northwest China, among which its cultivation is most prevalent in northeast China. The ethanolic extract of wild golden cherry is a modern traditional Chinese medicine. The ethanolic extract, which is derived from the dried mature fruit of *Rosa laevigata* Michx., has strong antioxidant activity, anti-aging effect, as well as preventive and therapeutic effects on various diseases and can exert pharmacological activity by reducing the level of reactive oxygen species and inhibit various pathogenic factors. The results of the present study showed that the ethanolic extract of wild golden cherry could significantly inhibit the

increase in intracellular reactive oxygen species, downregulate the activity of several intracellular signaling pathways, regulate cell metabolism and gene expression, as well as significantly reduce the level of inflammatory factor IL-1β [9-11].

4.2. Conclusion

In recent years, the morbidity and mortality of chronic kidney disease (CKD) have been on the rise. Hypertension, dyslipidemia, and hyperuricemia are the main mechanisms of which CKD occurs. As an important metabolic organ in the human body, the kidney undertakes an important task of removing excess water and toxins from the blood and maintaining normal circulation and metabolism in the body. The NLRP3 inflammasome pathway of renal intrinsic cell is one of the key links in the body's anti-inflammatory response and plays a vital role in autoimmune diseases, such as IgA nephropathy and nonalcoholic steatohepatitis. The results of the present study showed that the therapeutic effect of the ethanolic extract on the rat model of glomerulonephritis was significantly better than that observed in the blank control group and the DN group; the ethanolic extract of wild golden cherry also demonstrated a significant inhibitory effect on the expression levels of inflammatory factors IL-1β and IL-18 in kidney tissues. The activation of NLRP3 inflammasome pathway leads to the secretion of a large amount of proinflammatory factors by glomerular mesangial cells, which activate nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt, and other pathways to produce a large amount of profibrotic factors. These profibrotic factors promote the development of renal fibrosis. The ethanolic extract of wild golden cherry inhibits NLRP3 inflammasome activity and thus inhibits NLRP3 inflammasome pathway and NF-κBp65 entry into the nucleus to achieve anti-inflammatory effects.

NLRP3 inflammasomes play an important role in regulating autoimmune diseases and the body's inflammatory response. Recent studies have revealed that NLRP3 inflammasomes are involved in the development of kidney diseases [12,13]. It has been demonstrated that NLRP3 messenger RNA (mRNA) and protein expressions are low in normal kidney tissues but significantly higher in diseased kidney tissues, thus suggesting that NLRP3 inflammasomes may be involved in the development of kidney diseases. In addition, it has been reported that the activation of NLRP3 inflammasome pathway participates in the development of kidney injury by promoting the production of IL-13 and IL-6, both of which are important inflammatory mediators in kidney injury. Wang *et al.* [14] showed that the inhibition of NLRP3 inflammasome activation can reduce kidney injury and fibrosis in patients with chronic kidney disease. In addition, a study has shown that caspase-1 protease inhibitors and NLRP3 knockdown can inhibit neutrophil infiltration in acute kidney injury caused by sepsis [15]. However, the role of NLRP3 inflammasomes in renal tissue injury among rats with mesangial proliferative glomerulonephritis has not been investigated. In the present study, we found that the expression of NLRP3 protein was significantly higher in the renal tissues of rats with mesangial proliferative glomerulonephritis compared with the control group; moreover, the expression of NLRP3 positive cells in renal tissues was also significantly higher in rats with mesangial proliferative glomerulonephritis. However, the amount of NLRP3 protein and positive cells in renal tissues significantly decreased after treatment with the ethanolic extract of wild golden cherry. Therefore, we speculate that NLRP3 inflammasomes may be involved in the occurrence of renal injury in mesangial proliferative glomerulonephritis and suggest that ethanolic extract of wild golden cherry could alleviate renal tissue injury by inhibiting NLRP3 expression.

NLRP3 is a deacetylase-like inflammatory factor that regulates various processes, such as aging, cancer, glucose metabolism, and energy homeostasis. An imbalance in NLRP3 expression increases the risk for inflammatory diseases and autoimmune disorders. NLRP3 is widely expressed in renal cells, including glomerular mesangial cells. NLRP3 overexpression has been found to promote damage by a variety of factors, including oxidative stress, apoptosis, and renal inflammatory stimuli. By promoting transforming

growth factor-β1 (TGF-β1) signaling, shortening fibrosis progression, and increasing proteinuria, NLRP3 exerts a significant renal damaging effect. Studies have shown that NLRP3 promotes the expression of TGF-β1 and fibronectin (a key component of extracellular matrix deposition); in addition, it accelerates diabetic kidney injury and delays the fibrotic process. NF-κB is one of the important transcription factors involved in inflammatory response and an important component of the NLRP3 inflammasome pathway that induces the expression of a large number of inflammatory cytokines released extracellularly as early endogenous alerts of inflammation after injury. NF-κB is released earlier than other pro-inflammatory cytokines and acts as an "early mediator" of sepsis. Blocking NF-κB activity reduces mortality in animal models of endotoxemia. Unactivated NF-κB is present as a polymer with IκB or as two polymers with precursor proteins. In response to stimulation by inflammation-inducing factors, NF-κBp65 is transferred from the cytoplasm to the nucleus to regulate the expression of inflammatory cytokines.

Our results showed that the ethanolic extract of wild golden cherry has a protective effect on renal intrinsic cells and may be related to NLRP3 inflammasome pathway, suggesting a potential protective effect of wild golden cherry

against inflammatory damage in renal intrinsic cells. NLRP3 inflammasomes are involved in the development of various chronic inflammatory diseases, such as acute and chronic glomerulonephritis and renal fibrosis. As a natural herbal medicine with high anti-inflammatory, antioxidant, and anti-tumor activities, wild golden cherry provides a new path for further exploration of its physiological functions and mechanisms of action in various diseases. For example, wild golden cherry has been used in the treatment of cardiovascular diseases, tumors, *etc*.; the active ingredient in its extract has preventive and alleviating effects on lesions of the cardiovascular system, urinary system, and nervous system. The ethanol extract of wild golden cherry may play an important role in the protection of kidney cells by improving microcirculation, inhibiting the release of inflammatory factors and oxidative stress, as well as regulating immune function.

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Disclosure statement

The authors declare no conflict of interest.

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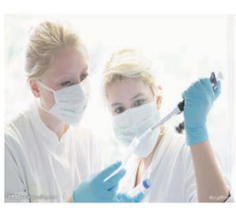

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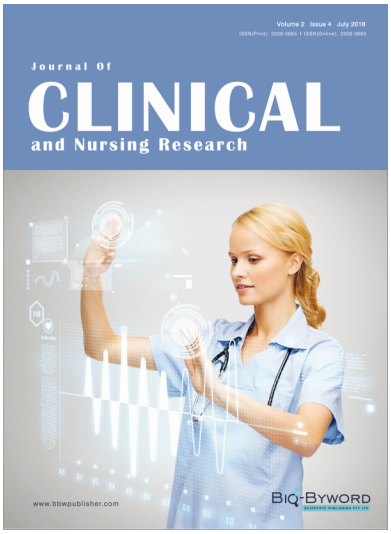

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- Electronic Materials
- Electronics and Communications Engineering
- Power Systems and Power Electronics
- Signal Processing
- Telecommunications Engineering
- Wireless and Mobile Communication