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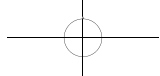
50 Clarence Street

SYDNEY NSW 2000

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ISSN (ONLINE): 2208-3553

ISSN (PRINT): 2208-3545



Proceedings of Anticancer Research

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Website: www.bbwpublisher.com Email: info@bbwpublisher.com

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Transcriptomic Analysis of Organ-Specific Metastasis Regulation in Breast Cancer

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Abstract: *Objective:* This study aimed to investigate the underlying mechanisms of breast cancer metastasis patterns through a transcriptomic analysis of breast cancer with organ-specific metastasis. *Methods:* (1) Breast cancer cell lines capable of specific lung and bone metastasis were constructed using the PyMT-1 cell line. (2) The IVIS imaging system was employed to screen cell lines with robust specific metastatic capabilities. (3) Transcriptome sequencing was conducted on the resultant breast cancer cell lines exhibiting specific metastasis. (4) The transcriptome sequencing data were analyzed to identify pathways influencing specific metastasis in breast cancer. (5) Validation of pathway-related genes through PCR detection. (6) Clinical validation of differentially expressed genes in paraffin specimens. *Results:* (1) The IVIS system confirmed the specific metastatic abilities of PyMT-1, Py-L1, Py-L2, Py-B1 and Py-B2 tumors. PyMT-1, Py-L1 and Py-B1 were chosen for further experiments. (2) Following transcriptome sequencing, differentially expressed genes were identified and enrichment analysis was conducted. Pairwise comparisons among the three cell lines revealed 917 differentially expressed genes between PyMT-1 and Py-L1 (including 641 upregulated and 276 downregulated genes); 4131 differentially expressed genes between PyMT-1 and Py-B1 (including 2158 upregulated and 1973 downregulated genes). *Conclusions:* This study analyzed the genetic background related to breast cancer-specific lung and bone metastasis at the transcriptome level. Obtained the corresponding differential expression profiles and potential functional pathways involved. Demonstrated the promoting roles of CCN2 and ITGB6 in breast cancer lung metastasis, and the promoting roles of CD44 and THBS1 in breast cancer bone metastasis.

Keywords: Breast cancer; Specific metastasis; Transcriptome sequencing

Online publication: August 11, 2026

1. Introduction

According to the epidemiological data statistics on various types of cancer released by the International Agency for Research on Cancer (IARC) under the World Health Organization in April 2024, breast cancer

(with an incidence rate of 11.6%) is the most common cancer among women and the fourth leading cause of cancer-related deaths. The incidence of breast cancer among Chinese women is increasing year by year ^[1]. According to the latest statistics, the age-standardized incidence rate of breast cancer in China is 39.10 per 100,000 ^[2]. Compared to the past, with improvements in early diagnosis and treatment of breast cancer, the disease-free survival period and overall survival period of patients have significantly increased. Although the mortality rate of cancer has decreased ^[3], the incidence of breast cancer is on the rise, with distant metastasis being the primary cause of high mortality in breast cancer patients, accounting for over 90% of cancer deaths due to metastasis of breast cancer.

Due to the current poor efficacy of metastatic breast cancer treatment and the lack of early prediction methods to determine which organs are prone to metastasis, patients with metastatic breast cancer often have a poor prognosis. Therefore, exploring the mechanisms driving breast cancer-specific metastasis is of great significance for identifying new biomarkers and therapeutic targets.

2. Materials and methods

2.1. Experimental materials

PPyMT-1, Py-L1, Py-L2, Py-B1, and Py-B2 cells were obtained from the School of Translational Medicine, Zhejiang University. Female C57BL/6 mice aged 8 weeks were purchased from Jiangsu Jicui Kang Biotechnology Co., Ltd., with an experimental animal production license number: SCXK (Su) 2018-0008. The experimental protocol was approved by the Experimental Animal Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine, with ethics approval number: (2024) Experimental Animal Fast Review No. (687).

With the approval of the ethics committee, clinical and pathological information from 38 patients with invasive ductal carcinoma (IDC) at Baoding First Central Hospital from 2016 to 2023 was collected, with ages ranging from 30 to 68 years and an average age of 51.6 years. Paraffin-embedded tissues included both adjacent and cancerous tissues: 25 cases without distant metastasis, 7 cases with lung metastasis, and 6 cases with bone metastasis. All sections were reviewed and verified by two senior pathologists according to the latest edition of the “WHO Classification of Tumours of the Breast.”

2.2. Acquisition of Py-L1, Py-L2, Py-B1, and Py-B2 tumor cells

In vitro cultured and amplified PPyMT-1 tumor cells were inoculated into immunodeficient mice (nude mice): cell lines capable of lung metastasis (Py-L1, Py-L2) were screened through tail vein injection, and cell lines capable of bone metastasis (Py-B1, Py-B2) were screened through left ventricular injection. The tumor-specific metastatic ability of Py-L1, Py-L2, Py-B1, and Py-B2 cells was verified using the IVIS system. Groups with better tumor formation were selected for subsequent experiments.

2.3. Transcriptome sequencing of Py-L1, Py-L2, Py-B1, and Py-B2 tumor cells

Total RNA was extracted from Py-L1, Py-L2, Py-B1, and Py-B2 tumor cells for sequencing and information analysis. The acquired whole transcriptome expression files required processing steps such as raw data filtering, sequencing error rate checking, and GC content distribution checking, with the final data summarized.

2.4. Transcriptome data analysis

2.4.1. Differential gene screening

First, genes were filtered based on the mean count values, retaining only those with mean counts > 2 in each group for subsequent analysis. The R package DESeq2 was used to normalize the count data across all samples and to calculate the fold changes. Differential expression between the two groups was then statistically tested. Finally, protein-coding genes were selected as differentially expressed based on the fold change and significance test results. In this study, the screening criteria were defined as adjusted *P*-value (q-value) < 0.05 and $|\log_2(\text{fold change})| > 0.5$. The differentially expressed genes were visualized using volcano plots and heatmaps.

2.4.2. Functional analysis of differential genes

ClusterProfiler software was used to perform GO enrichment analysis and functional annotation of differential genes to investigate the biological roles of significantly differentially expressed genes in the study. KEGG pathway analysis was also conducted to identify the most important pathways with significant changes in the study samples. The differential gene set consisted of genes that were significantly differentially expressed and annotated to the GO or KEGG databases, while the background gene set consisted of all genes subjected to significant differential analysis. To assess pathway differences among different subsets, Gene Set Enrichment Analysis (GSEA) was also performed.

2.5. Validation of differentially expressed genes

The qPCR method was used to validate the expression of differential genes in cell wax blocks from 38 patients at Baoding First Central Hospital.

3. Results

3.1. Validation of cell metastatic ability

PyMT-1, Py-L1, Py-L2, Py-B1, and Py-B2 cell lines were injected into mice via tail vein injection, and the growth of cancer foci in mice was observed at 7, 14, 21, and 28 days. The results showed that the fluorescence signal intensity and range at the tumor formation sites in mice in the Py-L1 and Py-B1 groups were significantly higher than those in the Py-L2 and Py-B2 groups (**Figure 1**). Therefore, Py-L1 and Py-B1 were used as breast cancer-specific metastatic cell lines for subsequent transcriptomic analysis in subsequent experiments.

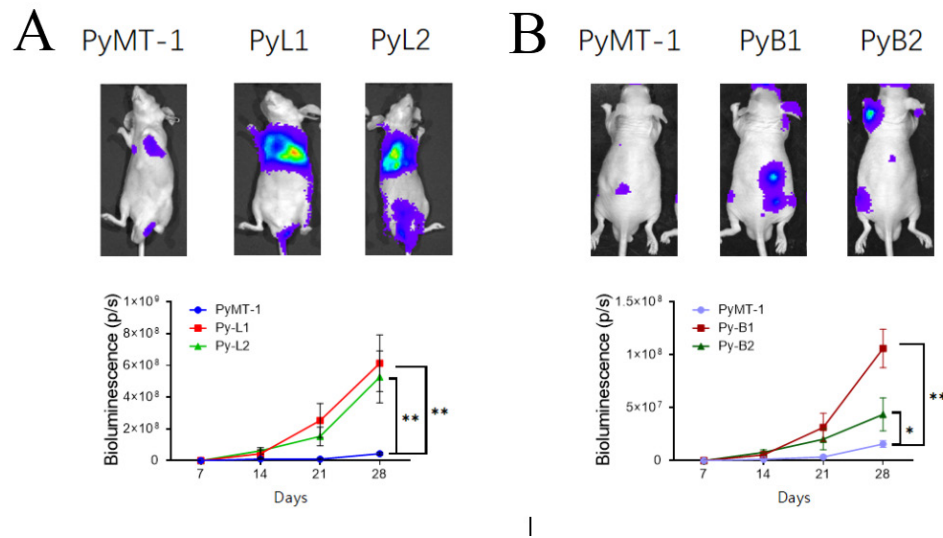


Figure 1. Verification of the metastatic ability of Py-L1 and Py-B1 cells.*indicates $P < 0.05$, ** indicates $P < 0.01$.

3.2. Transcriptome analysis results of PyMT-1, Py-L1, and Py-B1

3.2.1. Transcriptome data analysis results and validation of PyMT-1 and Py-L1

3.2.1.1. Analysis of differentially expressed genes

In this study, the DESeq2 R package (v3.6.1) was used to analyze differentially expressed genes from the quality-controlled transcriptome data. A total of 917 differentially expressed genes were identified, including 641 upregulated genes and 276 downregulated genes. The visual volcano plot is presented below (**Figure 2**).

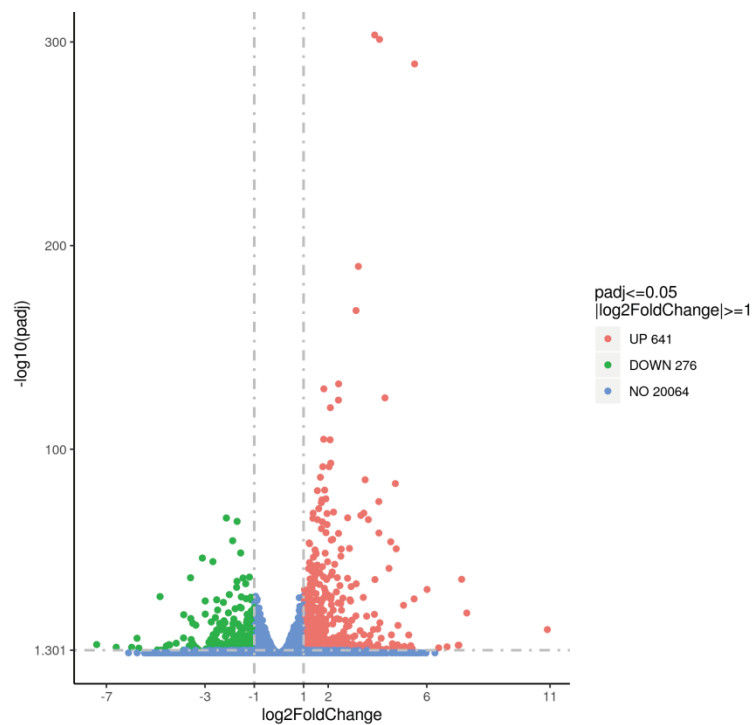


Figure 2. Volcano plot of differentially expressed genes between PyMT-1 and Py-L1.

3.2.1.2. Enrichment analysis of differentially expressed protein-coding RNAs

As mentioned earlier, a total of 917 differentially expressed protein-coding RNAs were identified between the PyMT-1 and Py-L1 groups. GO, KEGG enrichment analyses, and GSEA were performed on the differential gene sets.

3.2.1.2.1. Results of GO functional enrichment analysis

After screening for differentially expressed genes, GO functional enrichment analysis was performed and described from three aspects: biological process (BP), cellular component (CC), and molecular function (MF). A threshold of $P < 0.05$ was used to identify significantly enriched GO terms. The enrichment results were visualized as bubble plots, as shown in **Figure 3**.

Compared with PyMT-1 cells, the upregulated (**Figure 3A**) and downregulated (Figure 3B) GO pathways in Py-L1 cells are presented. The x-axis represents GeneRatio, defined as the number of genes enriched in a specific pathway divided by the total number of genes in the input gene set. The y-axis represents the specific biological pathways. The size of each bubble indicates the gene count (i.e., the number of genes enriched in the GO term), with larger bubbles representing more genes. The color of each bubble represents the significance of the pathway, expressed as the negative logarithm of the P value ($-\log_{10}(P \text{ value})$), with redder bubbles indicating more significant P values.

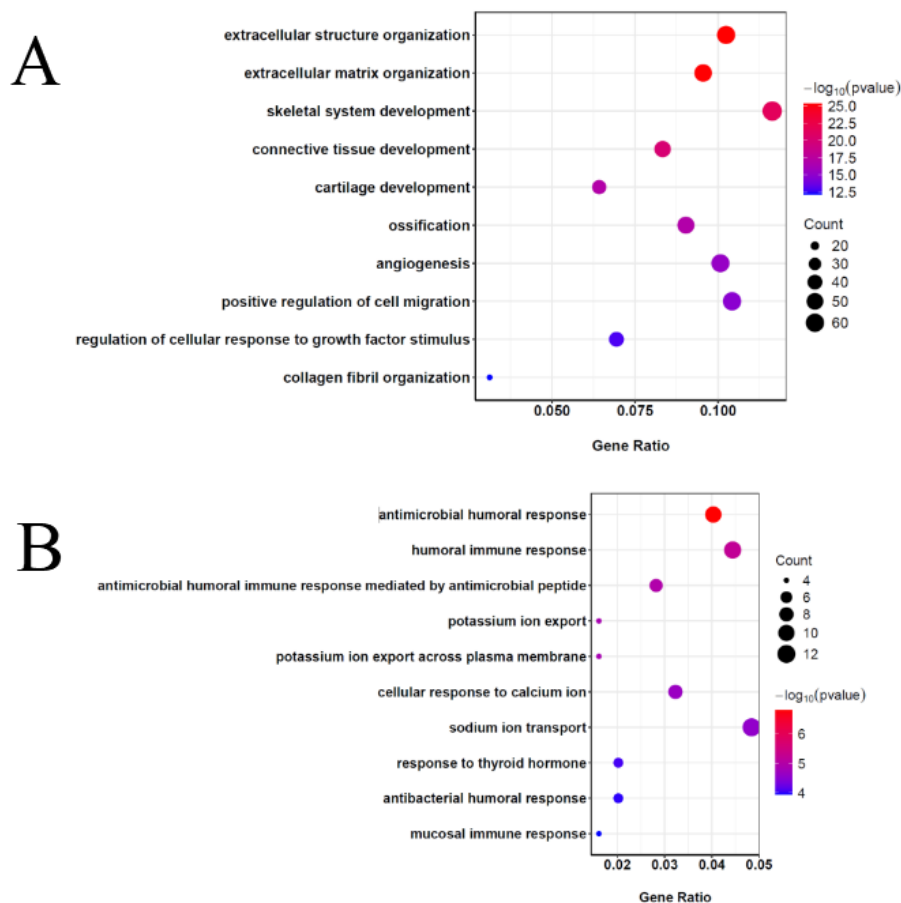


Figure 3. GO pathway analysis of differential genes in PyMT-1 and Py-L1 cells.

3.2.1.2.2. Results of KEGG pathway enrichment analysis

KEGG is a comprehensive database that integrates genomic, chemical, and systemic functional information. KEGG^[4] pathway enrichment analysis can identify significantly affected metabolic and signal transduction pathways. A significance enrichment threshold of $P < 0.05$ was used for KEGG pathway enrichment, and the top 10 significantly enriched pathways were selected. The visualization of the enrichment results in a bubble plot is shown below (Figure 4).

Compared to PyMT-1, the KEGG pathways that are upregulated (Figure 4A) and downregulated (Figure 4B) in Py-L1 cells are depicted; the x-axis represents the ratio of the number of differentially expressed genes annotated to the KEGG pathway to the total number of differentially expressed genes, while the y-axis represents the KEGG pathways. The size of the bubbles indicates the number of genes enriched in the KEGG pathway.

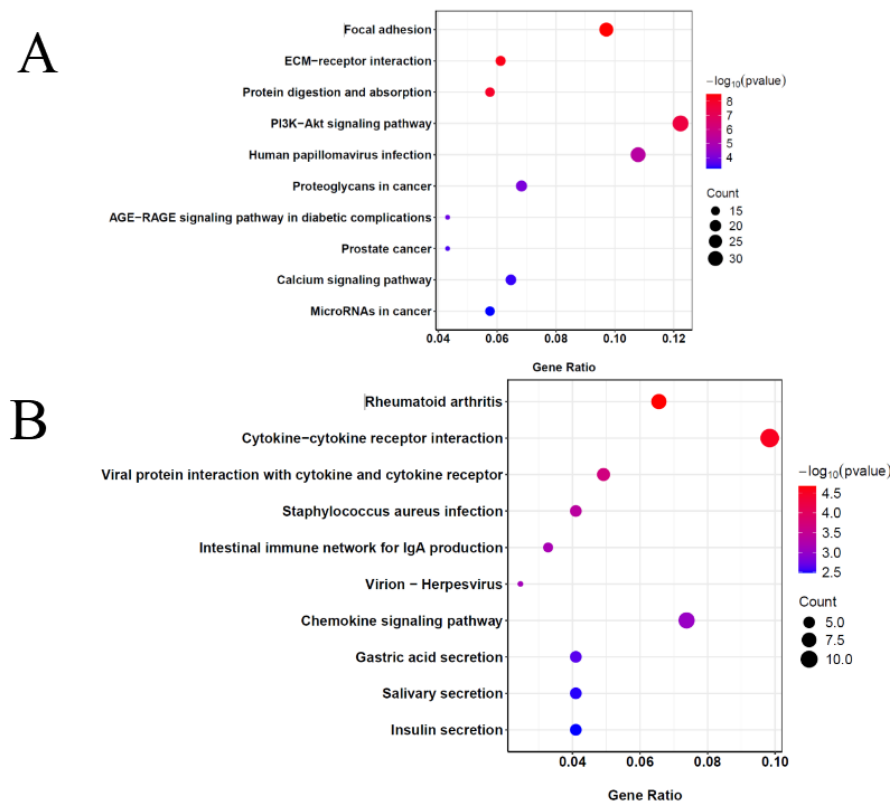


Figure 4. KEGG pathway analysis of differential genes in PyMT-1 and Py-L1 cells.

Based on the aforementioned pathway analysis, it can be observed that the differential genes between PyMT-1 and Py-L1 are primarily associated with cellular activities such as extracellular structural organization, extracellular matrix, as well as signaling pathways including PI3K-Akt, focal adhesion, and ECM-receptor interactions.

GO functional enrichment analysis of the differential genes revealed that among the genes upregulated in Py-L1, the most representative functional genes are primarily involved in biological processes such as extracellular matrix organization (ECM), skeletal system development, connective tissue development, positive regulation of cell migration, and angiogenesis. Conversely, the downregulated genes are mainly

associated with processes such as antimicrobial humoral response, antimicrobial peptide-mediated humoral immune response, humoral immune response, sodium-potassium-calcium ion transport, and mucosal immune response.

In KEGG pathway enrichment analysis, the upregulated differential genes were primarily associated with pathways such as focal adhesion (an important connection point between cells and the extracellular matrix, involved in signal transduction and processes like cell migration and growth), ECM-receptor interaction (the interaction between the extracellular matrix and cell surface receptors is crucial for cell adhesion, migration, and signal transduction), protein digestion and absorption, PI3K-Akt signaling pathway, human papillomavirus infection, cancer-associated proteoglycans, and calcium signaling pathway. The downregulated differential genes were mainly associated with pathways such as cytokine-cytokine receptor interaction, *Staphylococcus aureus* infection, herpesvirus infection, and chemokine signaling pathway.

3.2.1.2.3. Pathways upregulated in Py-L1 cells as revealed by GSEA analysis

GSEA analysis results indicated that the differentially expressed genes were primarily enriched in pathways related to extracellular tissue structure and connective tissue development (**Figure 5**).

When analyzing the enrichment degree of gene sets, the peak of the ES (Enrichment Score) can be observed. The peak of the green curve reflects the maximum enrichment degree of the gene set. A positive peak indicates a positive enrichment of gene sets related to extracellular tissue structure and connective tissue development in Py-L1 cells, with the core genes of the relevant pathways being those preceding the peak.

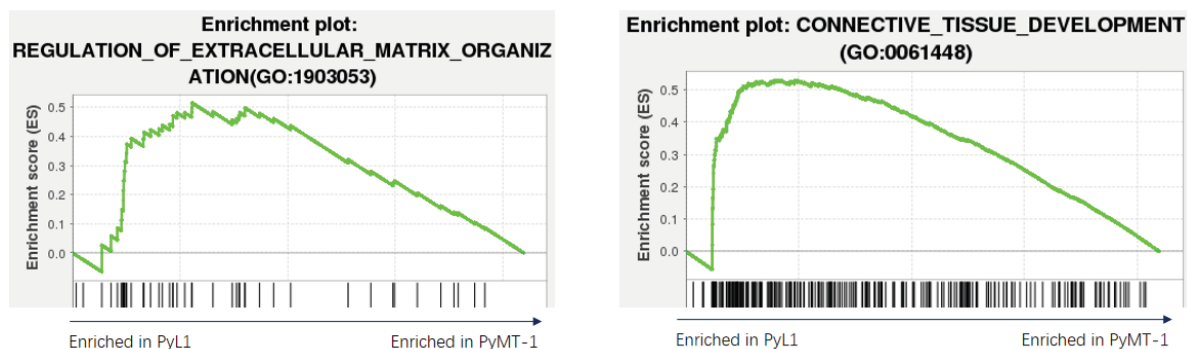


Figure 5. Up-regulated pathways in Py-L1 cells compared to PyMT-1.

3.2.1.3. Real-time fluorescent quantitative PCR detection of differential gene expression levels in PyMT-1 and Py-L1

To further validate the transcriptional levels of genes related to the ECM-receptor interaction and cytokine-cytokine receptor interaction pathways in breast cancer-specific lung metastasis, qPCR experimental techniques were employed to detect differences in the transcriptional levels of genes associated with these two pathways in PyMT-1 and Py-L1. The results are as follows (**Figure 6**). In **Figure 6A**, compared to PyMT-1, the expression levels of genes related to the ECM-receptor interaction pathway, namely Cellular Communication Network Factor 2 (CCN2), COL1A1, and ITGB6, were all elevated in the Py-L1 cell line. In **Figure 6B**, compared to PyMT-1, the expression levels of genes related to cytokine-cytokine receptor interaction, namely CXCL5, TNF, and CXCR4, were all decreased in the Py-L1 cell line.

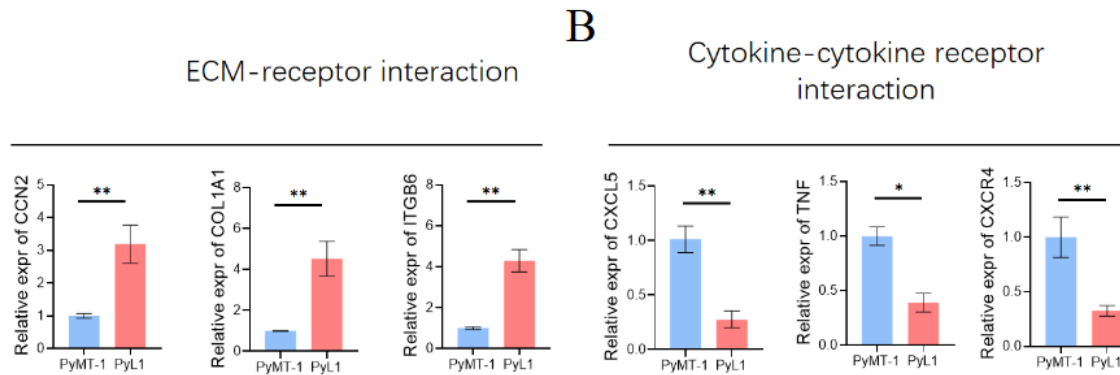


Figure 6. PCR validation results of up-regulated and down-regulated pathways in Py-L1 cell lines compared with PyMT-1.*indicates $P < 0.05$, ** indicates $P < 0.01$.

3.2.1.4. Clinical validation of differentially expressed genes

Among the 38 cases of IDC tissues, the significantly highly expressed genes CCN2 and ITGB6 identified in Py-L1 cells were validated. The results are as follows (Figure 7): Compared to breast cancer tissues without metastasis, the expression levels of the lung-specific metastatic genes CCN2 and ITGB6 were significantly elevated in breast cancer tissues with lung metastasis ($P < 0.05$), while there was no significant change in their expression levels in breast cancer tissues with bone metastasis.

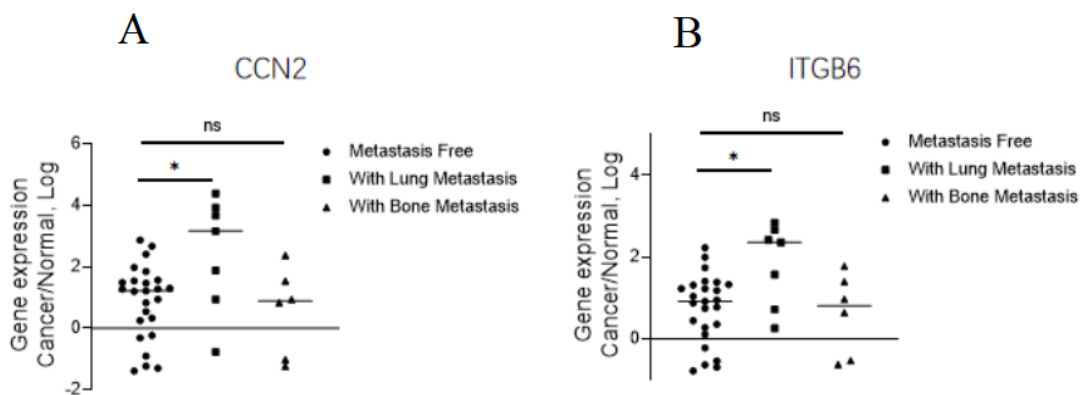


Figure 7. The expression levels of CCN2 and ITGB6 are elevated in breast cancer with lung metastasis.*indicates $P < 0.05$, and ns indicates no statistical difference.

3.2.2. Results and validation of transcriptome data analysis for PyMT-1 and Py-B1

3.2.2.1. Analysis of differentially expressed genes

A total of 4, 131 differentially expressed genes were identified, including 2, 158 upregulated genes and 1, 973 downregulated genes. The visualized volcano plot is shown below (Figure 8).

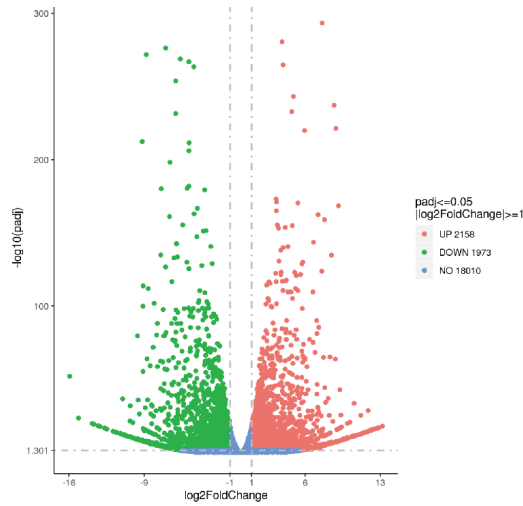


Figure 8. Volcano plot of differentially expressed genes between PyMT-1 and Py-B1.

3.2.2.2. Enrichment analysis of differentially expressed protein-coding RNAs

As previously mentioned, a total of 4, 131 differentially expressed protein-coding RNAs were identified between the PyMT-1 and Py-B1 groups. GO, KEGG pathway enrichment analysis, and GSEA analysis were performed on the set of differentially expressed genes.

3.2.2.2.1. Results of GO functional enrichment analysis

After screening the differentially expressed genes, the visualized bubble plot of the GO functional enrichment analysis results is shown below (**Figure 9**).

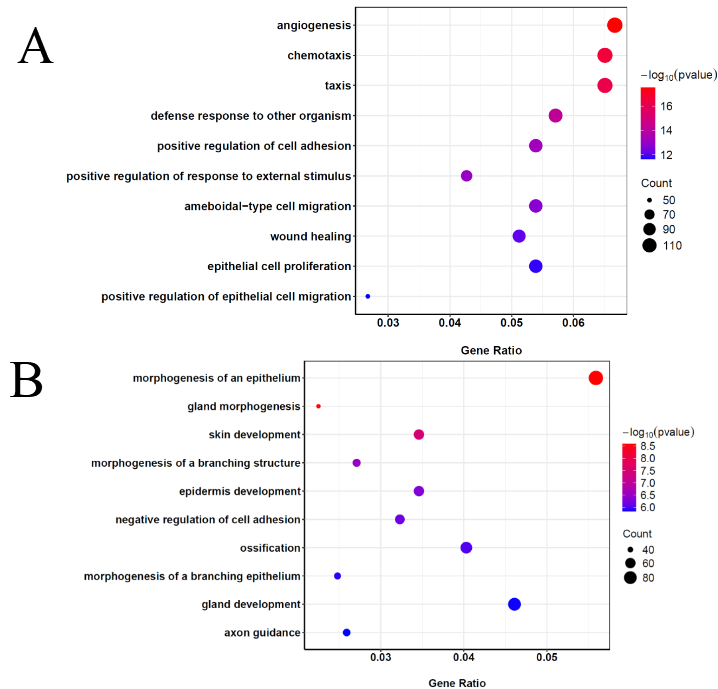


Figure 9. GO pathway analysis of differential genes in PyMT-1 and Py-B1 cells.

3.2.2.2.2. Results of KEGG pathway enrichment analysis

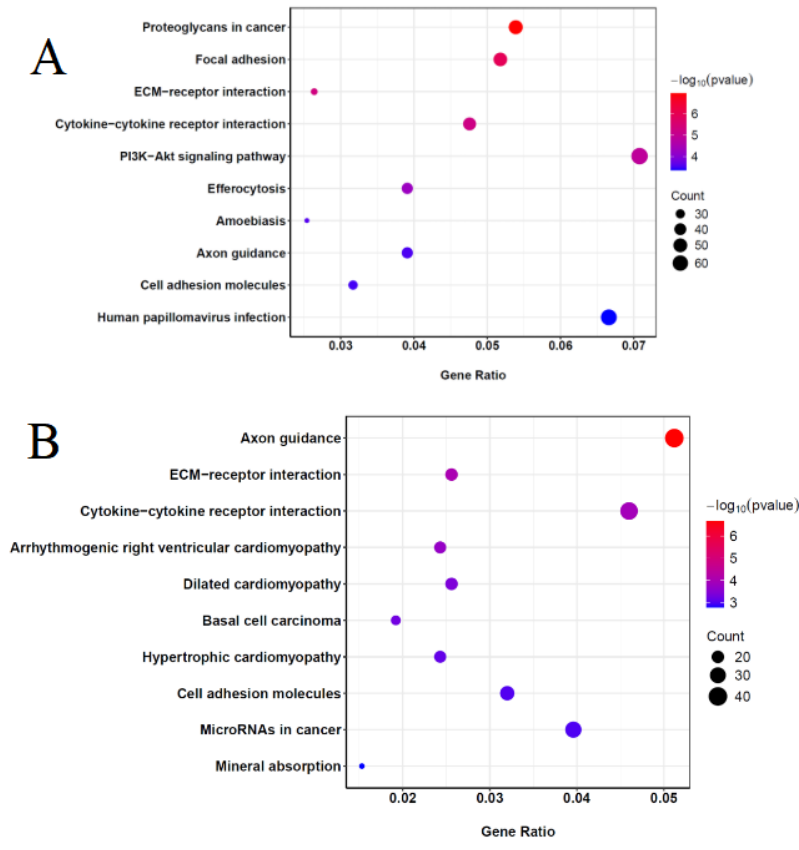


Figure 10. KEGG pathway analysis of differential genes in PyMT-1 and Py-B1 cells.

Through the aforementioned pathway analysis, it can be observed that the differentially expressed genes between PyMT-1 and Py-B1 are primarily associated with cellular activities such as angiogenesis, chemotaxis, positive regulation of cell adhesion, epithelial cell proliferation, and positive regulation of epithelial cell migration, as well as pathways including proteoglycans in cancer, focal adhesion, ECM-receptor interaction, cytokine-cytokine receptor interaction, and the PI3K-Akt signaling pathway.

GO functional enrichment analysis of the differentially expressed genes revealed that among the genes upregulated in Py-B1, the most representative functional genes are primarily involved in biological processes such as angiogenesis, chemotaxis, positive regulation of cell adhesion, epithelial cell proliferation, and positive regulation of epithelial cell migration. Conversely, the downregulated genes are mainly associated with processes such as epithelial morphogenesis, gland morphogenesis, negative regulation of cell adhesion, ossification, and axon guidance.

In KEGG pathway enrichment analysis, the upregulated differentially expressed genes are primarily related to pathways such as proteoglycans in cancer, focal adhesion, ECM-receptor interaction, cytokine-cytokine receptor interaction, PI3K-Akt signaling pathway, efferocytosis, axon guidance, and cell adhesion molecules. The downregulated differentially expressed genes are mainly associated with pathways such as synaptic guidance, ECM-receptor interaction, cytokine-cytokine receptor interaction, cell adhesion

molecules, and microRNAs in cancer.

3.2.2.2.3. Pathways upregulated in Py-B1 cells as revealed by GSEA analysis

GSEA analysis results indicate that the differentially expressed genes are primarily enriched in pathways related to embryonic skeletal system development and fibroblast growth response (**Figure 11**).

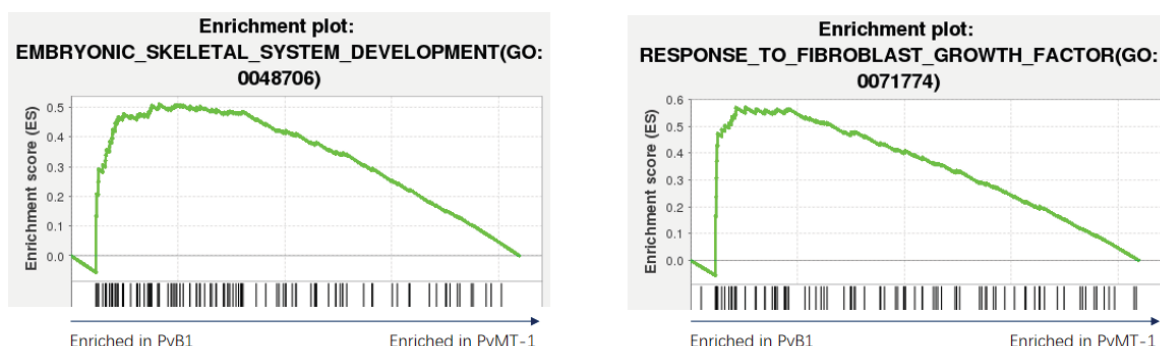


Figure 11. Up-regulated pathways in Py-B1 cells compared to PyMT-1.

3.2.2.3. Real-time fluorescent quantitative PCR analysis of differential gene expression levels in PyMT-1 and Py-B1

To further validate the expression levels of genes related to the proteoglycans in cancer and miRNA pathways in cancer, specifically in the context of breast cancer-specific bone metastasis, qPCR experimental techniques were employed to detect the transcriptional differences of genes associated with these two pathways in PyMT-1 and Py-B1. The results are presented as follows (**Figure 12**). In **Figure 12A**, compared to PyMT-1, the expression levels of genes related to the upregulated proteoglycans in cancer pathway, namely CD44, THBS1, and CAV1, are all elevated in the Py-B1 cell line. In **Figure 12B**, compared to PyMT-1, the expression levels of genes related to the downregulated miRNA pathway in cancer, specifically ST14, DNMT3A, and DNMT3B, are all decreased in the Py-B1 cell line.

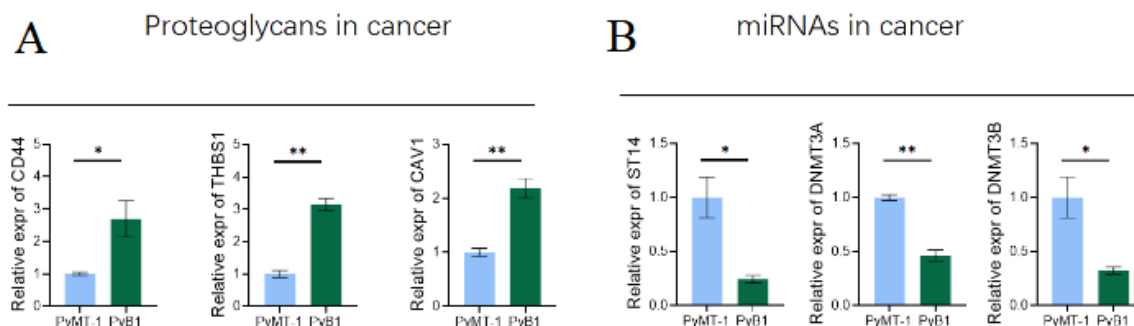


Figure 12. PCR validation results of up-regulated and down-regulated pathways in Py-B1 cell lines compared with PyMT-1.*indicates $P < 0.05$, ** indicates $P < 0.01$.

3.2.2.4. Clinical validation of differentially expressed genes

In 38 cases of invasive ductal carcinoma (IDC) tissues, the significantly highly expressed genes CD44 and

THBS1 identified in Py-B1 cells were validated. The results are as follows (**Figure 13**): Compared to breast cancer tissues without metastasis, the expression levels of the bone-specific metastasis genes CD44 and THBS1 were elevated in breast cancer tissues with bone metastasis ($P < 0.05$), while their expression levels remained unchanged in breast cancer tissues with lung metastasis.

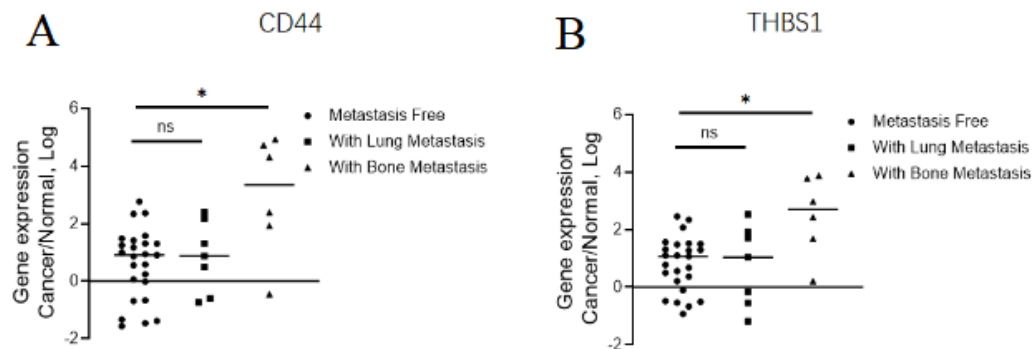


Figure 13. The expression levels of CD44 and THBS1 are elevated in breast cancer with bone metastasis.*indicates $P < 0.05$, ns indicates no statistical difference.

4. Discussion

According to the epidemiological data statistics for various types of cancer released by the International Agency for Research on Cancer (IARC) in April 2024, breast cancer stands as the most prevalent cancer among women and ranks fourth as a cause of cancer-related deaths. Distant metastasis is the primary contributor to the high mortality rate associated with breast cancer, with over 90% of cancer deaths attributed to metastatic spread. The expression of specific molecular mediators at both primary and metastatic sites determines the propensity of tumor cells to metastasize to particular target organs. Breast cancer exhibits metastatic heterogeneity, favoring different organs. Recent studies have identified bone, lung, and liver as common sites for distant metastasis in breast cancer, a phenomenon termed organ-specific metastasis. This study focuses on mouse models of breast cancer with specific bone and lung metastases, employing transcriptomic analysis to explore the underlying mechanisms of breast cancer-specific metastasis.

The efficiency of the metastatic process is exceedingly low, with less than 0.01% of tumor cells from the primary tumor capable of completing distant metastasis and forming secondary lesions at distant sites ^[5]. Clinically established patterns of organ-specific metastasis indicate that the sites of distant metastasis are not random but are influenced by the microenvironment of the target organ.

4.1. Biological function analysis of coding genes related to PyMT-1 and Py-L1

By comparing the PyMT-1 and Py-L1 cell lines, we identified differential genes potentially associated with lung metastasis in breast cancer. Enrichment analysis revealed relevant signaling pathways regulating lung metastasis in breast cancer.

Based on previous results, a total of 917 differential genes were identified between PyMT-1 and Py-L1, including 641 upregulated and 276 downregulated genes. These differential genes underwent GO functional enrichment analysis, KEGG pathway enrichment analysis, and GSEA analysis. Pathway enrichment analysis

indicated that the differential genes were primarily associated with cellular activities such as extracellular structure organization, ECM, skeletal development system, connective tissue development, and angiogenesis, as well as pathways like focal adhesion, PI3K-Akt signaling pathway, and ECM-receptor interaction. The activation or alteration of these pathways suggests their potential involvement in the specific lung metastasis of breast cancer.

Subsequent PCR validation confirmed the upregulation of genes related to ECM-receptor interaction in the upregulated pathway, namely CCN2, COL1A1, and ITGB6, which were significantly elevated in Py-L1 compared to PyMT-1 cell lines. Conversely, genes associated with cytokine-cytokine receptor interaction in the downregulated pathway, such as CXCL5, TNF, and CXCR4, were downregulated in Py-L1. Further validation in clinical samples demonstrated significantly elevated expression levels of CCN2 and ITGB6 in breast cancer tissues with lung metastasis, with no significant changes observed in IDC tissues without metastasis or with bone metastasis. This further suggests that CCN2 and ITGB6 may promote specific lung metastasis in IDC.

The crucial regulatory role of ECM in breast cancer has been widely acknowledged. Compared to normal breast tissue, ECM undergoes significant changes in composition and organization during breast cancer progression. Studies have shown that proteomic analysis of ECM in breast tumors of xenograft mouse models reveals distinct ECM compositions between tumors with high and low metastatic potential [6]. Current research on ECM in breast cancer metastasis is relatively thorough. For instance, Tenascin C (TNC), a cellular matrix protein in ECM, interacts with cell surface receptors and ECM proteins. In the breast, TNC expression is associated with mammary gland involution and breast cancer development [7]. In breast cancer cells, TNC is regulated by microRNA miR335 [8,9]. Metastatic breast cancer cells with a high propensity for lung metastasis often exhibit a loss of miR335 expression, leading to TNC upregulation. The mechanism may involve TNC sequentially activating macrophages and endothelial cells and generating a pro-metastatic vascular niche in the lungs [10]. Therefore, TNC from different sources in the tumor microenvironment promotes the metastatic progression of breast cancer, a finding further validated by this study. Simultaneously, inhibiting ECM components that promote tumor progression and metastasis may represent an effective strategy for treating breast cancer.

CCN2 regulates key biological activities such as cell growth, invasion, and migration. It modulates the breast tumor microenvironment by promoting angiogenesis, activating cancer-associated fibroblasts (CAFs), and inducing inflammation. The activity of CCN2 depends on various factors and varies by breast cancer type. This factor has been shown to promote tumor progression by activating multiple signaling pathways in breast cancer and regulating the expression of genes that facilitate migration, invasion, and chemotherapy resistance. Studies indicate that CCN2 can promote the expression of glucose transporter 3 (GLUT3), facilitating cell survival and metabolism, and upregulate VEGF expression to drive angiogenesis, supporting tumor growth [11,12]. This study found that CCN2 expression was upregulated only in IDC with lung metastasis, suggesting its potential as a specific lung metastasis marker.

The pre-metastatic niche (PMN) generated by primary tumors in specific target organs is crucial for the subsequent colonization and metastasis of tumor cells, involving vascular permeability and angiogenesis, immunosuppression, inflammation, and organ specificity [13]. The initial stage of PMN formation is characterized by angiogenesis and vascular permeability. Several PMN biomarkers related to angiogenesis and vascular permeability have been reported to aid in the diagnosis and treatment of metastatic cancer.

For example, cancer-associated fibroblasts (CAFs) are the primary stromal component of the tumor microenvironment ^[14]. CAFs not only promote cancer growth and metastasis by secreting cytokines and exosomes and synthesizing and remodeling the extracellular matrix ^[15,16] but also play a major role in angiogenesis by secreting cytokines ^[17,18]. AKT is a key factor in the PI3K-Akt signaling pathway. Three different AKT isoforms, namely AKT1, AKT2, and AKT3, are currently known. The PI3K-Akt signaling pathway is frequently altered in breast cancer patients. AKT typically promotes proliferation by regulating the cell cycle and prevents apoptosis by inhibiting pro-apoptotic proteins and promoting anti-apoptotic signals. Some studies have confirmed the anti-metastatic and anti-migratory abilities of AKT1, independent of breast cancer subtype ^[19,20].

4.2. Biological function analysis of coding genes related to PyMT-1 and Py-B1

By comparing the PyMT-1 and Py-B1 cell lines, we identified differential genes potentially associated with bone metastasis in breast cancer. Enrichment analysis revealed relevant signaling pathways regulating bone metastasis in breast cancer.

Based on previous results, a total of 4131 differential genes were identified between PyMT-1 and Py-B1, including 2158 upregulated and 1973 downregulated genes. These differential genes underwent GO functional enrichment analysis, KEGG pathway enrichment analysis, and GSEA analysis. Pathway enrichment analysis indicated that the differential genes were primarily associated with cellular activities such as angiogenesis, chemotaxis, positive regulation of cell adhesion, epithelial cell proliferation, and positive regulation of epithelial cell migration, as well as pathways like proteoglycans in cancer, focal adhesion, ECM-receptor interaction, cytokine-cytokine receptor interaction, and PI3K-Akt signaling pathway. Genes with altered expression levels in these two cell lines may be correlated with specific bone metastasis in breast cancer.

Subsequent PCR validation confirmed the upregulation of genes related to proteoglycans in cancer in the upregulated pathway, namely CD44, THBS1, and CAV1, which were significantly elevated in Py-B1 compared to PyMT-1 cell lines. Conversely, genes related to miRNA function in cancer in the downregulated pathway, such as ST14, DNMT3A, and DNMT3B, were downregulated in Py-B1. Further validation in clinical samples demonstrated significantly elevated expression levels of CD44 and THBS1 in breast cancer tissues with bone metastasis, with no significant changes observed in IDC tissues without metastasis or with lung metastasis. This further suggests that CD44 and THBS1 may promote specific bone metastasis in IDC. Studies have shown that CD44 can enhance the adhesion of metastatic breast cancer cells to bone marrow endothelial cells ^[21], consistent with this study's suggestion that increased CD44 expression levels may promote specific bone metastasis in breast cancer.

In other pathways, periostin (POSTN) plays a crucial role in adult skeletal, dental, and cardiac development, as well as tissue repair ^[22]. POSTN is known to regulate intracellular tyrosine kinase signaling, such as the PI3K-Akt signaling pathway ^[23]. Increasing evidence indicates that POSTN plays a significant role in breast cancer progression and metastasis. Overexpression of POSTN in human breast cancer cells induces primary tumor growth and angiogenesis in xenograft models by enhancing the expression of vascular endothelial growth factor receptor 2 (VEGFR2) in endothelial cells ^[24]. In mouse xenograft models, mice with bone metastasis exhibit high POSTN expression in the stroma and plasma surrounding metastatic tumors ^[25]. POSTN has been identified as a key ECM component of the metastatic niche ^[26], with studies

suggesting its use as a novel biomarker in combination with CA153 and CEA for breast cancer diagnosis and metastasis prediction ^[27].

By comparing the differential genes between PyMT-1 with Py-L1 and between PyMT-1 with Py-B1, we found that angiogenesis, ECM-receptor interaction, and the PI3K-Akt signaling pathway were enriched in both GO functional enrichment analysis and KEGG pathway enrichment analysis, suggesting that these cellular activities and pathways may promote both lung and bone metastasis in breast cancer to some extent.

This study conducted transcriptomic sequencing and protein-coding RNA analysis on three cell lines: PyMT-1, Py-L1, and Py-B1. Using GO, KEGG, and GSEA enrichment analyses, we predicted the biological functions of differential genes and performed preliminary validation of some upregulated and downregulated pathway-related genes. By collecting clinical data, we further validated the expression levels of the identified differential genes, gaining a basic understanding of the genetic background of specific lung and bone metastasis in breast cancer. Subsequent research can employ proteomics and metabolomics approaches to more comprehensively investigate the specific mechanisms of breast cancer-specific metastasis, providing a theoretical basis for the clinical treatment of metastatic breast cancer.

This study has several limitations. First, based on the findings of this study, further functional exploration of the important differential genes is indeed necessary, followed by an in-depth investigation of the underlying molecular mechanisms. Second, the breast cancer-specific metastasis model constructed in this study is based on single-cell transcriptomic data, which describes cellular function at the RNA level but does not specifically explore how the identified differential genes and related pathways function in organ-specific metastasis of breast cancer. According to the central dogma of “DNA-RNA-protein,” there is a lack of data on protein expression levels, such as single-cell proteomics data.

5. Conclusion

The upregulated enriched pathways among the differential genes between PyMT-1 and Py-L1, such as ECM, focal adhesion, and the PI3K-Akt signaling pathway, may be associated with lung metastasis in breast cancer. The upregulated enriched pathways among the differential genes between PyMT-1 and Py-B1, such as angiogenesis, chemotaxis, positive regulation of cell adhesion, and epithelial cell proliferation, may be associated with bone metastasis in breast cancer.

The expression levels of CCN2 and ITGB6 are elevated in breast cancer tissues with lung metastasis, while the expression levels of CD44 and THBS1 are elevated in breast cancer tissues with bone metastasis.

Funding

Hebei Health Commission Project (Project No.: 20240287); Hebei Natural Science Foundation of China (Key Program, Project No.: H2024104001).

Disclosure statement

The authors declare no conflict of interest.

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Research on the Application of Acupoint Embedding Thread in Comprehensive Tumor Treatment

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Abstract: Acupoint embedding therapy is a traditional Chinese external treatment method that, under the guidance of the theory of meridians and acupuncture in traditional Chinese medicine, implants absorbable thread bodies into specific acupoints and exerts therapeutic effects through continuous, gentle, long-lasting stimulation. In recent years, as the comprehensive treatment model for tumors has shifted from simply “prolonging survival” to “improving quality of life”, acupoint embedding, as an important part of integrated traditional Chinese and Western medicine treatment, has shown unique advantages in alleviating cancer pain, reducing adverse reactions of radiotherapy and chemotherapy, improving gastrointestinal function and regulating immune function. Based on relevant studies at home and abroad, this article reviews the mechanism of action, clinical application status and existing problems of acupoint embedding in adjuvant treatment of tumors, with the aim of providing references for clinical practice and in-depth research.

Keywords: Acupoint embedding; Tumor; Cancer pain; Chemoradiotherapy adverse reactions; Immune regulation

Online publication: August 11, 2025

1. Introduction

Malignant tumors have become a serious threat to human health. According to global cancer statistics, the incidence and mortality rates of malignant tumors continue to rise year by year. The current clinical treatment mainly consists of surgery, radiotherapy, chemotherapy, targeted therapy and immunotherapy. Although these treatment methods significantly prolong the survival period of patients, adverse reactions such as pain, nausea and vomiting, bone marrow suppression, fatigue, gastrointestinal dysfunction and psychological disorders are often accompanied during the treatment process, seriously affecting the treatment compliance and quality of life of patients.

With the transformation of the tumor treatment model from simply “prolonging survival” to “improving

quality of life”, the combination of traditional Chinese and Western medicine has gradually gained attention. The unique advantages of traditional Chinese medicine in “strengthening the body and enhancing the root” and “reducing toxicity and increasing efficacy” make it an indispensable part of comprehensive tumor treatment. Acupoint embedding thread therapy, as an extension and development of acupuncture therapy, involves embedding absorbable thread bodies into acupoints to continuously stimulate meridians in the body. It has the characteristics of long stimulation time, low treatment frequency and good compliance. In recent years, the therapy has been widely used in the field of chronic disease and tumor support treatment, and has made certain progress in improving the symptoms of tumor patients. This article provides a systematic review of the mechanism of action, clinical application and existing problems of acupoint embedding in comprehensive tumor treatment, with the aim of providing a reference for the clinical promotion and in-depth study of this therapy.

2. Overview of acupoint embedding therapy

2.1. Basic principles

Acupoint embedding therapy, under the guidance of acupuncture theory, involves implanting absorbable protein threads (such as sheep intestine thread, polyglycolic acid thread, etc.) into relevant acupoints. Through the degradation and absorption process of the thread body, a continuous stimulating effect is produced to achieve the purpose of regulating qi and blood, unblocking meridians, and strengthening the body and eliminating pathogenic factors^[1].

From the perspective of modern medicine, the mechanism of action can be summarized into three levels: At the physical level, the continuous mechanical stimulation of the thread on the acupoints and nerve endings produces a long-lasting needle sensation similar to “retention”, and the stimulation effect lasts for several hours to several days, much longer than the immediate effect of ordinary acupuncture; At the neuro-humoral level, acupoint stimulation is transmitted through afferent nerves to the central nervous system, which regulates the neuro-endocrine-immune network, promotes the release of endogenous opioid peptides to exert central analgesic effects, and regulates the expression of inflammatory factors to improve the systemic inflammatory state; At the local tissue level, the filament degradation process induces immune cell infiltration and cytokine release in the local microenvironment, improves microcirculation and tissue metabolism, and promotes the repair of damaged tissues. The thread usually takes 7 to 30 days to fully degrade and absorb in the body. During this period, it continuously provides gentle and effective stimulation to the acupoint, and this “long-lasting needle sensation” feature is the core difference between acupoint embedding thread and ordinary acupuncture.

2.2. Characteristics of action

The core advantage of acupoint embedding over traditional acupuncture lies in its “long-lasting stimulation, low-frequency treatment” feature. The thread body is slowly degraded in the body, and a single treatment can maintain a continuous stimulating effect for 7 to 30 days. The treatment frequency is usually once every 1 to 2 weeks, significantly lower than the daily or alternate treatment of ordinary acupuncture^[2]. This feature offers a triple advantage: first, it reduces the frequency of patient visits, lowering time and economic costs; Second, it improves treatment compliance, especially for cancer patients who have difficulty moving or live in remote areas; Third, continuous acupoint stimulation is more conducive to the long-term management

of chronic symptoms and is highly consistent with the need for long-term rehabilitation support for cancer patients ^[3].

3. The mechanism of acupoint embedding thread in the comprehensive treatment of tumors

3.1. Immunomodulatory effects

Tumorigenesis and development are closely associated with immune dysfunction. The body's anti-tumor immunity mainly depends on the functional integrity of immune effector cells such as T lymphocytes and natural killer cells (NK cells). Studies have shown that acupuncture and acupoint stimulation can regulate the proportion of T lymphocyte subsets, increase NK cell activity, and enhance the body's immune surveillance function.

By continuously stimulating tonifying acupoints such as Zusanli, Guanyuan and Qihai, acupoint embedding can improve the immunosuppressive state of cancer patients and enhance the body's disease resistance. A randomized controlled study by Zhang et al. ^[2] on 140 patients with spleen and lung qi deficiency syndrome of non-small cell lung cancer after chemotherapy showed that after treatment with acupoint thread and Jianpi Qingfei Decoction, the CD4⁺/CD8⁺ ratio and interferon - γ (IFN- γ) levels in the study group were significantly higher than those in the control group, while the interleukin-10 (IL-10) level was lower than that in the control group. It was suggested that acupoint embedding could effectively improve the immune function of patients after chemotherapy. Another study observed the effect of acupoint embedding on T lymphocyte subsets in the peripheral blood of colorectal cancer patients and found that it could maintain the normal distribution of T lymphocyte subsets in the peripheral blood of patients during chemotherapy, thereby better maintaining the immune status of patients ^[4]. Animal experiments further revealed the mechanism by embedding sheep intestinal thread into the Zusanli point in rats, which induced an increase in the levels of macrophage CD68, tumor necrosis factor - α (TNF- α), and interleukin-1 β (IL-1 β) in the local microenvironment ^[5]. The findings suggest that acupoint embedding may activate immune cells by inducing local aseptic inflammatory responses at the acupoint, thereby initiating systemic immunomodulatory effects.

3.2. Analgesic effects

Cancer pain is one of the most common and unbearable symptoms for cancer patients. According to the World Health Organization, about 60% to 90% of patients with advanced cancer suffer from cancer pain to varying degrees. The analgesic mechanisms of acupoint embedding mainly include: activating the endogenous analgesic system and promoting the release of endogenous opioid peptides such as β -endorphin and enkephalin; Inhibiting the expression of pain mediators such as prostaglandins, substance P, etc. Regulate pain conduction in the central nervous system and raise the pain threshold; Relieve pain indirectly by regulating inflammatory factors. Relevant studies have shown that back-shu acupoint embedding can relieve cancer pain, improve the quality of life of patients, and at the same time improve anxiety and depression, demonstrating the comprehensive regulatory advantage of acupoint embedding in pain relief ^[6].

3.3. Improve gastrointestinal function

Patients with tumors, especially those with digestive system tumors, often experience a series of gastrointestinal dysfunction symptoms such as nausea, vomiting, loss of appetite, abdominal distension,

constipation, etc. These symptoms not only affect the patient's nutritional intake, but also seriously reduce the quality of life. Acupoint embedding can promote the recovery of gastrointestinal motility and improve digestive function by stimulating acupoints related to the stomach meridian, spleen meridian and Ren meridian such as Zusanli, Zhongwan and Tianshu. Studies have found that it has a good sustained effect in improving abdominal pain and quality of life. Studies on patients after colorectal cancer surgery have shown that acupoint embedding therapy can significantly accelerate intestinal function recovery and shorten the time to first flatulence and defecation after surgery^[7]. Acupoint embedding also shows certain application value in the prevention and treatment of delayed-type diarrhea caused by irinotecan^[8].

3.4. Regulating the neuroendocrine system

Long-term dual stress of disease and treatment in cancer patients can lead to neuroendocrine dysfunction, mainly manifested as dysfunction of the hypothalamic-pituitary-adrenal axis (HPA axis), abnormal increase in sympathetic nerve tension, etc.^[9] Acupoint embedding can improve the overall condition of patients by regulating the function of the HPA axis, alleviating anxiety, depression and cancer-related fatigue. Clinical studies of Wuzangshu acupoint embedding for the treatment of cancer-related fatigue in breast cancer patients have shown that the therapy can effectively relieve fatigue symptoms, improve quality of life and immune function^[10]. For the "chemotherapy brain" (chemotherapy-related cognitive impairment) that occurs in breast cancer patients after chemotherapy, the study suggests that acupoint thread therapy can restore neurological function, regulate neural reflexes, and inhibit the release of inflammatory factors^[11].

4. Clinical application of acupoint embedding in comprehensive treatment of tumors

4.1. Cancer pain treatment

Cancer pain has a significant impact on the quality of life of patients and is one of the core elements of palliative care for tumors. Although the three-step analgesic principle is the standard regimen, long-term use of opioids is prone to problems such as nausea, vomiting, constipation, drug resistance and addiction risk, so adjuvant analgesic treatment has received extensive attention.

A number of randomized controlled studies have shown that acupoint embedding is one of the effective adjunctive methods for controlling cancer pain. Studies have found that acupoint embedding combined with three-step pain relief treatment can significantly increase the efficiency of pain relief and improve the quality of life of patients. Gou Jiao et al. conducted a randomized controlled study on 80 patients with moderate to severe bone metastatic cancer pain and found that acupoint embedding combined with paracetamol and oxycodone tablets could significantly relieve pain, reduce the dosage of painkillers, lower the incidence of drug-related adverse reactions, and significantly improve the quality of life of patients^[12]. A study on patients with lung cancer pain also confirmed the analgesic effect of acupoint embedding. Common acupoints include Ashi, Shenshu, Pishu, Weishu, Zusanli, Sanyinjiao, Yanglingquan, etc. The principle is to select acupoints based on syndrome differentiation combined with local location.

4.2. Reduce adverse reactions of radiotherapy and chemotherapy

4.2.1. Nausea and vomiting

Chemotherapy-related nausea and vomiting are among the most common and feared adverse reactions for

patients. In clinical practice, acupoints such as Neiguan, Zusanli, Zhongwan and Gongsun are often selected for thread embedding treatment. Continuous stimulation can improve gastrointestinal nerve function, relieve nausea and vomiting, and increase patients' food intake and treatment compliance. A randomized controlled study of patients with non-small cell lung cancer undergoing chemotherapy showed that moxibustion combined with acupoint embedding could significantly improve chemotherapy-related nausea and vomiting and improve the quality of life of patients ^[13]. Another study observed the intervention effect of acupoint thread on chemotherapy-induced nausea and vomiting in patients with malignant hematological diseases and found that it could improve gastrointestinal motility hormone indicators and quality of life in patients ^[14].

4.2.2. Myelosuppression

Myelosuppression manifestations such as leukopenia, thrombocytopenia and anemia often occur after chemotherapy, which not only increase the risk of infection and bleeding, but also often lead to delayed or reduced chemotherapy dosage. Some clinical studies have found that stimulating acupoints such as Zusanli, Guanyuan, and Pishu can promote the production of qi and blood and improve the state of bone marrow suppression. A clinical study involving 72 patients with advanced non-small cell lung cancer found that acupoint embedding combined with GP chemotherapy could lower the grade of adverse reactions and reduce the degree of bone marrow suppression ^[15].

4.2.3. Cancer-related fatigue

Cancer-related fatigue is an important long-term symptom in patients with cancer, characterized by persistent, subjective fatigue that is closely related to the tumor itself and treatment and is difficult to relieve with rest. Acupoint embedding can improve fatigue and enhance activity and quality of life by strengthening the body and tonifying the spleen and kidneys. The clinical study of Wuzangshu acupoint embedding for the treatment of cancer-related fatigue in breast cancer patients has achieved positive results, providing clinical evidence for the application of acupoint embedding in this field ^[16].

4.3. Postoperative rehabilitation of tumors

Patients who have undergone tumor surgery are prone to problems such as gastrointestinal dysfunction, delayed flatulence and defecation, loss of appetite, and slow recovery of physical strength, which seriously affect the postoperative recovery process. Acupuncture for the recovery of gastrointestinal function has been widely studied in recent years. A randomized controlled study involving 80 patients after laparoscopic rectal cancer surgery showed that warming the kidney, strengthening the spleen and benefiting qi combined with embedding thread at Zusanli point could effectively promote the recovery of gastrointestinal function and improve immune function in patients ^[16]. In patients after colorectal cancer surgery, acupoint embedding therapy also showed the effect of accelerating intestinal function recovery ^[18]. Common acupoints include Tianshu, Zusanli, Shangjuxu, Zhongwan, Dachangshu, etc., based on the principle of regulating the gastrointestinal tract and strengthening the body.

4.4. Improve the quality of life of cancer patients

With the obvious trend of cancer becoming chronic, improving quality of life has become one of the important goals of cancer treatment. Studies have shown that acupoint embedding can improve the quality of life of cancer patients in multiple ways: relieve pain, improve sleep, increase appetite, relieve anxiety and

depression, and improve physical condition. Some studies have found that patients' Karnofsky Performance Status Score (KPS) and Quality of Life Core Scale score (QLQ-C30) improved significantly after thread embedding treatment. Acupoint embedding, through the comprehensive regulation of multiple symptoms such as pain, digestive tract symptoms, fatigue and mood, demonstrates the advantages of the holistic concept and syndrome differentiation and treatment of traditional Chinese medicine.

5. Common acupoints and their selection patterns

According to current literature reports, thread embedding treatment for cancer patients often follows the principle of combining syndrome differentiation with disease differentiation, and the selection of acupoints shows certain regularity.

The main acupoints for strengthening the body are Zusanli, Guanyuan, Qihai, Pishu and Shenshu, which are mainly used to enhance immunity, improve physical condition and relieve fatigue. Among them, Zusanli is the most frequently used acupoint, embodying the classic theory of “retaining the three Li points in the abdomen” in the “Four General Acupoint Songs”.

- (1) Pain-relieving acupoints: mainly ashi, Yanglingquan, Sanyinjiao, Hegu, Taichong, mainly used for the treatment of cancer pain and bone metastasis pain, embodying the therapeutic concept of “if there is no flow, there is pain; if there is no flow, there is no pain”.
- (2) Acupoints for regulating the spleen and stomach: Zhongwan, Tianshu, Neiguan, Gongsun, Shangjuxu are the main acupoints for improving digestive tract symptoms such as nausea, vomiting, constipation and abdominal distension.
- (3) Emotional regulation acupoints: Mainly Shenmen, Baihui, Yintang, Taichong, mainly used for regulating emotional problems such as anxiety, depression, and sleep disorders.

Overall, the selection of acupoint embedding in tumor supportive treatment centers on Zusanli, taking into account both syndrome differentiation and local selection of acupoints, embodying the therapeutic principle of “strengthening the body and eliminating pathogenic factors, treating both symptoms and root causes”.

6. Conclusion

To sum up, acupoint embedding, as a safe and convenient traditional Chinese medicine external treatment method, has shown unique auxiliary value in the field of tumor supportive treatment. Existing clinical evidence supports its application in alleviating cancer pain, reducing nausea, vomiting and bone marrow suppression caused by radiotherapy and chemotherapy, promoting postoperative gastrointestinal function recovery, improving cancer-related fatigue, etc., and shows comprehensive advantages of multi-dimensional regulation in improving the quality of life of patients. The mechanism of action involves the overall regulation of the neuro-endocrine-immune network, and the hypothesis that linear degradation products induce local aseptic inflammatory responses and activate Toll-like receptor signaling pathways provides a new direction for subsequent studies.

However, there are still many deficiencies in this field at present. First, studies are mostly single-center, small-sample, with most trials having a sample size of 60 to 150 cases, limited statistical power, and the particularity of acupoint embedding operations makes it difficult to perform complete blinding for both

the operator and the patient, which may introduce implementation bias and measurement bias. Secondly, different studies used different efficacy evaluation indicators (such as VAS, NRS, KPS, QLQ-C30, etc.), and there was a lack of unified standards for operation elements such as thread materials (sheep intestine thread, PGA thread, etc.), acupoint combinations, treatment frequencies, and course Settings, resulting in poor comparability and repeatability between studies and limiting the conduct of high-quality meta-analyses. At the same time, existing mechanism studies mostly remain at the level of clinical efficacy observation, and there is a lack of systematic exploration of the molecular mechanisms by which acupoint embedding regulates the neuro-immune-endocrine network and reshapes the tumor microenvironment. At the same time, the vast majority of studies did not use “false thread embedding” (only the skin was pricked without thread embedding) as a placebo control, making it difficult to eliminate the interference of psychological comfort effect on the assessment of subjective symptoms (fatigue, pain); There was also a lack of long-term systematic follow-up for rare adverse events such as thread migration, deep infection, and allergic reactions.

In response to these limitations, future research should focus on the following directions: conducting multicenter, large-sample, high-quality randomized controlled studies and promoting the inclusion of acupoint embedding in tumor supportive treatment guidelines; Establish standardized operation norms covering thread materials, acupoint combinations, treatment frequency and course of treatment to enhance comparability and repeatability among studies; Explore in depth the molecular mechanisms by which acupoint embedding regulates the tumor immune microenvironment, especially the hypothesis that “thread degradation products act as adjuvants to activate the TLR pathway” deserves particular attention; Actively promote the combined application research of acupoint embedding with modern anti-tumor approaches such as immunotherapy and targeted therapy. Animal experiments have preliminarily confirmed that acupoint embedding can enhance the anti-tumor efficacy of anti-PD-1 inhibitors^[19] and has significant potential for clinical translation. Based on the above research, an expert consensus on acupoint embedding for tumor support therapy was ultimately formed to provide standardized guidance for clinical promotion.

Funding

Xianyang Science and Technology Bureau Qinchuangyuan Traditional Chinese Medicine Industry Innovation Agglomeration Area Project (Project No.: L2024-QCY-ZYYJJQ-300)

Disclosure statement

The authors declare no conflict of interest.

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Publisher's note

Bio-Byword Scientific Publishing remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Research Progress on the Efficacy of PD-1 Inhibitors Combined with Chemotherapy in Advanced Gastric Cancer and the Regulatory Mechanism of Th1/Th2 Immune Balance

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Abstract: Advanced gastric cancer is highly malignant, and traditional chemotherapy has limited efficacy. Currently, combining PD-1 inhibitors with chemotherapy represents a significant breakthrough in immunotherapy for this disease. This article reviews clinical evidence on the efficacy of PD-1 inhibitors combined with chemotherapy in advanced gastric cancer and the regulatory mechanism of Th1/Th2 immune balance. It explores the advantages of the combination strategy in terms of objective response rate (ORR), progression-free survival (PFS), and overall survival (OS). The combination therapy can harness cytotoxic effects to release tumor antigens, actively reshape the tumor immune microenvironment, and regulate key molecules such as MFSD2A. However, the predictive value of biomarkers is limited, and strategies to reverse the Th2-dominant microenvironment are immature, necessitating the integration of multi-omics data to construct precise predictive models. Future efforts should focus on developing novel combination regimens, establishing a multidimensional biomarker system, and optimizing individualized immune regulatory strategies to further enhance long-term survival benefits for patients with advanced gastric cancer.

Keywords: Advanced gastric cancer; PD-1 inhibitors; Combined chemotherapy; Th1/Th2 immune balance; Immune microenvironment

Online publication: August 11, 2026

1. Introduction and research value positioning

1.1. Clinical challenges and immunotherapy needs in advanced gastric cancer

Advanced gastric cancer (AGC) is characterized by high heterogeneity, concealed early symptoms, and frequent distant metastasis at diagnosis, resulting in a poor prognosis with a 5-year survival rate of less than 30%. Traditional chemotherapy regimens based on platinum and fluorouracil can provide some relief

but have limited ORR and are prone to drug resistance and disease progression ^[1]. In recent years, with a deeper understanding of the tumor immune microenvironment, immune checkpoint inhibitors, particularly programmed death receptor-1 (PD-1) inhibitors, have been approved for second-line treatment of advanced gastric cancer and have demonstrated significant potential for survival benefits in multiple clinical trials ^[2]. For example, the KEYNOTE-859 Phase III clinical trial confirmed that pembrolizumab combined with chemotherapy significantly improved OS (HR = 0.78, $p < 0.0001$) in previously untreated patients with locally advanced or metastatic HER2-negative gastric cancer, with manageable safety, suggesting that this combination strategy has the potential to become a first-line standard treatment ^[3]. These advances highlight the urgent clinical need for immunotherapy in advanced gastric cancer, especially in overcoming the limitations of traditional chemotherapy, prolonging survival, and improving quality of life.

1.2. The central role of the PD-1/PD-L1 pathway in tumor immune escape

The PD-1/PD-L1 signaling pathway is one of the key mechanisms by which tumors achieve immune escape. Tumor cells evade immune surveillance by overexpressing PD-L1, which binds to PD-1 on the surface of T cells, inhibiting T cell activation, proliferation, and cytotoxic function. Abnormal activation of this pathway is particularly prominent in gastric cancer. Studies have found that the IKZF4/NONO-RAB11FIP3 axis can enhance membrane surface expression of PD-L1 by promoting its endosomal recycling, thereby strengthening immunosuppressive effects; inhibiting RAB11FIP3 or IKZF4 can significantly promote CD8⁺ T cell infiltration and enhance their cytotoxicity, effectively inhibiting tumor progression ^[4]. Notably, although PD-1 monoclonal antibodies have shown significant efficacy in some patients, not all patients with high PD-L1 expression benefit, indicating that the functional regulation of this pathway is far more complex than simple protein expression and involves multiple mechanisms such as endocytic recycling, gene polymorphisms, and microenvironment interactions. Therefore, in-depth analysis of the dynamic regulatory network of the PD-1/PD-L1 pathway in gastric cancer immune escape is crucial for optimizing immunotherapy strategies.

1.3. The association between Th1/Th2 immune balance and antitumor efficacy

The polarization state of helper T cell (Th) subsets, particularly the balance between Th1 and Th2, is a core factor determining the efficacy of antitumor immune responses. Th1-type immune responses, characterized by the secretion of pro-inflammatory cytokines such as IFN- γ and TNF- α , can activate CD8⁺ T cells and macrophages, exerting potent antitumor effects; whereas Th2-type responses, dominated by IL-4 and IL-10, tend to induce immune tolerance and promote tumor progression. For example, in patients with advanced gastroesophageal adenocarcinoma, pembrolizumab combined with chemotherapy can induce the formation of an “immune hub” in the tumor microenvironment containing CXCL13⁺ tumor-reactive T cells and interferon-stimulated gene programs, significantly increasing CD8⁺ T cell infiltration, suggesting the establishment of a Th1-dominant state ^[5]. Similarly, neoadjuvant chemotherapy alone can also reduce the proportion of FOXP3⁺ Tregs, increase CD8⁺ T cell levels, and be accompanied by increased TCR diversity, indicating that chemotherapy itself has the ability to reshape the Th1/Th2 balance ^[6].

2. Clinical efficacy evaluation of PD-1 inhibitors combined with chemotherapy

2.1. Clinical evidence of objective response rate and survival benefits

In recent years, multiple high-quality clinical studies have confirmed that PD-1 inhibitors combined with

chemotherapy can significantly improve ORR and prolong patient survival in advanced gastric cancer treatment. In the CheckMate 649 study, nivolumab combined with chemotherapy significantly improved OS and PFS in patients with a PD-L1 combined positive score (CPS) ≥ 5 , establishing this regimen as a first-line standard treatment ^[7]. In a randomized, double-blind, placebo-controlled Phase III clinical trial conducted in China (NCT03745170), cemiplimab combined with XELOX chemotherapy significantly improved median OS in patients with unresectable locally advanced or metastatic gastric/gastroesophageal junction cancer (overall population: 15.2 months vs 12.3 months, HR 0.77; PD-L1 CPS ≥ 5 subgroup: 18.4 months vs 12.9 months, HR 0.66) ^[8]. In patients with HER2-negative unresectable advanced or metastatic gastric cancer, a Phase 1b/2 study showed that cadonilimab combined with chemotherapy achieved an ORR of 52.1% (95% CI 41.6–62.5) and a median OS of 17.48 months (95% CI 12.35–26.55), with even higher values of 20.32 months in patients with PD-L1 CPS ≥ 5 ^[9]. For HER2-positive patients, the KEYNOTE-811 study confirmed that adding pembrolizumab to trastuzumab combined with chemotherapy significantly improved ORR and induced partial complete responses ^[10]. These data collectively indicate that PD-1 inhibitors combined with chemotherapy not only enhance tumor regression but also translate into statistically and clinically significant survival advantages.

2.2. Comparative efficacy studies with traditional chemotherapy regimens

In the Chinese population, the cemiplimab combined with XELOX regimen also demonstrated superior OS benefits compared to placebo combined with chemotherapy. Notably, even in the overall population without biomarker screening, PD-1 inhibitors combined with chemotherapy can still provide survival benefits. For example, global multicenter observational data and clinical practice guidelines indicate that for patients with advanced gastric cancer expressing PD-L1, combination therapy can prolong survival by approximately 3 months compared to chemotherapy alone ^[11]. In addition, anti-PD-1 combined with chemotherapy has been recommended as a first-line treatment option for unselected advanced gastric/gastroesophageal junction adenocarcinoma, reflecting its broad applicability and superiority over traditional chemotherapy ^[12]. However, not all combination regimens involving immune checkpoint inhibitors can surpass chemotherapy alone. For example, in the PRODIGE 59-FFCD 1707-DURIGAST study, FOLFIRI combined with durvalumab (a PD-L1 inhibitor) or further combined with tremelimumab (a CTLA-4 inhibitor) was used in patients who progressed after platinum-based chemotherapy. Although some PFS advantages were observed in the PD-L1 TPS $\geq 1\%$ subgroup, the overall study did not meet the primary endpoint, suggesting that simply adding immunotherapy in second-line or later treatment may not replicate the benefits of first-line combination therapy ^[13]. This highlights the critical impact of treatment line, patient selection, and drug combination on efficacy.

2.3. Efficacy differences among different combination regimens (e.g., DCF regimen)

Currently, research data on the efficacy differences between PD-1 inhibitors combined with different chemotherapy backbones (such as FLOT, SOX, XELOX, DCF, etc.) are insufficient, but preliminary explorations have been conducted. A Phase II study (NEOSUMMIT-01) using toripalimab combined with oxaliplatin-based perioperative chemotherapy significantly improved the TRG 0/1 rate (44.4% vs 20.4%, $P = 0.009$) ^[14].

In China, the SOX regimen (S-1 combined with oxaliplatin) as perioperative chemotherapy has been shown to be non-inferior to FOLFOX (3-year OS rate 75.2% vs 67.8%) ^[15], suggesting that oxaliplatin-

containing regimens are well tolerated and effective in the Asian population and may provide an ideal backbone for PD-1 inhibitor combinations. In addition, neoadjuvant/conversion therapy with camrelizumab combined with apatinib and S-1±oxaliplatin achieved a pCR rate of 15.8% in locally advanced gastric cancer with cT4a/bN+, and efficacy was associated with microsatellite instability (MSI), PD-L1 expression, and tumor mutation burden ^[16].

Although DCF (docetaxel + cisplatin + 5-FU) is an effective chemotherapy regimen for advanced gastric cancer, no large-scale studies have specifically evaluated the efficacy of PD-1 inhibitors combined with DCF in gastric cancer. Current evidence mostly focuses on FLOT, XELOX, or oxaliplatin/fluorouracil-containing regimens. Notably, in the KEYNOTE-585 study, pembrolizumab combined with platinum-based doublet chemotherapy (usually 5-FU + cisplatin or capecitabine + oxaliplatin) significantly improved the pCR rate (12.9% vs 2.0%, $P < 0.00001$) and prolonged event-free survival in neoadjuvant/adjuvant treatment ^[17]. This suggests that platinum-based doublet chemotherapy backbones have synergistic potential when combined with PD-1 inhibitors, but the choice of specific regimens needs to consider efficacy, toxicity, and regional medication habits.

In summary, PD-1 inhibitors combined with different chemotherapy regimens have shown superior clinical benefits compared to chemotherapy alone, with the most substantial evidence supporting regimens based on platinum-based doublets (such as XELOX and FLOT). Future head-to-head comparative studies are needed to identify the optimal chemotherapy backbone further to achieve a balance between maximizing efficacy and minimizing toxicity.

3. Regulatory mechanisms of Th1/Th2 immune balance

3.1. Differentiation and functional characteristics of Th1/Th2 cells

Helper T cells (Th) can be divided into two major subsets, Th1 and Th2, based on their cytokine profiles and functions. These subsets play distinct roles in anti-tumor immunity. Th1 cells primarily secrete interferon-gamma (IFN- γ), interleukin-2 (IL-2), and tumor necrosis factor-beta (TNF- β), promoting cellular immune responses and activating macrophages and cytotoxic CD8⁺ T cells, thereby enhancing the clearance of tumor cells. Conversely, Th2 cells predominantly produce cytokines such as IL-4, IL-5, IL-6, and IL-10, which tend to induce humoral immune responses and, in certain contexts, suppress Th1-mediated anti-tumor effects, even promoting tumor immune evasion ^[18]. In the tumor microenvironment, shifts in the Th1/Th2 balance directly influence the efficacy of immunotherapy: a Th1-dominant state is generally associated with favorable anti-tumor responses, whereas a Th2-dominant state often correlates with immune suppression and disease progression. For instance, in patients with advanced gastric cancer, elevated levels of Th2-type cytokines such as IL-4, IL-6, and IL-10 are associated with small intestinal injury and activation of inflammatory pathways, suggesting that Th2 polarization may exacerbate immune imbalance ^[21].

3.2. Impact of PD-1 signaling on Th1/Th2 polarization

The PD-1/PD-L1 signaling pathway not only directly inhibits T cell activation but may also influence the Th1/Th2 immune balance by regulating Th cell polarization. Although existing literature does not explicitly describe the direct regulatory mechanisms of PD-1 signaling on Th1/Th2 differentiation, multiple indirect lines of evidence support its crucial role in this process. For example, in untreated patients with metastatic melanoma, baseline levels of Th1 cytokines (e.g., IFN- γ) are significantly lower than those in healthy

controls, whereas responders to PD-1 inhibitor therapy exhibit a marked increase in IFN- γ levels by week 6, suggesting that PD-1 blockade promotes Th1 polarization ^[19]. In advanced gastric cancer, the IKZF4/NONO-RAB11FIP3 axis mediates immune evasion by enhancing PD-L1 membrane expression, while inhibiting this axis increases CD8⁺ T cell infiltration and promotes T cell proliferation, indirectly supporting the restoration of Th1-type immune responses. Notably, in the gastric cancer microenvironment, lactate accumulation can upregulate PD-1 expression on regulatory T cells (Tregs), causing PD-1 inhibitors to preferentially activate immunosuppressive Tregs rather than effector T cells, potentially weakening Th1 responses ^[20]. This suggests that the impact of PD-1 signaling on the Th1/Th2 balance is highly dependent on the metabolic state of the microenvironment.

3.3. Dynamic changes in key cytokines (IFN- γ , IL-4, etc.)

IFN- γ and IL-4, as core effector molecules of Th1 and Th2 responses, respectively, serve as important indicators for assessing the Th1/Th2 balance during immunotherapy. In the context of PD-1 inhibitor therapy, elevated IFN- γ levels typically correlate positively with treatment response. Various intervention strategies can enhance IFN- γ production: for example, FX-11@PEG-Ce6 promotes CD8⁺ T cell secretion of IFN- γ by alleviating tumor microenvironment acidity, synergistically enhancing anti-PD-1 efficacy ^[21]; high expression of MFSD2A reduces T cell exhaustion by inhibiting the COX2-prostaglandin-TGF β 1 axis, thereby improving IFN- γ secretion capacity ^[22]. Conversely, elevated levels of Th2-type cytokines such as IL-4 are often associated with poor prognosis. The REGOMUNE study found that in patients with advanced gastric cancer who did not respond to regorafenib combined with avelumab, peripheral blood IL-4 levels were significantly elevated, accompanied by increased infiltration of M2 macrophages, suggesting that a Th2-type inflammatory environment may drive immunotherapy resistance ^[23]. Therefore, monitoring the dynamic changes in cytokines such as IFN- γ and IL-4 not only helps assess the Th1/Th2 balance but also serves as a potential biomarker for predicting the efficacy of PD-1 inhibitors combined with chemotherapy.

4. PD-1/PD-L1 pathway and immune microenvironment remodeling

Tumor-associated macrophages (TAMs), as a major component of the tumor microenvironment (TME), not only participate in establishing an immunosuppressive state but also play a key role in regulating the efficacy of PD-1/PD-L1 inhibitors ^[24]. Multiple studies have shown that TAMs can highly express PD-L1 and inhibit T cell function by binding to PD-1 on the surface of T cells. In breast cancer patients, single-cell transcriptomic and spatial multiplex immunofluorescence analyses reveal that PD-L1⁺ TAMs exhibit mature and immunostimulatory characteristics and are spatially proximal to CD8⁺ T cells; higher density or proportion of these cells correlates with better clinical prognosis. Notably, PD-L1 expression can spontaneously upregulate during monocyte-to-macrophage differentiation without exogenous IFN- γ stimulation and can promote CD8⁺ T cell proliferation and cytotoxic function ^[25]. Additionally, in esophageal squamous cell carcinoma (ESCC), PD-L1⁺ TAMs also correlate positively with clinical benefit, and in vitro PD-L1 blockade induces TAM reprogramming toward a pro-inflammatory phenotype ^[26]. However, TAMs exhibit highly heterogeneous functions. For example, in cholangiocarcinoma, PD-L1 is primarily expressed by M2-type TAMs, mediating CD8⁺ T cell exhaustion and forming an immunosuppressive microenvironment; in contrast, the density of non-exhausted PD-1⁺EOMES⁺CD8⁺ effector T cells correlates significantly with longer survival ^[27]. Further

research has found that targeting TAMs can enhance the efficacy of PD-1/PD-L1 blockade. For instance, in the Mycn-nGEMM neuroblastoma mouse model, although tumor cell PD-L1 expression is low, macrophages highly express PD-L1, and anti-PD-L1 therapy effectively depletes anti-inflammatory macrophages and increases T cell infiltration; combining this with a CD40 agonist antibody further improves survival rates ^[28]. Similarly, PI3K γ inhibitors combined with anti-PD-1/PD-L1 therapy can reprogram TAMs by regulating the PI3K γ -AKT-NF- κ B pathway, reducing myeloid-derived suppressor cells, increasing CD8⁺ T cell numbers and pro-inflammatory cytokine levels, thereby reversing the immunosuppressive microenvironment ^[29]. However, strategies targeting TAMs also face challenges. A phase I clinical study showed that although the CSF-1R inhibitor pexidartinib reduces CD14⁺CD16⁺ monocytes in peripheral blood, it also impairs dendritic cell differentiation and function, potentially limiting its anti-tumor activity ^[30]. These findings suggest that TAMs play a dual role in PD-1/PD-L1 pathway-mediated immune microenvironment remodeling, and their regulation requires balancing pro-inflammatory and immunostimulatory functions.

5. Current controversies and challenges in research

5.1. Limitations of biomarker predictive efficacy

Although PD-1 inhibitors combined with chemotherapy have demonstrated clinical benefits in treating advanced gastric cancer, existing biomarkers exhibit significant limitations in predicting efficacy. Commonly used predictive indicators such as PD-L1 combined positive score (CPS), microsatellite instability (MSI) status, and tumor mutation burden (TMB) show poor predictive consistency across different patient populations ^[31]. However, not all patients with high TMEscore achieve ideal outcomes, indicating that single-dimensional biomarkers cannot fully capture the complexity of the tumor immune microenvironment. Additionally, although high expression of HSPA4 correlates with a favorable response to chemotherapy combined with anti-PD-1 therapy, it predicts poor prognosis in patients undergoing surgery alone, reflecting contradictory predictive values of this marker across different treatment contexts ^[32]. Similarly, although lymphoid radiomic scores (LRS) and myeloid radiomic scores (MRS) constructed based on CT imaging can assess immune microenvironment status, objective response rates vary significantly among patients with different imaging subtypes (type 1: 27.3%, type 2: 53.3%, type 3: 10.2%, type 4: 30.0%), further highlighting the heterogeneity and uncertainty of current predictive models ^[33]. Although multidimensional tumor-infiltrating immune cell (TIIC) signatures constructed via multiplex immunohistochemistry aid in predicting responses to anti-PD-1/PD-L1 therapy, their clinical adoption remains limited by issues of technical standardization and reproducibility ^[34]. Therefore, there is an urgent need to integrate multi-omics data to construct more robust and universally applicable predictive systems.

5.2. Regulatory challenges in Th2-dominant microenvironment formation

A Th2-type immune-dominant microenvironment is considered one of the key factors limiting the efficacy of PD-1 inhibitors, but its formation mechanisms and intervention strategies remain challenging. Although high expression of MFSD2A can reduce TGF β 1 release by inhibiting COX2-prostaglandin synthesis, thereby enhancing CD8⁺ T cell activation and improving Th1/Th2 imbalance, the clinical targetability of this pathway remains to be validated. On the other hand, PRDM15 may indirectly promote Th2 polarization by activating the PVR/TIGIT axis to inhibit T cell function, but its specific regulatory role in Th1/Th2 balance remains unclear ^[35]. Therefore, effectively reversing or preventing the formation of a Th2-dominant

microenvironment remains a core challenge in optimizing current combination therapy strategies.

6. Future research directions

6.1. Exploration of novel combination therapy strategies

Although current PD-1 inhibitors combined with chemotherapy have shown significant clinical benefits in treating advanced gastric cancer, a substantial proportion of patients still do not benefit or develop resistance. Therefore, exploring novel combination therapy strategies with stronger synergistic effects is an important future research direction. In recent years, multi-mechanism interventions targeting immunosuppressive pathways have gradually gained attention. For example, inhibiting RAB11FIP3 or IKZF4 can block PD-L1 endosomal recycling, enhancing T cell infiltration and cytotoxicity, suggesting that this axis may serve as a novel target for gastric cancer immunotherapy. Additionally, PRDM15 inhibits T cell function by activating the PVR/TIGIT axis, providing a theoretical basis for combining TIGIT inhibitors ^[36]. The development of nanomedicine platforms also offers new ideas for combination therapy. For instance, the FX-11@PEG-Ce6 nanosystem promotes dendritic cell maturation and enhances CD8⁺ T cell function by alleviating acidic microenvironments, thereby improving PD-1 inhibitor efficacy; another class of mesoporous polydopamine-based nanoparticles loaded with oxaliplatin and manganese dioxide, combined with mild photothermal therapy, can induce immunogenic cell death, activate the cGAS-STING pathway, and significantly enhance anti-tumor immune responses. Furthermore, fecal microbiota transplantation (FMT) combined with PD-1 inhibitors has shown preliminary efficacy in previously resistant patients, with an objective response rate of 20%, suggesting that modulating the gut microbiota may represent a novel strategy for overcoming immunotherapy resistance ^[37]. These cutting-edge explorations lay the foundation for constructing multidimensional, multi-layered combination therapy systems.

6.2. Construction of multi-omics biomarker systems

Although PD-L1 expression (e.g., CPS score) has been used to guide PD-1 inhibitor therapy, its predictive efficacy remains limited, necessitating the integration of multi-omics data to construct more precise biomarker systems. Studies have shown that high expression of MFSD2A correlates with a favorable response to anti-PD-1 therapy, with mechanisms involving inhibition of COX2-prostaglandin synthesis, reduced TGFβ1 release, and enhanced CD8⁺ T cell activation, suggesting that MFSD2A may serve as a potential predictive marker ^[38]. Although high expression of HSPA4 correlates with poor prognosis in patients undergoing surgery alone, it correlates with a better treatment response in those receiving PD-1 inhibitors combined with chemotherapy, further highlighting the dynamic nature of biomarkers in the context of combination therapy ^[39]. Additionally, PD-L1 gene polymorphisms (e.g., the P146R mutation caused by rs17718883) can weaken PD-1/PD-L1 interactions, enhance IFN-γ secretion, and inhibit tumor growth, but patients with this mutant type do not derive additional benefits from PD-1 monoclonal antibodies, suggesting that this polymorphism may be used to exclude ineffective treatments ^[40]. Immune microenvironment classification systems (e.g., TIME-inflamed, TIME-desert, TIME-excluded) also exhibit predictive value, with patients in the TIME-inflamed subtype more likely to benefit from pembrolizumab combined with chemotherapy ^[41]. Future research should integrate multi-dimensional data from genomics, transcriptomics, proteomics, and immune cell atlases, combined with artificial intelligence algorithms, to establish dynamic, quantifiable comprehensive predictive models for precise stratification and individualized treatment decision-making.

6.3. Optimization of individualized immune regulation strategies

The core of individualized immune regulation lies in customizing treatment plans based on patients' tumor immune microenvironment characteristics, host immune status, and molecular subtypes. Studies have shown that EBV-positive gastric cancer exhibits higher CD8⁺ T cell infiltration and interferon-induced gene expression, whereas EBV-negative gastric cancer highly expresses genes related to the COX-2/PGE2 pathway, suggesting that different molecular subtypes may respond differently to immunotherapy. The status of *Helicobacter pylori* infection may also influence the efficacy of PD-1 inhibitors, and although existing research conclusions remain controversial, its interactions with the gut microbiota and immune infiltration warrant further exploration. Additionally, single-cell sequencing reveals that after neoadjuvant PD-1 inhibitor therapy combined with chemotherapy, CD3⁺, CD8⁺ T cells, and NK cells significantly increase in the tumor microenvironment of patients with major pathological response (MPR), whereas regulatory T cells (FOXP3⁺) are predominantly enriched in non-MPR patients, indicating that dynamic changes in Treg cells can serve as efficacy monitoring indicators. Chemotherapy itself can also remodel the immune microenvironment, such as reducing FOXP3⁺ Treg proportions and increasing CD8⁺ T cell levels after neoadjuvant chemotherapy, accompanied by increased TCR diversity, providing a basis for optimizing the timing of combination therapy. Future strategies should be based on patients' baseline immune characteristics (e.g., Th1/Th2 cytokine profiles, T cell exhaustion status, myeloid cell composition) combined with dynamic immune monitoring during treatment (e.g., changes in peripheral blood cytokines) to optimize dosing schedules, doses, and drug combinations, achieving a transition from a "one-size-fits-all" to a "tailored" treatment model.

7. Summary and clinical translation significance

In recent years, significant progress has been made in understanding the core mechanisms of PD-1 inhibitors combined with chemotherapy for treating advanced gastric cancer. Multiple studies have demonstrated that this combination strategy not only enhances antigen release by directly killing tumor cells but also significantly remodels the tumor immune microenvironment, promoting Th1-type immune responses and suppressing Th2-dominant states. For example, neoadjuvant PD-1 inhibitor therapy combined with chemotherapy significantly increases CD3⁺, CD8⁺ T cell, and NK cell infiltration in the tumor microenvironment while reducing FOXP3⁺ regulatory T cell proportions, thereby improving the Th1/Th2 balance. Additionally, chemotherapy itself has been shown to increase TCR diversity and enhance CD8⁺ T cell activity, laying an immunological foundation for PD-1 inhibitor efficacy. At the molecular level, high expression of MFSD2A can enhance CD8⁺ T cell function and reduce exhaustion by inhibiting the COX2-prostaglandin pathway to decrease TGFβ1 release, improving anti-PD-1 treatment response; the IKZF4/NONO-RAB11FIP3 axis regulates T cell cytotoxicity by influencing PD-L1 membrane expression, highlighting its key role in Th1/Th2 polarization. These findings collectively reveal the synergistic mechanisms by which PD-1 inhibitors combined with chemotherapy modulate the immune microenvironment, reverse T cell exhaustion, and optimize the Th1/Th2 balance through multiple dimensions.

Funding

Study on the Efficacy of Immunotherapy (PD-1 Inhibitors) Combined with Chemotherapy in Advanced

Disclosure statement

The authors declare no conflict of interest.

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Inferring Tumor Spatial Gene Expression from Routine H&E Images: Pan-Cancer Validation and Spatial Profiling of Primary Breast Tumors and Lymph Node Metastases

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Abstract: Spatial transcriptomics (ST) enables gene expression profiling while preserving tissue architecture, but its high cost and limited throughput constrain large-scale applications. This study evaluated the biological utility of VirtualST, a conditional diffusion model for inferring spatial gene expression from H&E images, in colon adenocarcinoma (COAD), primary invasive ductal carcinoma of the breast (IDC), and breast cancer lymph node metastasis (LYMPH_IDC). Twelve samples with paired H&E images and ground-truth ST data were evaluated using leave-one-sample-out cross-validation. Inference started from pure noise, with ground-truth expression used only for post hoc evaluation, and the final prediction for each sample was obtained by averaging ten sampling runs. The mean gene-wise Pearson correlation coefficients (PCCs) were 0.467, 0.439, and 0.496 for COAD, IDC, and LYMPH_IDC, respectively, with better reconstruction of tumor epithelial, secretory, and proliferative programs. Using ground-truth ST epithelial scores from the held-out samples as an independent molecular reference, the predicted and ground-truth scores achieved within-sample Pearson correlations of 0.79 and 0.66 in IDC and LYMPH_IDC, respectively, with corresponding hotspot Dice coefficients of 0.64 and 0.44. These results indicate that VirtualST can recover within-sample spatial epithelial signals but is not suitable for absolute quantification across tissues or direct pathological region detection.

Keywords: Spatial gene expression prediction; H&E histology; Conditional diffusion; Breast cancer; Lymph node metastasis

Online publication: August 11, 2026

1. Introduction

Tumor tissues consist of malignant cells, immune cells, fibroblasts, and other components of the tumor

microenvironment. The non-random spatial distribution of these cell populations creates substantial spatial heterogeneity and is closely associated with tumor growth, local invasion, and treatment response. Spatial transcriptomics (ST) captures gene expression information while preserving tissue coordinates, providing an important tool for characterizing tumor cell compartments and their microenvironments^[1]. However, the high experimental cost, complex sample-processing procedures, and limited throughput of ST hinder its direct application to large clinical cohorts. In contrast, routine hematoxylin and eosin (H&E)-stained sections are inexpensive, readily accessible, and extensively accumulated in clinical pathology workflows. Inferring spatial gene expression from H&E images may therefore offer a cost-effective approach to scaling up spatial molecular analyses.

In recent years, several computational methods have been developed to infer spatial gene expression from H&E images. ST-Net uses convolutional neural networks to extract histological image features and predict expression levels at different spatial locations^[2]. HisToGene further introduces an attention mechanism to enhance the modeling of relationships among tissue regions^[3], whereas Hist2ST combines a Transformer with a graph neural network to jointly incorporate morphological features and spatial neighborhood information^[4]. These studies demonstrate that tissue morphology contains molecular cues that can be used to infer spatial expression. However, existing evaluations generally focus on overall correlation metrics, with limited investigation into the predictability of specific gene programs or whether model-inferred expression retains biological consistency across different tissue contexts, such as primary tumors and metastatic lesions.

In this study, the study employed VirtualST, a conditional diffusion model that uses morphological features extracted by a pathology foundation model, cell composition derived from nuclei segmentation, and spatial neighborhood information as conditioning inputs to generate predictions in gene-expression space progressively. Unlike deterministic regression models, diffusion-based inference is inherently stochastic. If noisy ground-truth expression is used as the initial input during testing, test information may leak into the inference process and lead to overestimated performance. Therefore, all predictions in this study were generated through a leakage-free sampling procedure starting from pure Gaussian noise. Ground-truth ST expression was used only for evaluation after inference, thereby maintaining independence between model predictions and the molecular reference.

Accordingly, the study evaluated the biological utility of VirtualST using leave-one-sample-out cross-validation across 12 samples with paired H&E images and ground-truth ST data from three cohorts: COAD, IDC, and LYMPH_IDC. The study addressed three main questions. First, the study assessed the overall fidelity of spatial expression reconstruction across different tissue contexts. Second, the study compared the predictability of tumor epithelial, secretory, proliferative, hormone-receptor-related, and immune-related gene programs. Third, the study used ground-truth ST epithelial scores from held-out samples, which were not involved in inference, as an independent molecular reference to evaluate the spatial consistency of the predicted epithelial programs. Importantly, the IDC and LYMPH_IDC samples were not patient-matched; therefore, this study compared spatial recovery across two tissue contexts without concluding metastatic evolution or molecular mechanisms.

2. Materials and methods

2.1. Data sources and preprocessing

This study used three tumor cohorts from the HEST-bench dataset^[5]: colon adenocarcinoma (COAD), primary invasive ductal carcinoma of the breast (IDC), and breast cancer lymph node metastasis (LYMPH_IDC). Each cohort contained four samples with paired H&E images and ground-truth ST data, yielding a total of 12 samples. H&E image regions corresponding to individual spatial locations were used as morphological inputs, whereas ground-truth ST expression served as the training target and as a post hoc evaluation reference for held-out samples. Gene-expression values were first transformed using $\log(1+x)$ and then independently min-max normalized within each sample. Because normalization parameters were determined separately for each sample, this study evaluated only relative within-sample spatial patterns and did not perform absolute expression comparisons across samples or tissue types.

2.2. VirtualST model

VirtualST uses H&E images as its primary information source and performs conditional diffusion directly in gene-expression space. First, a frozen Phikon-v2 pathology foundation model extracts morphological features from the image region corresponding to each spatial location^[6]. HoVer-Net is then used to segment and classify nuclei and to calculate the proportions of different cell types at each location^[7]. The morphological features and cell-composition profiles jointly condition the diffusion process, guiding the model to progressively generate spatial gene-expression predictions from random noise^[8]. The model output is further refined through a residual spatial graph module, in which each location is connected to its six nearest spatial neighbors to integrate local tissue-structure information. The trained graph-refinement module was applied to IDC and LYMPH_IDC, whereas the corresponding module in COAD remained an identity mapping because it was not separately trained.

2.3. Leakage-free cross-validation and model inference

Leave-one-sample-out cross-validation was conducted separately within each of the three cohorts. Because each cohort contained four samples, four independent folds were generated: three samples were used for training and the remaining sample was held out for testing in each fold, until every sample had served once as the test sample. The last checkpoint obtained at the end of training was consistently used for inference in all folds. During testing, the model followed a leakage-free sampling procedure, starting from pure Gaussian noise with the same dimensionality as the target expression and generating predicted expression through reverse diffusion. Ground-truth expression from the held-out sample was not used for noise initialization, reverse diffusion, or spatial graph refinement and was used only for performance evaluation after inference. To reduce stochastic variation in diffusion sampling, ten predictions were independently generated for each test sample using ten fixed random seeds, and their average was used as the final model-inferred output.

2.4. Evaluation metrics and statistical analysis

Overall reconstruction performance was evaluated using the mean gene-wise Pearson correlation coefficient (PCC) and Spearman rank correlation coefficient. For each gene (g), the correlation between predicted expression $\hat{x}_{ig}$ and ground-truth expression x_{ig} was calculated across all spatial locations within a sample. The correlations were then averaged across all genes in the evaluation set to obtain the mean gene-wise

correlation for that sample. In addition to the complete evaluation gene set, performance was separately assessed for highly expressed genes (HEGs), highly variable genes (HVGs), and cancer-specific marker genes. To prevent gene-selection leakage, HVGs in each cross-validation fold were identified using only the expression variances of the three training samples and were subsequently evaluated in the held-out sample. All metrics are reported as the mean $\pm$ standard deviation across four held-out samples, with the sample treated as the statistical unit ($n = 4$ per cohort).

The breast cancer epithelial program score was constructed using EPCAM, KRT8, and KRT7, which were shared across both cohorts. For each spatial location i , the epithelial score was defined as the mean normalized expression of the three genes:

$$S_i = \frac{X_{i,EPCAM} + X_{i,KRT8} + X_{i,KRT7}}{3} \quad (1)$$

The predicted epithelial score $\hat{S}_i$ and ground-truth ST epithelial score S_i were calculated separately, and their Pearson and Spearman correlations were evaluated within each sample. Spatial locations falling within the top 20% of predicted and ground-truth scores were further defined as the high-value hotspot sets H_{pred} and H_{ST} , respectively. Hotspot overlap was quantified using the Dice coefficient:

$$\text{Dice} = \frac{2 | H_{pred} \cap H_{ST} |}{| H_{pred} | + | H_{ST} |} \quad (2)$$

Because the ground-truth ST epithelial scores were not involved in model inference for the held-out samples, they served as an independent molecular reference. However, these scores should not be considered equivalent to tumor regions annotated by pathologists.

3. Results

3.1. Overall reconstruction fidelity across tissue contexts

Across the 12 held-out samples from the three cohorts, the final VirtualST outputs showed interpretable within-sample spatial concordance with ground-truth ST data (**Table 1**). The mean gene-wise PCCs were 0.467 ± 0.054 , 0.439 ± 0.076 , and 0.496 ± 0.127 for COAD, IDC, and LYMPH_IDC, respectively, corresponding Spearman correlations of 0.475 ± 0.057 , 0.456 ± 0.080 , and 0.471 ± 0.142 . Although the mean overall with PCC in LYMPH_IDC was not lower than that in IDC, its larger standard deviation indicated greater variability in model performance across lymph node metastasis samples. Because each cohort contained only four samples, these findings were interpreted descriptively and were not used to infer statistically significant differences between cohorts.

Across the evaluated gene subsets, the HEG, HVG, and marker-gene correlations in COAD were 0.587 ± 0.069 , 0.669 ± 0.061 , and 0.557 ± 0.067 , respectively, with the training-fold-defined HVGs showing the highest correlation. The corresponding correlations in IDC were 0.596 ± 0.075 , 0.564 ± 0.078 , and 0.537 ± 0.093 , indicating moderate concordance across all three subsets. In LYMPH_IDC, the HEG, HVG, and marker-gene correlations were 0.468 ± 0.130 , 0.412 ± 0.102 , and 0.473 ± 0.138 , respectively. These values were generally lower than the corresponding results in COAD and IDC and showed greater between-sample variability. Collectively, these findings indicate that VirtualST recovered part of the spatial expression structure across all three tissue contexts, although performance varied according to

both the gene subset and tissue background. **Table 1** reports the final model outputs after sparse gating and spatial graph refinement; the graph-refinement module in COAD was an identity mapping.

Table 1. Spatial expression reconstruction fidelity of VirtualST across the three cohorts

Cohort	PCC	Spearman	HEG	HVG	Marker genes
COAD	0.467 ± 0.054	0.475 ± 0.057	0.587 ± 0.069	0.669 ± 0.061	0.557 ± 0.067
IDC	0.439 ± 0.076	0.456 ± 0.080	0.596 ± 0.075	0.564 ± 0.078	0.537 ± 0.093
LYMPH_IDC	0.496 ± 0.127	0.471 ± 0.142	0.468 ± 0.130	0.412 ± 0.102	0.473 ± 0.138

3.2. Predictability of specific gene programs

Gene-wise analysis revealed substantial differences in the spatial predictability of distinct biological programs. In COAD, genes associated with tumor epithelium, secretion, and proliferation showed relatively strong performance. The mean PCCs for CEACAM5, GPX2, CEACAM6, and MKI67 were 0.84, 0.83, 0.76, and 0.73, respectively, whereas the intestinal stem-cell and tumor-associated markers LGR5 and OLFM4 achieved PCCs of 0.63 and 0.52. In comparison, the PCCs for ACTA2, MS4A1, and VWF were 0.26, 0.30, and 0.40, respectively. A similar pattern was observed in IDC, where the epithelial markers EPCAM and KRT8 achieved PCCs of 0.79 and 0.77, while ERBB2 and the proliferation marker TOP2A achieved PCCs of 0.67 and 0.65. These results indicate that VirtualST more reliably reconstructed tumor epithelial and proliferative programs with relatively clear morphological correlates.

Compared with epithelial programs, hormone-receptor- and immune-related genes were less predictable in IDC. The mean PCCs for ESR1, PGR, and CD8A were 0.42, 0.38, and 0.33, respectively. This weaker performance may be related to their more diffuse spatial distributions, narrower dynamic ranges, or expression variation that is less directly reflected in routine tissue morphology, although these interpretations require validation in larger cohorts. In LYMPH_IDC, KRT18, KRT8, and EPCAM each achieved a PCC of approximately 0.60, indicating that VirtualST recovered part of the breast cancer epithelial program within the lymph node tissue background. The immunoglobulin-related genes IGKC, IGHG1, and IGLC2 achieved PCCs of 0.42, 0.41, and 0.35, respectively, whereas ERBB2 achieved a PCC of 0.33, lower than that observed in IDC. Overall, VirtualST showed more consistent reconstruction of tumor epithelial, secretory, and proliferative programs, whereas its inference of hormone-receptor- and some immune-related programs was more limited.

3.3. Spatial recovery in primary breast cancer and lymph node metastasis

In representative samples, VirtualST predictions exhibited epithelial-marker spatial patterns similar to those observed in ground-truth ST data (**Figure 1**). In the IDC sample TENX95, the prediction-to-ground-truth ST correlations for EPCAM and ERBB2 were 0.86 and 0.85, respectively. In the LYMPH_IDC sample NCBI684, the corresponding correlations were 0.75 and 0.65.

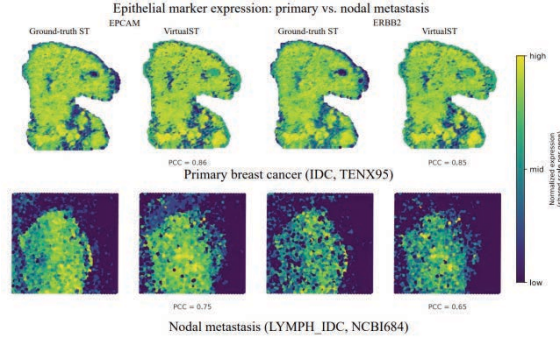


Figure 1. Spatial expression of EPCAM and ERBB2 in primary breast cancer and lymph node metastasis.

The study further used ground-truth ST data from the held-out samples, which were not involved in the inference process, as an independent molecular reference. The Pearson correlations between predicted and ground-truth epithelial scores were 0.79 ± 0.10 in IDC and 0.66 ± 0.11 in LYMPH_IDC. The corresponding Dice coefficients for the top 20% high-value hotspots were 0.64 ± 0.15 and 0.44 ± 0.08 , respectively (Table 2). These results indicate that VirtualST recovered the relative spatial patterns of epithelial programs in both tissue contexts.

Table 2. Spatial concordance between predicted and ground-truth ST epithelial-marker scores

Metric	Primary IDC	LYMPH_IDC lymph node metastasis
Pearson correlation	0.79 ± 0.10	0.66 ± 0.11
Spearman correlation	0.74 ± 0.14	0.61 ± 0.13
Top-20% hotspot Dice	0.64 ± 0.15	0.44 ± 0.08

The correlations between predicted EPCAM expression and the proportion of neoplastic cells in representative IDC and LYMPH_IDC samples were 0.59 and 0.52, respectively.

4. Discussion and conclusion

This study evaluated the biological utility of VirtualST using leakage-free sampling across 12 held-out samples from the COAD, IDC, and LYMPH_IDC cohorts. The model preserved moderate and interpretable within-sample spatial expression signals, with relatively strong prediction performance for genes associated with tumor epithelial, secretory, and proliferative programs. Using ground-truth ST data that were not involved in inference as an independent molecular reference, the predicted and ground-truth epithelial scores achieved Pearson correlations of 0.79 in IDC and 0.66 in LYMPH_IDC. These findings indicate that VirtualST recovered epithelial spatial patterns to a certain extent in both breast cancer tissue contexts. Therefore, VirtualST may serve as an exploratory spatial molecular prior when ST data are scarce, but it should not be considered a replacement for ground-truth ST measurements.

This study has several limitations, including the small sample size, the absence of patient-matched IDC and LYMPH_IDC samples, independent normalization of each sample, and saturation in model predictions. Because HoVer-Net-derived cell composition was used as a model input, the corresponding correlation

analysis reflects only directional consistency between the model output and its conditioning information and does not constitute independent validation. Moreover, ground-truth ST epithelial scores are not equivalent to tumor regions annotated by pathologists. Therefore, the current results are not suitable for absolute expression comparisons across tissues or direct pathological tumor-region detection. Future studies should evaluate VirtualST in larger independent cohorts, incorporate pathological region annotations and AUROC, AUPRC, and Dice metrics, and perform retraining-based ablation without cell-composition conditioning.

Disclosure statement

The authors declare no conflict of interest.

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Research Progress and Evidence-Based Evaluation of Chromoendoscopy in Colonoscopy

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Abstract: Colorectal cancer is a high-incidence malignant tumor of the digestive tract worldwide. Colonoscopic screening and complete resection of early lesions serve as the core strategies to reduce its morbidity and mortality. Conventional white-light endoscopy (WLE) exhibits a high missed diagnosis rate for minute adenomas, flat lesions, laterally spreading tumors (LSTs), and inflammatory bowel disease (IBD)-associated dysplasia, which fails to meet the demands of precise early diagnosis. Chromoendoscopy (CE) enhances the fine mucosal microstructure, crypt morphology, and lesion boundaries via topical mucosal spraying of staining agents, thereby significantly improving lesion detection efficacy. Based on evidence-based medical data, this study systematically summarizes the mechanism of action, characteristics of commonly used staining agents, standardized operating procedures, and quality control key points of CE. By integrating randomized controlled trials (RCTs), meta-analyses, and domestic and international authoritative guidelines, this paper hierarchically evaluates its clinical value in adenoma detection, early colorectal cancer diagnosis, LST assessment, IBD-related dysplasia screening, and postoperative follow-up. It further conducts an evidence-based comparison of the diagnostic efficacy among CE, WLE, virtual chromoendoscopy, magnifying endoscopy, and artificial intelligence (AI)-assisted endoscopy. Additionally, the current limitations of existing studies are objectively summarized, and future research directions of this technology are prospected, aiming to provide evidence-based references for the standardized implementation of enhanced colonoscopy and the construction of a stratified early screening strategy for colorectal cancer.

Keywords: Chromoendoscopy; Colonoscopy; Colorectal neoplasms; Adenoma detection rate; Evidence-based evaluation; Research progress

Online publication: August 11, 2026

1. Introduction

According to the 2024 global cancer statistics, new cases and deaths of colorectal cancer consistently rank among the top three of all malignant tumors worldwide, with a rising incidence in young and middle-aged populations year by year, leading to an increasing burden of disease prevention and control ^[1]. Colonoscopy,

with multiple functions including screening, targeted biopsy, and minimally invasive endoscopic treatment, is regarded as the gold standard for colorectal cancer screening and surveillance ^[2]. As the primary precancerous lesion of colorectal cancer, adenoma can reduce the canceration risk by more than 70% through early detection and complete resection. However, missed diagnosis remains a prominent problem in clinical practice with WLE: the missed diagnosis rate of minute adenomas (≤ 5 mm in diameter) and flat adenomas in the right colon reaches 20%–30%, and missed lesions constitute the leading cause of interval cancer ^[3,4]. Therefore, optimizing endoscopic enhancement techniques and improving the visualization of subtle mucosal lesions has become a key research direction in the field of digestive endoscopy.

Chromoendoscopy (CE) has been developed for more than 30 years. It highlights mucosal crypts, orifice structures, and lesion contours through physical contrast or biochemical staining, enabling fine mucosal observation without magnifying equipment, and stands as a traditional enhanced endoscopic technique with sufficient evidence-based support ^[5]. In recent years, virtual enhancement techniques such as narrow-band imaging (NBI), flexible spectral imaging color enhancement (FICE), and AI-assisted endoscopy have been rapidly popularized. Some clinicians hold the view that CE is cumbersome to operate and tends to be replaced. Nevertheless, multiple meta-analyses and real-world studies have confirmed that CE retains irreplaceable diagnostic advantages in high-risk population screening, IBD surveillance, and detection of depressed early tumors, with the additional strengths of low consumable costs and high accessibility in primary hospitals ^[6].

From an evidence-based evaluation perspective, this study integrates high-quality clinical studies, systematic reviews, and domestic and international digestive endoscopy guidelines published in the past decade, comprehensively summarizes the basic technical essentials and clinical application evidence of CE, and compares different endoscopic techniques horizontally. Existing shortcomings are analyzed and future research directions are proposed, aiming to provide theoretical support for the standardized application of chromocolonoscopy and the improvement of the stratified screening system for colorectal cancer.

2. Basic principles, staining agents, and operational quality control of chromoendoscopy

2.1. Diagnostic mechanism

CE improves mucosal imaging through three pathways: physical deposition, epithelial uptake, and chemical reaction, with four core diagnostic advantages. First, contrast enhancement effect. Non-absorbable staining agents only deposit in mucosal grooves and crypt depressions without staining normal flat mucosa, and the light-dark contrast enables rapid identification of flat elevated and minute depressed lesions. Second, visualized pit pattern classification. Staining clearly displays crypt morphology, distinguishing normal, hyperplastic, adenomatous, and cancerous crypts to realize endoscopic “optical biopsy”. Third, accurate lesion boundary delineation. For LSTs and large-area flat adenomas, staining clearly defines lesion margins to guide preoperative planning of endoscopic submucosal dissection (ESD). Fourth, reduced inter-observer variability. Standardized staining objectifies mucosal structure, minimizing missed diagnosis and misjudgment caused by insufficient clinical experience among junior physicians ^[7].

2.2. Evidence-based characteristics of clinically commonly used staining agents

2.2.1. Indigo carmine

As the clinically preferred contrast staining agent, indigo carmine is non-absorbable by mucosal epithelium, only depositing in mucosal depressions. It features stable staining, no cytotoxicity, and extremely rare systemic adverse reactions. With a standard concentration of 0.2%–0.4%, it is suitable for total colon screening, preliminary screening of LSTs, and lesion boundary determination, serving as the most promotable staining protocol in primary hospitals^[8]. A prospective controlled study conducted by Paiva et al.^[9] enrolled 203 participants, who received re-examination via pure WLE and indigo carmine CE, respectively. The results demonstrated that the indigo carmine group had a significantly higher number of newly detected polyps and total polyp detection volume, showing a prominent screening enhancement effect.

2.2.2. Methylene blue

Methylene blue is an absorbable staining agent that can be taken up by hyperproliferative intestinal epithelium. Adenomas and early cancers exhibit significantly deeper staining than normal mucosa. It is mostly combined with magnifying endoscopy for precise pit pattern classification, with higher sensitivity for flat minute adenomas than indigo carmine. However, a small amount of the agent can be absorbed into the blood, so cautious use is required in patients with severe renal insufficiency^[10]. Currently, the mainstream clinical methylene blue staining methods include topical lesion spraying, submucosal injection, and oral integral staining. Traditional topical spraying is complicated in operation, high in consumable consumption, and time-consuming for endoscopy, resulting in limited clinical application and gradual replacement by submucosal injection staining. In contrast, oral methylene blue staining has been widely applied in the screening and diagnosis of colorectal lesions with the updating of endoscopic techniques, due to its simple operation and stable and objective staining effect.

A large-sample double-blind RCT conducted by Repici et al.^[11] further verified the clinical value of this technique. A total of 1205 subjects were enrolled and randomly divided into the oral methylene blue experimental group and the placebo control group at a 1:1 ratio. The results showed that the experimental group yielded significantly higher detection rates of non-polypoid lesions, overall adenomas, and minute adenomas (≤ 5 mm) than the control group (35.07% vs 43.92%, OR=1.66, 95%CI: 1.21–2.26; 47.81% vs 56.29%, OR=1.46, 95%CI: 1.09–1.96; 30.90% vs 37.11%, OR=1.36, 95%CI: 1.01–1.83). These findings confirm that oral methylene blue staining can effectively improve the detection efficacy of various colorectal lesions, especially for minute adenomas that are easily missed in clinical practice.

2.2.3. Acetic acid

Acetic acid staining is a commonly used auxiliary technique to improve polyp detection rate in colonoscopy, with the advantages of low cost, convenient access, and simple operation. Recent clinical studies have shown that the combined application of acetic acid staining and NBI endoscopy can further optimize the diagnostic accuracy of colorectal polyps with prominent clinical value. As a reactive temporary staining agent, 1%–3% acetic acid induces transient dehydration and whitening of epithelial cells with temporary highlighting of crypt structures, which fades naturally within 30–60 seconds without flushing, enabling time-efficient outpatient rapid screening. Nevertheless, its short staining duration limits its application in large-scale and long-term lesion evaluation^[12]. A prospective single-arm study by Wiessner et al.^[13] enrolled 55 patients, who first underwent WLE examination followed by acetic acid combined with NBI re-examination, with a

total of 63 polyps detected. The study showed no significant difference in lesion diagnostic accuracy between the two methods (85.5% vs 80.6%, $P=0.450$). However, the combined mode significantly improved the clarity of lesion boundary display, with a clear boundary visualization rate of 98.4%, which was remarkably higher than 75.8% of pure WLE ($P < 0.001$).

2.2.4. Special staining agents

Crystal violet and eosin are only used for precise classification under high-magnification endoscopy. They provide durable staining but cause slight mucosal irritation, thus rarely applied in routine screening.

3. Evidence-based evaluation of clinical applications of chromoendoscopy

3.1. Improvement of adenoma detection rate (ADR)

ADR is the core indicator for evaluating colonoscopy screening quality. Multiple meta-analyses involving tens of thousands of subjects published from 2020 to 2022 consistently confirmed that total colon indigo carmine CE increased the overall ADR by 18%–34% compared with pure WLE, with the most prominent enhancement for minute adenomas (≤ 5 mm) and a nearly 40% increase in the detection of flat adenomas in the right colon^[14,15].

Stratified population analysis showed that average-risk screening populations obtain moderate diagnostic benefits, while high-risk populations (with a history of adenomas or family history of colorectal cancer) gain significantly higher diagnostic benefits from CE, with a GRADE Ia evidence level. The underlying mechanism is that WLE is prone to missing flat non-protruding adenomas in the right colon, while CE intuitively visualizes subtle mucosal depressed structures and substantially reduces interval cancers caused by missed diagnosis.

3.2. Differential diagnosis of early colorectal cancer and intraepithelial neoplasia

Combined magnifying CE enables clear pit pattern classification: type I and II indicate normal/hyperplastic mucosa, type IIIs, IIIL, and IV suggest adenomas, and type V is highly suggestive of intramucosal or submucosal invasive cancer. Systematic review data showed that magnifying CE achieves a sensitivity of 91.2% and a specificity of 88.7% in the diagnosis of high-grade intraepithelial neoplasia and early colorectal cancer, with a Kappa value of 0.82 for consistency with postoperative pathological results, superior to single WLE and pure NBI^[16].

For clinically challenging depressed early cancers, which only present with slight mucosal redness and erosion under WLE and are easily misdiagnosed as inflammation, indigo carmine staining clarifies depressed boundaries. Combined with pit pattern analysis, it can rapidly assess invasion depth and guide minimally invasive endoscopic resection or surgical treatment.

3.3. Detection and preoperative evaluation of laterally spreading tumors (LSTs)

LSTs grow horizontally along the mucosa with unobvious protrusion and higher malignant transformation potential than ordinary polyps, constituting a high-risk type of missed diagnosis in colonoscopy. A meta-analysis including 12 RCTs revealed that CE increases the LST detection rate by 42% and accurately distinguishes granular and non-granular LSTs. Non-granular LSTs have a higher risk of submucosal invasion; CE clearly identifies subtle marginal depressions of lesions, provides boundary markers for complete ESD dissection, and reduces the risk of postoperative residual lesions and recurrence^[17].

3.4. Surveillance of inflammatory bowel disease (IBD)-associated dysplasia

Patients with long-term ulcerative colitis and extensive Crohn's disease have an 8–15 times higher risk of colorectal cancer than the general population, and conventional random WLE biopsy misses more than 60% of dysplastic lesions. Guidelines from the European Crohn's and Colitis Organization (ECCO) and the IBD Group of the Chinese Society of Gastroenterology strongly recommend CE-guided targeted biopsy as the standard protocol for long-term IBD surveillance (evidence level Ia).

Evidence-based data indicate that the dysplasia detection rate of CE-guided targeted biopsy is 2.3–4.8 times that of random WLE biopsy. It accurately identifies flat and minute low/high-grade dysplastic mucosal lesions, avoids massive invalid random biopsies, shortens the pathological diagnosis cycle, and reduces the long-term canceration risk ^[18].

3.5. Post-adenoma resection follow-up and surveillance

Regular colonoscopic follow-up is required 3–5 years after complete adenoma resection, with residual minute adenomas and occult recurrent lesions as the key surveillance targets. Prospective cohort studies show that CE for postoperative follow-up increases the recurrent lesion detection rate by 27% compared with WLE. It can identify minute occult adenomas around surgical scars, enable timely intervention, and reduce the risk of interval cancer, making it suitable for high-risk follow-up populations after resection of high-grade adenomas, multiple adenomas, and LSTs.

4. Evidence-based comparison of chromoendoscopy with other enhanced endoscopic techniques

4.1. Chromoendoscopy vs Conventional White-Light Endoscopy

All published systematic reviews draw consistent conclusions: CE is comprehensively superior to WLE in the detection of adenomas, minute flat lesions, LSTs, and IBD-associated dysplasia, with no contradictory high-quality evidence. As a simple and low-cost technique to improve the diagnostic performance of basic colonoscopy, it is suitable for popularization in hospitals at all levels.

4.2. Chromoendoscopy vs Virtual Chromoendoscopy (NBI/FICE)

NBI requires no spraying of a staining agent, with a simpler operation process and higher compliance in routine outpatient screening. However, the two techniques have distinct dominant clinical scenarios. Meta-analyses show that NBI and indigo carmine CE have no statistically significant difference in the diagnosis of ordinary protruding polyps. In contrast, CE presents higher image contrast and diagnostic accuracy for depressed early cancers, minute flat adenomas in the right colon, and IBD-associated mucosal dysplasia ($P < 0.05$) ^[19].

Health economic evaluation demonstrates that the consumable cost of CE is only 1/20 of the upgrade cost of virtual chromoendoscopy equipment. It delivers significantly higher cost performance in primary medical institutions and large-scale population screening. The two techniques are complementary rather than substitutive.

4.3. Combined application of chromoendoscopy and magnifying endoscopy

The combination of magnifying endoscopy and CE is currently the gold standard for pit pattern classification diagnosis. Single CE only observes gross lesion morphology, while combined magnifying endoscopy enables accurate interpretation of mucosal crypt microstructures. This combined technique is applied for the

evaluation of early cancer invasion depth and precise preoperative ESD assessment, serving as an essential technical combination in tertiary hospital digestive endoscopy centers.

4.4. Combined application of chromoendoscopy and artificial intelligence-assisted endoscopy

Artificial intelligence can automatically identify and mark suspected intestinal mucosal polyps, while insufficient contrast of conventional white-light images easily leads to missed detection. CE significantly enhances lesion boundaries and texture features, which improves the sensitivity of AI automatic identification and reduces false-negative results when input into AI models. As an emerging research hotspot in recent years, small-sample studies have confirmed that the combined application can further reduce the adenoma missed diagnosis rate.

5. Conclusion

With sufficient grade Ia evidence-based medical support, chromoendoscopy features high safety, low consumable cost, and wide accessibility. It can significantly improve the detection rates of minute colorectal adenomas, flat lesions, LSTs, and IBD-associated dysplasia, possessing unique clinical value in the differential diagnosis of early colorectal cancer, preoperative lesion evaluation, and postoperative follow-up surveillance. Compared with virtual chromoendoscopy, CE has prominent diagnostic advantages in detecting depressed lesions and monitoring high-risk populations, and the two techniques can be applied complementarily.

Current limitations of CE include time-consuming operation, insufficient standardized training, and lack of long-term prognostic data. Future research should unify operating specifications, improve the training system for primary physicians, promote research on targeted staining agents, intelligent spraying equipment, and AI-assisted combined diagnosis, and conduct long-term real-world cohort follow-up studies. These efforts will further explore the application value of CE in early screening and diagnosis of colorectal cancer and optimize the stratified prevention and control system for digestive tract tumors in China.

Funding

Intramural Research Projects of Foshan Greater Bay Area General Hospital (Project No.: FX2025016)

Disclosure statement

The authors declare no conflict of interest.

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Publisher's note

Bio-Byword Scientific Publishing remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Current Research Status and Molecular Mechanisms of Traditional Chinese Medicine Combined with Anti-Tumor Therapy Targeting Tumor-Associated Macrophages

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Abstract: Tumor-associated macrophages (TAMs) are a key component of the tumor immune microenvironment, and their function depends on their polarization state. M1 macrophages are generally associated with anti-tumor immune activity, while M2 macrophages are linked to immunosuppression, tumor progression, and metastasis. Recent studies have shown that Traditional Chinese Medicine (TCM) may regulate TAM polarization in cancer. Both experimental and clinical evidence suggest that TCM monomers and formulas can influence this process, although the strength of evidence varies. Compounds such as artesunate, matrine, and celastrol may shift macrophages toward the M1 phenotype, involving pathways including PI3K/Akt/mTOR, STAT6, and NF- κ B. TCM formulas such as Bufei Decoction and Danggui Buxue Decoction have also been reported to enhance chemotherapy or immunotherapy effects, possibly through reducing immunosuppressive interactions in the tumor microenvironment. Clinically, Jianpi Yangzheng Xiaozhong Decoction has shown potential survival benefits in gastric cancer and has been included in integrated treatment guidelines. However, current evidence remains limited, with issues such as small sample sizes and lack of standardization. In addition, poor bioavailability of some TCM components continues to limit clinical application. Overall, TCM may regulate TAM polarization and reshape the tumor immune microenvironment through multiple targets, but further well-designed clinical studies are still needed for translation into practice.

Keywords: TAMs; TCM; Traditional Chinese Medicine anti-tumor therapy; Integrated Traditional Chinese and Western Medicine anti-tumor therapy; Research status; Molecular mechanisms

Online publication: August 11, 2026

1. Introduction

Tumor-associated macrophages (TAMs) are a major immune cell population in the tumor microenvironment. They are involved in several processes related to cancer, including tumor growth, metastasis, and anti-

tumor immune responses. Because of these roles, TAMs have been studied more frequently in recent years, although their functions are still not fully understood in different tumor types.

At the same time, there has also been increasing interest in how Traditional Chinese Medicine (TCM) might affect these cells. Some studies have reported that extracts and active compounds from TCM can influence macrophage behavior, especially the polarization process of TAMs, which may be related to anti-tumor effects ^[1].

Research on the relationship between TAMs and tumor progression is still developing. In this context, this review looks at what is currently known about TAMs in cancer, and then focuses on how TCM may regulate them. Studies involving both single compounds and herbal formulas are considered, particularly those related to signaling pathways in macrophage polarization. Some work has also explored combining TCM with chemotherapy or immunotherapy, and this topic has gained attention recently, although the evidence is still limited.

Overall, while the idea of targeting TAMs is becoming more discussed, many mechanisms are still unclear. Even so, it may provide a possible direction for integrating traditional Chinese and Western medicine in cancer treatment.

2. Research progress on core molecular mechanisms of tumor-associated macrophages

2.1. Relationship between TAM polarization phenotypes (M1/M2) and tumor progression

Tumor-associated macrophages (TAMs) are generally described as existing in two main polarization states, M1 and M2, although in reality the situation may be more flexible and not always strictly divided. The balance between these two types is thought to influence tumor progression, but this balance can look different depending on tumor context.

M1-type macrophages are usually linked with anti-tumor immune activity. They are known to produce inflammatory mediators such as CD86, IL-1 β , TNF- α , and IL-12, which are involved in activating Th1-type immune responses and supporting recruitment of CD8⁺ T cells into tumor tissues. However, the strength of this response can vary in different tumor environments.

On the other hand, signals in the tumor microenvironment tend to push macrophages toward an M2-like state. Cytokines such as IL-4, IL-13, and IL-10 are often mentioned in this process. These M2-like macrophages show higher expression of markers including CD206, Arg1, Fizz1, and Ym1, and they also release immunosuppressive factors like IL-10 and TGF- β . Once this phenotype becomes dominant, effector T-cell activity tends to decrease, and tumor cells can more easily survive and expand. In addition, processes such as angiogenesis and invasion are promoted, partly through VEGF and matrix metalloproteinases (MMPs) ^[2].

Clinical observations more or less support this general pattern. Higher infiltration of M2-type TAMs has been reported in cancers such as lung cancer, gastric cancer, and hepatocellular carcinoma, and these cases are often associated with poorer outcomes. Still, the relationship is not completely identical across all tumor types, and different studies report some variation. Overall, it is generally accepted that the M1/M2 balance is related to tumor progression, even if the exact mechanism is not fully consistent across cancers.

2.2. Key signaling pathways of TAM-mediated immune suppression and their roles in tumor proliferation and metastasis

The regulation of TAM polarization is not controlled by a single pathway, but rather by several interconnected signaling networks. PI3K/Akt/mTOR is often mentioned first in this context, mainly because it is closely associated with M2 polarization. When cytokines such as IL-4 are present, this pathway becomes activated and M2-related genes are upregulated. There are also reports showing that blocking PI3K γ can shift macrophages back toward a more inflammatory state, partly restoring M1-like activity.

STAT6 is usually discussed together with IL-4 signaling. After phosphorylation, it enters the nucleus and triggers transcription of genes such as Arg1 and Fizz1. Some experimental studies have shown that when STAT6 is inhibited, tumor metastasis can be reduced, although the exact effect seems to depend on tumor type and model used.

Beyond the two major pathways mentioned above, PI3K γ seems to play a wider regulatory role in macrophages. It has been reported to suppress NF- κ B activity while at the same time enhancing C/EBP β signaling, which overall pushes macrophages toward a more immunosuppressive state. IL-10 produced by TAMs may further strengthen this tendency. Some studies suggest that IL-10 can upregulate PD-L1 expression on tumor cells, and this then reduces CD8⁺ T-cell activity through the PD-1/PD-L1 axis. There is also another pathway that has been discussed more recently: the NLRP3/Caspase-1/IL-1 β pyroptosis pathway. The situation here does not seem completely straightforward. In some experimental models, overactivation of this pathway may cause pyroptosis in M1 macrophages, which could weaken anti-tumor immune responses. On the other hand, inhibition of NLRP3 has been reported to help maintain M1 function, and this might improve the effect of immunotherapy, although this is still being explored ^[3].

Apart from immune-related regulation, TAMs also have more direct effects on tumor cells themselves. M2 macrophages can release growth factors such as EGF, TGF- β , and IL-6, and these factors activate several downstream signaling pathways in tumor cells, including MAPK, PI3K/Akt, and Wnt/ β -catenin. This is generally associated with increased proliferation and maintenance of stem-like characteristics, although the strength of these effects may vary in different tumor models.

During metastasis, molecules like TGF- β and IL-10 are often mentioned in relation to epithelial-mesenchymal transition (EMT), which is linked to increased migration and invasion ability. At the same time, processes such as matrix degradation and angiogenesis also play a role. MMPs contribute to the breakdown of the extracellular matrix, while VEGF supports the formation of new blood vessels. All of this together makes metastatic spread easier in many cases. TAMs can also reduce CD8⁺ T-cell infiltration and function, mainly through IL-10 and PD-L1-related mechanisms ^[4]. Figure 1 gives a general overview of these pathways and their effects.

Overall, TAMs are involved in different stages of tumor development, including immune escape, growth, and metastasis. Because of this, they are often considered a potential target in cancer therapy. Recently, TCM has been discussed in this context since it may influence several signaling pathways at the same time rather than acting on just one target.

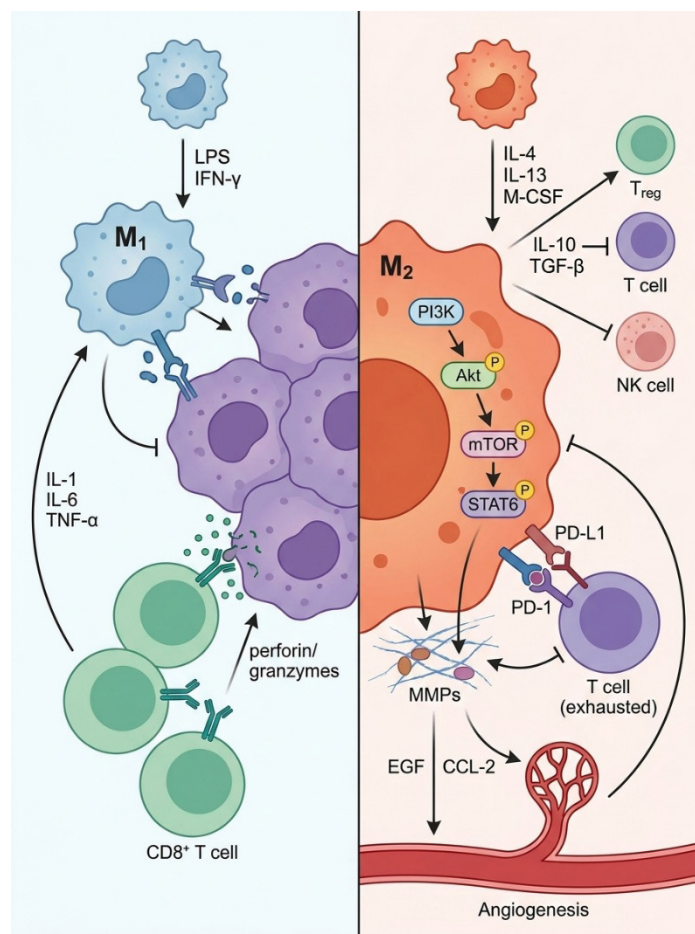


Figure 1. TAMs polarization.

2. Molecular mechanisms of anti-tumor effects of traditional Chinese medicine by regulating TAMs

2.1. Regulatory effects of TCM monomers on macrophage polarization

There is increasing evidence that several compounds derived from Traditional Chinese Medicine (TCM) can influence the polarization state of tumor-associated macrophages (TAMs). Most of the studies point in a similar direction, that is, shifting macrophages from the M2 phenotype toward M1, although the specific pathways involved are not always the same.

For instance, a water extract containing ginseng and Astragalus was reported to increase M1-related markers while reducing M2-type polarization. In that study, immune activity within the tumor environment appeared to be enhanced after treatment ^[5]. Compound Kushen Injection shows a somewhat similar trend, but through a different mechanism. It activates the TNFR1/NF-κB/p38MAPK signaling pathway, and this activation is accompanied by increased proliferation and cytotoxic function of CD8⁺ T cells ^[6].

Some single compounds have also been studied at the molecular level. Lobeline, for example, has been shown to bind MAPK14 and regulate the p53/Slurp1 pathway. This does not directly act only on macrophages, but seems to indirectly push the balance toward M1 polarization, and there are also reports that it may work better when combined with anti-PD-1 therapy ^[7]. Bezoar (Calculus bovis) has been reported to

inhibit M2 polarization, possibly through suppression of the Wnt/ β -catenin signaling pathway, although the exact downstream process is still not fully clear.

Matrine has been studied in Lewis lung cancer models and is often described as suppressing M2-type TAM polarization. The effect is generally linked with inhibition of the PI3K/Akt/mTOR pathway. In the same context, changes in epithelial–mesenchymal transition (EMT)-related proteins have also been observed, including increased E-cadherin and decreased N-cadherin, Vimentin, and Snail. These changes correspond with reduced migration and invasion of tumor cells, suggesting a broader anti-tumor effect ^[8].

Pulsatilla saponins (PS) mainly act through the STAT6 pathway. In IL-4-induced macrophage models, PS were found to reduce M2 polarization and downregulate markers such as Arg1, Fizz1, Ym1, and CD206 ^[9]. One study on vinegar-processed frankincense (PF) water extract reported a different pattern. Instead of acting directly on macrophages, its effect seems to be linked with the gut microbiota and related metabolites. This microbiota–immune interaction may also influence TAM behavior, although the mechanism is still not well defined. It adds a slightly different angle to how TCM might regulate immune responses in tumors ^[10].

2.2. Reprogramming of TAMs by TCM compounds

Compared with single compounds, more recent studies have started to focus on TCM formulas and how they affect TAM reprogramming in a more complex way. Some of the findings suggest that these formulas may not only act on signaling pathways, but also influence macrophage metabolism, which in turn affects the tumor immune environment.

One example is the Wenxia Changfei Formula. Yin et al. reported that it suppressed the activation of the PPAR- γ /CD36 pathway in M2 macrophages. As a result, fatty acid uptake and lipid accumulation were reduced, and fatty acid oxidation was also affected. These metabolic shifts were linked with a change in macrophage state, from M2-like toward M1-like phenotype. In the same experimental model, reduced migration and metastasis of Lewis lung cancer cells were also observed ^[11].

Xihuang Pills (XHP) show a somewhat different mechanism. Based on network pharmacology combined with metabolomic analysis, one study found that XHP decreased kynurenine levels in a mouse model of pancreatic ductal adenocarcinoma. This was accompanied by inhibition of AKT/mTOR signaling and promotion of M1 polarization, together with stronger anti-tumor immune responses. In that work, β -boswellic acid was identified as one of the key components that may be responsible for regulating both kynurenine metabolism and macrophage polarization ^[12].

Overall, these studies suggest that TCM formulas do not act through a single target. The effects seem to involve multiple components and different biological layers, especially metabolic regulation in macrophages. By shifting the balance from M2 toward M1 phenotype and changing the tumor immune environment, they may help slow down tumor progression, although the exact contribution of each pathway is still not fully clear.

3. Strategies and research progress of traditional Chinese medicine in anti-tumor therapy

3.1. Preclinical research applications of TCM combination treatment strategies

Combination therapy has become an important approach in cancer treatment because it can simultaneously target multiple biological pathways and may improve the limitations of single-agent therapy. Traditional

Chinese Medicine (TCM) has attracted considerable interest in this area, and an increasing number of experimental studies have examined its use together with chemotherapy, targeted therapy, or immunotherapy.

3.1.1. Lung cancer

Several studies in non-small cell lung cancer have suggested that TCM may enhance anti-tumor immunity through regulation of tumor-associated macrophages (TAMs). Hu et al. reported that artesunate promoted M1 polarization through activation of the STAT1/IRF1 signaling pathway, resulting in increased T-cell infiltration and improved responses to anti-PD-1 therapy in Lewis lung cancer models ^[13]. In another study, Zhan et al. showed that crude polysaccharides isolated from Danggui Buxue Decoction (DBDP) reduced deoxycytidine secretion by M2 macrophages and decreased cytidine deaminase (CDA) expression by promoting the transition from M2 to M1 macrophages, thereby alleviating TAM-associated resistance to gemcitabine ^[14]. Bufe Decoction (BFD) has also been reported to interfere with the TAM–IL-10–PD-L1 signaling axis, suppressing the malignant behavior of lung cancer cells and suggesting a possible role for combination treatment with immune checkpoint inhibitors ^[15].

3.1.2. Liver cancer

In hepatocellular carcinoma, *Polygonatum sibiricum* polysaccharide (PSP) has been shown to activate the TLR4/MyD88/NF- κ B pathway, promoting macrophage polarization toward the M1 phenotype and suppressing tumor growth ^[16]. Lu Linzhu and colleagues further reported that *Aconitum* polysaccharide (FPS), when combined with lenvatinib, enhanced the anti-tumor activity of lenvatinib by increasing M1 macrophage polarization, indicating that TCM polysaccharides may improve the efficacy of targeted therapy ^[17].

3.1.3. Intrahepatic cholangiocarcinoma

Yang et al. investigated the interaction between tumor metabolism and immune regulation in intrahepatic cholangiocarcinoma. Their study showed that celastrol reduced glycolysis in tumor cells through inhibition of the AKT/mTOR pathway, leading to lower lactate production. As lactate is an important driver of M2 polarization, this metabolic change was accompanied by reduced M2 polarization and inhibition of tumor progression ^[18].

3.1.4. Breast cancer

Different mechanisms have also been reported in breast cancer models. Using TAM membrane-coated magnetic nanoparticles together with LC-MS analysis, Yang et al. identified liquiritin as one of the major active components of Aidikang Formula (ADQ). Liquiritin increased cathepsin K (CTSK) expression, promoted lysosomal degradation of CXCL1 in TAMs, and reduced endothelial tube formation and tumor angiogenesis ^[19]. Jin et al. found that total glucosides of paeony (TGP) suppressed TAM recruitment through inhibition of the NF- κ B/CCL2 pathway while increasing memory T-cell populations, suggesting an improvement in anti-tumor immune responses ^[20]. In another study, dandelion extract regulated the IL-10/STAT3/PD-L1 pathway and promoted the transition of TAMs from the M2 phenotype to the M1 phenotype ^[21].

3.1.5. Esophageal cancer

Recent work has also examined the interaction between tumor metabolism and macrophage polarization in esophageal squamous cell carcinoma. Wang et al. reported that IL-4 released by tumor cells activated

the JAK2/STAT6 pathway in macrophages, promoting M2 polarization. These macrophages subsequently enhanced glycolysis in tumor cells, as reflected by increased expression of HK2, GLUT1, and LDHA together with elevated glucose uptake and lactate production. *Zuogui Yin* did not directly suppress glycolysis in tumor cells, but inhibited M2 polarization by targeting JAK2 in macrophages and blocking IL-4/JAK2/STAT6 signaling. This indirect effect was associated with reduced glycolytic activity in tumor cells. Mass spectrometry combined with molecular dynamics analysis further identified isoferulic acid and dehydrocorydaline as two active compounds capable of binding stably to JAK2 ^[22].

3.2. Clinical research applications of TCM characteristic treatment technologies

In clinical practice, several Traditional Chinese Medicine (TCM) formulas have been evaluated as adjunctive treatments for different types of cancer. *Jianpi Yangzheng Xiaozheng* Decoction (JPYZXZ), which was developed according to the principle of “invigorating the spleen, supporting healthy *qi*, resolving stasis, and dissipating masses,” has been used for many years in patients with gastric cancer. Clinical studies suggest that it may improve quality of life, prolong survival, reduce chemotherapy-related adverse effects, and enhance immune function, including macrophage activity ^[23]. Following multicenter clinical evaluation, the formula has been included in the Guidelines for Integrated Traditional Chinese and Western Medicine Diagnosis and Treatment of Gastric Cancer.

Fuzheng Jiedu Xiaoji Formula has been reported in the treatment of hepatocellular carcinoma (HCC). Some clinical data suggest that it may help relieve symptoms in patients and also slow down disease progression to a certain extent. In studies where it was used together with PD-1/PD-L1 inhibitors, an improvement in median survival has been observed. This effect is often discussed in relation to changes in the tumor immune microenvironment, especially the balance between M1 and M2 macrophages ^[24].

Fuzheng Yiliu Formula (FZYL) has also been used in prostate cancer, mainly as an adjunct therapy. Clinical observations are still relatively limited, but there are reports suggesting that it may delay progression and improve symptom control. These effects are thought to be related to regulation of tumor-associated macrophages as well as chemokine-related signaling ^[25]. In a similar way, *Gehua Jiecheng* Decoction (GHJCD) has been applied in lung cancer patients to relieve clinical symptoms. Both experimental and clinical findings point to possible involvement of immune regulation, reduction of inflammatory activity, and suppression of angiogenesis, although the mechanisms are not fully consistent across studies ^[26].

At the same time, it should be noted that clinical evidence for many of these interventions is still not strong enough. Most studies are either small-scale or not well-controlled, and large prospective trials combining TCM with immune checkpoint inhibitors are still rare. External therapies used in TCM practice, such as topical herbal applications combined with paraffin-based treatment, have been reported in some cases, but overall supporting data is quite limited.

Table 1 gives an overview of recent studies on TCM in anti-tumor applications. From preclinical research, it is generally suggested that TCM may affect tumor progression through several different routes, including regulation of TAM polarization, interference with immunosuppressive signaling, and metabolic-immune interactions. Some clinical reports also mention potential benefits from formulas such as *Jianpi Yangzheng Xiaozheng* Decoction and *Aidikang* Formula. However, how to translate these findings into standard clinical use is still not very clear. More work is needed, especially on how to combine TCM with chemotherapy or immunotherapy in a more rational way, and also on identifying which patient groups may

actually respond better.

Table 1. Research progress of TCM in anti-tumor therapy

TCM / Active Ingredient	Tumor Type	Regulatory Effect on TAMs	Core Molecular Mechanism	Reference
Bufei Decoction (BFD)	Non-small cell lung cancer	Inhibits IL-10 secretion by TAMs, downregulates PD-L1 expression	Regulation via IL-10/PD-L1 axis, inhibits immune escape	[15]
Jianpi Yangzheng Xiaozheng Decoction (JPYZXZ) and its decomposed formulas	Gastric cancer	Promotes TAMs M2→M1 polarization; inhibits EMT	JPYZ regulates polarization; XZSJ inhibits EMT	[23]
Gehua Jiecheng Decoction (GHJCD)	Liver cancer (DEN-induced)	Regulates immune cells and inflammatory factors	Inhibits NF-κB/WNT signaling pathway	[26]
Fuzheng Jiedu Xiaoji Formula (FZJDXJ)	Liver cancer	Inhibits tumor cell proliferation and migration	Regulates AKT/CyclinD1/p21/p27 pathway	[24]but its curative effect still requires extensive clinical research and mechanistic analysis. PURPOSE: To explore the effectiveness of FZJDXJ in HCC patients and investigate its biological function and mechanism underlying anticancer therapy. METHODS: This randomized controlled clinical trial enrolled 291 HCC patients receiving transcatheter arterial chemoembolization (TACE
Dandelion extract	Triple-negative breast cancer	Promotes M1 polarization, inhibits M2 polarization	Inhibits IL-10/STAT3/PD-L1 pathway	[21]
Total glucosides of paeony (TGP)	Breast cancer	Inhibits TAMs recruitment, increases T cell memory subsets	Inhibits NF-κB/CCL2 signaling axis	[20]
Crude polysaccharides of Danggui Buxue Decoction (DBDP)	Lung cancer	Reshapes TAMs (M2→M1); reverses chemoresistance	Inhibits dC secretion and CDA expression, combined with gemcitabine	[14]
Fuzheng Yiliu Formula (FZYL)	Prostate cancer	Inhibits TAMs/CCL5 pathway; inhibits cancer stem cell self-renewal	Inhibits cancer stem cell renewal, EMT and metastasis	[25]
Polygonatum sibiricum polysaccharide (PSP)	Liver cancer	Induces TAMs M2→M1 polarization	Activates TLR4-MyD88-NF-κB pathway	[16]
Aconitum polysaccharide (FPS) + Lenvatinib	Hepatocellular carcinoma	Promotes TAMs M1 polarization	Upregulates iNOS/IL-12b, downregulates Arg-1/IL-10; combined targeted therapy	[17]

TCM / Active Ingredient	Tumor Type	Regulatory Effect on TAMs	Core Molecular Mechanism	Reference
Celastrol	Intrahepatic cholangiocarcinoma	Inhibits tumor glycolysis; reverses lactate-driven M2 polarization	Inhibits AKT/mTOR pathway, reduces lactate production	[18]
Artesunate (AS) + anti-PD-1 antibody	Non-small cell lung cancer	Reprograms TAMs (M2→M1); enhances T cell activation	Mediated by STAT1/IRF1, synergistic with immunotherapy	[13]
Aidikang Formula (ADQ) / Liquiritin	Breast cancer	Promotes lysosomal degradation of CXCL1 in TAMs; inhibits angiogenesis	Mediates CXCL1 degradation via CTSK	[19]
Zuogui Yin (ZGY)	Esophageal squamous cell carcinoma	Blocks M2 polarization; indirectly inhibits tumor glycolysis	Inhibits IL-4/JAK2/STAT6 pathway	[22]

4. Limitations of current research and future directions

Although considerable progress has been made in recent years, several issues continue to limit the clinical application of TAM-targeted Traditional Chinese Medicine (TCM) therapies.

4.1. Technical challenges related to the bioavailability of TCM components

One of the main problems with many active components in Traditional Chinese Medicine (TCM) is that their bioavailability is not very high. This includes monomers, polyphenols, and polysaccharides. In most cases, this is related to poor water solubility, fast metabolism *in vivo*, and also limited accumulation in tumor tissues ^[27]. Because of these factors, the actual amount of drug reaching the target site can be quite low, which may in turn reduce therapeutic effect.

To address this issue, a number of drug delivery approaches have been explored. Nanotechnology-based systems, for example, liposomes and polymeric nanoparticles, are often mentioned. These systems are mainly designed to improve stability and also control drug release. Some pH-responsive nanocarriers are reported to release drugs more efficiently in acidic tumor environments, which may slightly improve local targeting ^[28]. Apart from this, there are also other experimental platforms such as exosome-based delivery systems and metal-organic frameworks, but most of them are still in early-stage research and not yet close to clinical use.

4.2. Standardization of integrated TCM-Western medicine therapy

In recent years, combining TCM with chemotherapy or immune checkpoint inhibitors has shown some promising results in clinical studies. Some reports mention improved survival outcomes and also changes in immune-related indicators within the tumor microenvironment ^[29]. However, it is also clear that the overall clinical evidence is still not strong enough at this stage.

One major issue is that treatment protocols are not consistent across studies. Different research groups use different dosages, schedules, and even different ways to evaluate efficacy. Because of this, it is very difficult to compare results directly. For example, there is still no clear agreement on how to choose doses or how to set treatment timing when TCM formulas are used together with immune checkpoint inhibitors.

Management of adverse reactions is also not standardized.

At present, most of these combination strategies are still at the exploratory stage. More clinical data, especially from multicenter studies, will probably help clarify which patient groups may benefit more and how these therapies should be used more consistently.

5. Conclusion

Current evidence suggests that tumor-associated macrophages (TAMs) have an important role in the tumor microenvironment, and changes in macrophage polarization are closely related to tumor growth, immune escape, and metastasis. A number of signaling pathways, including PI3K/Akt/mTOR, STAT6, NF- κ B, and IL-10/PD-L1, participate in these processes and have therefore become potential targets for anti-tumor therapy.

There are quite a few studies suggesting that Traditional Chinese Medicine (TCM) can influence tumor-associated macrophages (TAMs), but the way it works does not seem to be limited to one single pathway. Compounds like artesunate, matrine, and celastrol have all been mentioned in the literature, although each of them seems to act through different and sometimes overlapping mechanisms. The same kind of pattern is also reported in several formulas such as *Bufei* Decoction, *Danggui Buxue* Decoction, and *Aidikang* Formula.

In addition to effects on macrophage polarization itself, some studies also point out that TCM may affect how macrophages interact with other cells in the tumor microenvironment. This includes tumor cells, T cells, and endothelial cells, but the exact degree of each interaction is still not very clear.

There are also experimental and some clinical findings suggesting that TCM might enhance the effects of chemotherapy, targeted therapy, or immunotherapy when they are used together. A few formulas, for example *Jianpi Yangzheng Xiaozhong* Decoction, *Fuzheng Jiedu Xiaoji* Formula, and *Fuzheng Yiliu* Formula, have shown relatively positive results in clinical reports. But it has to be said that most of these studies are still small, and many come from single hospitals or limited datasets, so the evidence is not really strong yet.

At the same time, there are still many things that remain unclear. For example, pathways like STAT1/IRF1, TLR4-MyD88, and CTSK are often mentioned in experimental work, but how important they really are in actual human patients is still uncertain. It is also not very clear whether TCM works mainly by reshaping the tumor immune microenvironment or whether it just adds to the effect of existing treatments in a more general way. In addition, problems such as poor bioavailability of some compounds and the lack of unified treatment standards are still there and continue to limit clinical use.

Moving forward, progress will likely depend on how well experimental work and clinical studies can be linked together. It would be important to identify which components in effective formulas are actually responsible for the observed effects, and how they act at the molecular level. Drug delivery systems, including nanocarriers or exosome-based approaches, may help improve this, but most of these are still in early stages. On the clinical side, larger and better-designed multicenter studies are still needed to really understand how TCM should be combined with chemotherapy, targeted therapy, or immunotherapy. For now, the role of TCM in regulating the tumor immune environment is still being explored rather than clearly defined.

Disclosure statement

The authors declare no conflict of interest.

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Publisher's note

Bio-Byword Scientific Publishing remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Application of “Integrative Acupuncture and Moxibustion” in the Treatment of Lumbar Disc Herniation: A Clinical Case Example

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Abstract: *Objective:* To explore the clinical efficacy of Integrative Acupuncture and Moxibustion in the treatment of lumbar disc herniation. *Methods:* The concept and clinical application of “Integrative Acupuncture and Moxibustion” are elucidated through a case study. *Conclusion:* Integrative Acupuncture and Moxibustion, employing a stepped clinical treatment model, effectively empowers the promotion of comprehensive external treatment protocols of traditional Chinese medicine, holistically enhances the clinical efficacy of acupuncture and moxibustion, reduces the economic burden on patients, and fully leverages the advantages of acupuncture and moxibustion—simplicity, convenience, affordability, and efficacy—which is of great significance.

Keywords: Lumbar disc herniation; “Integrative Acupuncture and Moxibustion”; Clinical research

Online publication: August 11, 2026

1. Introduction

Low back pain is the leading cause of disability and productivity loss worldwide, and lumbar disc herniation (LDH) is one of the most common causes of low back pain ^[1]. Lumbar disc herniation is a clinical syndrome caused by degenerative changes of the intervertebral disc, rupture of the annulus fibrosus, and protrusion of the nucleus pulposus, which stimulates and compresses the nerve roots. Common symptoms include low back and leg pain ^[2]. This condition predominantly occurs in young and middle-aged men, and severe cases may result in disability ^[3]. For 80%–85% of patients, conservative treatment is the preferred approach ^[1]. Conservative treatment includes pharmacotherapy (such as non-steroidal anti-inflammatory drugs, corticosteroids, dehydrating agents, and neurotrophic agents), physical therapy, functional exercise, and traction ^[4].

Traditional Chinese medicine (TCM) has significant advantages in the treatment of this condition,

with diverse schools of thought ranging from etiology and pathogenesis to principles, methods, formulas, and medicinals. This article illustrates, through Integrative Acupuncture and Moxibustion and a stepped clinical treatment model, the holistic enhancement of the clinical efficacy of acupuncture and moxibustion, the reduction of patients' economic burden, and the full exploitation of the advantages of acupuncture and moxibustion—simplicity, convenience, affordability, and efficacy. This approach holds great significance in terms of patient satisfaction and acceptance.

2. Case presentation

The patient, surnamed Wei, a 40-year-old male, presented with recurrent low back pain for 2 years, with recurrence and aggravation for 1 week. The patient experienced low back pain, predominantly soreness, 2 years ago after overexertion. The pain worsened upon standing, walking, and physical activity. There was no difficulty with urination or defecation, no saddle anesthesia, and no numbness or weakness of the lower limbs. He visited a local community hospital, and lumbar spine imaging suggested “lumbar disc herniation”. His symptoms were relieved after pharmacotherapy, acupuncture, and other treatments. Subsequently, the low back pain recurred with similar characteristics. In mid-June, he experienced unbearable low back pain upon waking, accompanied by distending pain on the lateral aspect of the left lower limb and slight numbness on the dorsum of the left foot. From July 16 to July 19, he was admitted to the acupuncture and rehabilitation department of another hospital with a diagnosis of “lumbar disc herniation with sciatica. During hospitalization, lumbar MRI revealed: (1) Annular tears of the L4/L5 and L5/S1 intervertebral discs, L4/L5 disc bulging and herniation (left paramedian type) with compression of the ipsilateral nerve root at the corresponding level; (2) L5/S1 disc bulging and herniation (central type); (3) Endplate osteochondrosis at the opposing margins of the L4/L5 vertebral bodies; (4) Degenerative changes of the lumbar spine. Ultrasound examination showed a small cyst in the left kidney. His symptoms were markedly relieved after acupuncture and rehabilitation therapy. One week prior, the symptoms exacerbated after excessive exercise, including sexual activity and swimming. The symptoms did not improve significantly after resting at home, and he came to our department for further systematic treatment.

Current symptoms: low back pain, pronounced upon forward flexion, with slightly limited range of motion; pain on the lateral aspect of the left thigh; slight numbness of the left lower limb and dorsum of the foot, worse in the morning with difficulty getting out of bed; inability to sit for prolonged periods; aggravation with exertion and relief with rest. No weakness of the four limbs, no saddle anesthesia, no headache, normal appetite and sleep, normal bowel and bladder function. Tongue: dark purple with a slightly greasy yellow coating; pulse: wiry and slippery.

Specialist physical examination: Lumbar physiological curvature straightened, without obvious scoliosis; lumbar muscles stiff and bulging; slightly limited lumbar range of motion in all directions; tenderness on palpation of the bilateral para-spinous points of L3/L4, L4/L5, and L5/S1 (+), percussion pain (-); positive straight leg raise and augmentation test on the left side (+). Bilateral femoral nerve stretch test (±); Patrick's (figure-of-4) test (-). Muscle strength, muscle tone, skin and mucosa of the four limbs normal. Physiological reflexes normal and symmetrical, pathological reflexes not elicited. VAS score: 8.

Ancillary investigations: A lumbar MRI performed at our hospital today revealed: (1) Degenerative changes of the lumbar spine. Endplate osteochondrosis of L4–5 vertebral bodies (Type I); (2) L4/L5 disc bulging and herniation (paramedian type); L5/S1 disc herniation (central type).

TCM diagnosis: Lumbar impediment (qi stagnation and blood stasis pattern). Western medical diagnosis: (1) Lumbar disc herniation with sciatica; (2) Simple renal cyst (left); (3) Personal medical history (postoperative status for perianal abscess).

3. Treatment course

(1) Pricking and cupping bloodletting: Tender points over the lumbosacral region and the ipsilateral Weizhong (BL40) acupoint.

(a) Acupuncture: Baihui (GV20), Yintang (EX-HN3), Neiguan (PC6, bilateral), Yanglingquan (GB34, bilateral), and Yinqi Guiyuan (leading Qi back to its origin) needling technique.

(b) Moxibustion: Sihua points (Geshu [BL17] and Danshu [BL19]), Shenshu (BL23), and Mingmen (GV4).

(c) Consolidation: Intradermal needle embedding at Xinshu (BL15) and Danshu (BL19); auricular point pressing with seeds at Kidney (CO10) and Lumbar vertebrae (AH9).

(2) Procedure: The patient was first placed in the prone position. Small purple superficial venules were located at the tender points of the lumbar region. The affected areas were pricked with a three-edged needle, and a No. 5 glass cup was rapidly applied for flash cupping to drain the stagnant blood until the bleeding of morbid blood ceased. After removing the cups, routine skin disinfection of the acupoints was performed.

For acupuncture, the patient was placed in the supine position. Baihui (GV20) and Yintang (EX-HN3) were needled conventionally. After insertion, the patient was instructed to take six deep breaths through the nasal passage, rest for 1 minute, and then take another six deep breaths; this was repeated until needle removal. The remaining acupoints were needled conventionally with even supplementation and drainage manipulation; after obtaining Qi, the needles were retained for 25 minutes.

A small amount of Wanhua Oil (a traditional Chinese herbal oil) was applied to the Sihua points, Shenshu (BL23), and Mingmen (GV4) on the back, which served both as an adhesive and to prevent burns. Moxa cones the size of a grain of wheat were placed on the acupoints and ignited with an incense stick. When approximately two-thirds of the cone had burned and the patient felt a warming sensation, the unburned moxa was removed with forceps. Two cones were applied to each acupoint. After moxibustion, intradermal needles were embedded at Xinshu (BL15) and Danshu (BL19). Wangbuliuxing (Semen Vaccariae) seeds were taped to the auricular points of the Kidney and Lumbar vertebrae on one ear, with alternate ear pressing between the left and right ears.

(3) Intradermal needle embedding procedure: After routine skin disinfection, the skin around the acupoint was stretched taut with the thumb and index finger of the left hand. With the right hand, a wheat-grain-type intradermal needle was grasped with forceps, the needle tip was aimed at the acupoint and inserted flat into the dermis. A small piece of adhesive tape was placed between the needle handle and the skin, and then a larger piece of tape was applied over the needle handle to prevent displacement or detachment. The patient was instructed to remove the tape and needle by himself after 3 days.

(4) Auricular point pressing procedure: After routine disinfection of the skin of one ear, the reactive points corresponding to the Kidney and Lumbar vertebrae were located using an ear probe. The ear patch with Wangbuliuxing seeds was applied to the tender points. The patient was instructed to remove the ear patches by himself after 3 days. Auricular pressing was alternated between the left and right ears. The treatment regimen was twice weekly, with each session lasting approximately 1 hour.

4. Outcomes

Treatment was administered twice per week, with 6 sessions constituting one course. At the end of the first course, the VAS score for low back pain decreased from 8 to 4, and the patient was able to engage in moderate-intensity exercises such as weight-bearing activities and running. A telephone follow-up three months later revealed occasional low back soreness after prolonged sitting and overexertion, without numbness of the lower limbs or radiating pain to the left lower limb. The VAS score ranged from 0 to 2. The goal of conservative treatment was achieved, and the clinical outcome was effective.

5. Discussion

5.1 Modern medical understanding of the disease

With regard to diagnosis, it is essential to distinguish between lumbar disc herniation as a morphological finding and lumbar disc herniation syndrome. The former is merely a morphological or imaging definition; a diagnosis should not be made solely on the basis of MRI or CT examinations. The latter refers to a constellation of symptoms caused by the pathological disc, such as bowel and bladder dysfunction due to compression of the cauda equina, lower limb numbness, and local pain. A definitive diagnosis can only be established by integrating the patient's history, symptoms, physical signs and imaging findings.

In terms of treatment, modern medicine has already developed a mature and well-established therapeutic standard. Regarding the choice between conservative and non-conservative treatment, surgical intervention is the first choice when there is marked nerve root compression posing serious risks. In the context of postoperative rehabilitation, seeking a long-term effective regimen is a common goal shared by both clinicians and patients.

5.2. Traditional medical understanding of the disease

The understanding of this disease in traditional Chinese medicine (TCM) has a long history, and it is classified under the category of “lumbar Bi syndrome” (low back pain impediment). As stated in the *Suwen* (Plain Questions) · Bi Lun (Treatise on Bi Syndrome), “The combined invasion of wind, cold and dampness leads to Bi syndrome”, indicating that the invasion of these three pathogenic factors is a major external cause of Bi syndrome. Drawing upon previous research, various schools of thought have elaborated on this condition. For instance, Zhu Danxi, in his work *Danxi Xinfu* (Danxi's Experiential Therapy), emphasized kidney deficiency as a causative factor and proposed the viewpoints of “damp-heat, kidney deficiency, static blood, sprain and contusion, and phlegm accumulation”. Chao Yuanfang, in his *Zhubing Yuanhou Lun* (Treatise on the Causes and Manifestations of Diseases), also summarized the etiologies of low back pain, categorizing them into traumatic injury and overstrain, and further distinguishing between sudden low back pain and chronic low back pain. When there is a deficiency of vital Qi combined with the invasion of external pathogens, the interplay of internal and external factors and the intermingling of deficiency and excess lead to obstruction of the meridians and stagnation of Qi and blood, thereby giving rise to this disease.

5.2.1. TCM etiology and pathogenesis theory

The patient in the case was a middle-aged male, at the age of “five times eight” (forty years), when the kidney Qi begins to decline, resulting in hair loss and tooth withering. The low back is the residence of the kidneys; the kidneys govern the bones, produce marrow, and nourish the brain. The liver governs the sinews;

the liver and kidney share the same origin. Insufficient replenishment of Qi, blood, and body fluids leads to stagnation, and the chronic, recurrent course of the disease, when analyzed through the four diagnostic methods, corresponds to the category of lumbar Bi syndrome in TCM. Static blood obstructs the meridians of the lumbar region and lower limbs, resulting in impaired venous circulation and localized distension, pain, and numbness in the lower limbs. A dark purplish tongue with a slightly greasy yellow coating and a wiry, slippery pulse are all consistent with the pattern of Qi stagnation and blood stasis.

As stated in the *Lingshu* (Miraculous Pivot) · *Jing Mai* (Meridians), “The Gallbladder meridian is primarily responsible for diseases arising from the bones”. Zhang Jiebin of the Ming Dynasty noted in the *Leijing* (Classified Classic) · *Jue Syndromes of the Twelve Meridians*, “When there is reversal of the Shaoyang meridian, the joints become impaired; when the joints are impaired, the waist cannot move, and the neck cannot turn. The Shaoyang meridian of the foot is the Gallbladder meridian; the joints are the crucial meeting places of sinews and bones.” This indicates that the Shaoyang Gallbladder meridian itself can regulate disorders of the sinews and bones as well as low back pain caused by joint impairment. Furthermore, the invasion of wind, cold, and dampness tends to obstruct the local and systemic circulation of Qi and blood. Clinical practice should therefore focus on regulating the Qi dynamic. Since the liver governs the free flow of Qi throughout the body and is closely related to the gallbladder, once the Shaoyang pivot is turned, the Qi dynamic will naturally be regulated. Where the meridians traverse, there the therapeutic effect extends; malnutrition of Qi and blood leads to numbness and loss of sensation, precipitating this disease. The disease location is in the lumbar region, and it is closely associated with the liver, kidney, and gallbladder.

5.2.2. Analysis of the treatment protocol

As recorded in the *Suwen* (Plain Questions) · *Ci Yao Tong Lun* (Treatise on Acupuncture for Low Back Pain), “Puncture the resolving vessel... where there are nodes like millet grains at the popliteal crease; prick them to let out black blood until red blood appears”. Chronic diseases enter the collaterals, and chronic diseases invariably involve blood stasis. For the low back, the *Weizhong* point is sought; pricking *Weizhong* on the affected side to bleed can relieve local compression, alleviate pain, and unblock the collaterals to stop pain, a method repeatedly proven effective in clinical practice.

(1) Holistic regulation of the heart and mind as the main thread, with the heart-gallbladder axis as the core principle of treatment

The diversity of modern socioeconomic culture and the complexity of lifestyles determine the multifactorial nature of disease etiology. Normal mental activities are beneficial to physical and mental health, whereas loss of emotional control and disease caused by the seven emotions lead to disharmony of yin and yang, manifesting as psychosomatic disorders. Therefore, simultaneous regulation of the body and mind is our primary consideration in guiding clinical treatment.

(2) Selecting a few but refined acupoints, with preservation, treatment, and regulation of the spirit as the key

As stated in the *Suwen* (Plain Questions) · *Bao Ming Quan Xing Lun* (Treatise on Preserving Life and Body), “The essence of acupuncture lies first in regulating the spirit; only after the five zang organs are stabilized and the nine pulse regions are assessed can the needle be applied.” When the physician concentrates fully and the patient relaxes with eyes closed, perceiving the needling sensation, the therapeutic effect can only be maximized through coordinated cooperation between physician and patient. The *Lingshu* (Miraculous Pivot) · *Da Huo Lun* (Treatise on Great Confusion) says, “The heart is the dwelling of the spirit.”

The Du Meridian “enters the collaterals of the brain” and “ascends to connect with the heart.” Li Shizhen also proposed that “the brain is the seat of the primordial spirit.” Baihui (GV20), located at the vertex, is the meeting point of the three yang meridians of the hand and foot with the Du Meridian, closely associated with brain function, and is a key point for regulating brain function. The Zhen Jiu Da Cheng (Great Compendium of Acupuncture and Moxibustion) states that it is indicated for “vexation, palpitations, forgetfulness, absent-mindedness, and mental disorientation,” highlighting its marked spirit-regulating effect. Yintang (EX-HN3), an extra point on the Du Meridian pathway, can be needled to refresh the brain, regulate the spirit, calm the heart, and enhance mental acuity. Neiguan (PC6) is the luo-connecting point of the Pericardium Meridian; the pericardial network serves as the outer guard of the heart, receiving pathogenic factors on its behalf. As a confluent point of the eight extraordinary vessels communicating with the Yinwei Meridian, it treats disorders of the stomach, heart, and chest. The influential point of sinews, Yanglingquan (GB34), is the lower he-sea point of the Gallbladder Meridian; “the he points treat the internal fu organs,” and it also serves as a pivotal point for regulating the Qi dynamic as the he point of the Gallbladder Meridian. The combination of Neiguan and Yanglingquan treats low back pain from the perspective of the heart and gallbladder, achieving effects of calming the mind, tranquilizing, alleviating pain, and relaxing the sinews and joints.

(3) “One acupuncture, two moxibustion, three consolidation”: integrating acupuncture and moxibustion to ensure therapeutic efficacy

The points for “guiding Qi back to the origin” (Yinqi Guiyuan: Zhongwan CV12, Xiawan CV10, Qihai CV6, Guanyuan CV4) are all points on the Ren Meridian, which can regulate the Qi of the yin meridians. Meanwhile, Zhongwan, Qihai, and Guanyuan are all front-mu points, which can regulate the Qi of the six fu organs. The Ren, Du, and Chong meridians share a common origin with three divergent pathways; where the meridians traverse, they primarily treat diseases of the lumbar and abdominal regions.

The refined moxibustion (Jing Jiu) technique is characterized by concentrated heat, rapid thermal penetration, short duration, and strong stimulation. One cone of refined moxibustion can achieve the effect of 2–3 cones of ordinary wheat-grain-sized moxibustion, hence its name, signifying its refined yet highly effective nature. Its functions include warming the meridians and dispersing cold, supporting yang and preventing desertion, eliminating stasis and resolving masses, and disease prevention and health maintenance. It exerts benign regulatory effects on the nervous, metabolic, endocrine, immune, respiratory, digestive, and circulatory systems, and has notable analgesic, sedative, and antidepressant effects. The Huangdi Neijing (Inner Canon of the Yellow Emperor) emphasizes that “where acupuncture is not suitable, moxibustion is indicated”; the Yixue Rumen (Introduction to Medicine) points out that “where medicine fails and acupuncture cannot reach, moxibustion must be applied,” highlighting the importance of moxibustion.

The term “Sihua points” (Four Flower Points), first recorded in Gao Wu’s Zhen Jiu Ju Ying (Gathered Gems of Acupuncture and Moxibustion) of the Ming Dynasty, refers to the paired acupoints Geshu (BL17) and Danshu (BL19). The Zhen Jiu Da Cheng (Great Compendium of Acupuncture and Moxibustion) states: “Master Cui’s method of selecting Sihua points treats five-taxation and seven-impairment disorders, deficiency of Qi and weakness of blood, bone-steaming tidal fever, cough, phlegm, dyspnea, emaciation, and chronic intractable diseases in both men and women”. The Suwen (Plain Questions) · Tiao Jing Lun (Treatise on Regulating the Meridians) says, “The most valuable constituents of the human body are blood and Qi”. When Qi is abundant, the spirit flourishes; when blood is abundant, the body is strong. When Qi and blood are disharmonious, all diseases arise. As a classical point combination, Sihua points involve Danshu

regulating liver Qi and facilitating the flow of Qi throughout the body, combined with Gesu nourishing and activating the blood; thus, they can treat both Qi and blood. Moreover, Gesu governs blood, pertaining to yin, while Danshu governs Qi, pertaining to yang, forming a complementary yin-yang and blood-qi pair. Since the low back is the residence of the kidneys, supplementing the fire of the congenital life gate allows them to be utilized synergistically and enhance each other's effects.

From the perspective of traditional TCM theory, the therapeutic effect of intradermal needles (thumbtack needles) applied to the cutaneous regions is due to their ability to stimulate the body's defensive Qi (wei Qi) through shallow needling. Wei yang represents the body's protection and barrier; shallow needling of the cutaneous regions can excite and mobilize the wei yang Qi located on the body's surface, causing it to rapidly gather at the site of pathogenic invasion to resist pathogens, protect the body, and treat disease. Thus, stimulation of the back-shu points (Xinshu BL15, Shenshu BL23) with intradermal needles can supplement heart blood to nourish the sinews and bones, and invigorate kidney yang to unblock collaterals and stop pain. It has the effects of moving wei Qi, unblocking fine collaterals through shallow needling, and nurturing wei yang through prolonged needle retention.

The Lingshu (Miraculous Pivot) · Kou Wen Pian (Treatise on Oral Inquiry) says, "The ear is where all the meridians converge," indicating its close connection with the zang-fu organs and meridians. Persistent stimulation of auricular points (Lumbar, Kidney) can fully leverage the endocrine regulatory effects of the brain-heart axis, thereby achieving the therapeutic accomplishment of treating the root of the disease and consolidating the curative effect.

6. Conclusion and prospects

The "Integrative Acupuncture and Moxibustion" tiered protocol is a summary of the experience of Professor Fu Wenbin, a renowned TCM practitioner from Guangdong Province, and his team. Professor Fu believes that clinical diseases are often challenging, complex, and severe in nature, making it difficult to achieve ideal therapeutic effects with acupuncture therapy alone. Adopting the TCM-characteristic integrative treatment model of "one acupuncture, two moxibustion, three consolidation" can yield more significant clinical efficacy^[5].

In summary, the development of acupuncture and moxibustion requires both inheritance and innovation. The expanding spectrum of multidisciplinary diseases presents acupuncturists with unprecedented opportunities and challenges. Whether we can empower the development of traditional medicine with high quality requires continuous exploration and summarization by our generation. The tiered protocol of integrative acupuncture and moxibustion deserves multidisciplinary promotion and application.

Disclosure statement

The author declares no conflict of interest.

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Bio-Byword Scientific Publishing remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Research Progress on the Mechanisms of m6A Methylation in Neurodegenerative Cognitive Disorders

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Abstract: *Objective:* To explore the core role of N6-methyladenosine (m6A) methylation in neurodegenerative cognitive disorders, with a particular focus on its contribution to disease progression through the regulation of common mechanisms such as neuroimmune inflammation, and to discuss its clinical translational prospects. *Methods:* A systematic review of recent research literature on m6A methylation in neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease, was conducted, integrating analyses from molecular mechanisms, cellular phenotypes, to systemic pathology. *Results and Conclusions:* As a key epitranscriptomic regulatory mechanism, m6A methylation precisely regulates neural synaptic plasticity, autophagic clearance, and neuroimmune homeostasis through a dynamic network comprising “writer”, “eraser”, and “reader” proteins. Its homeostatic imbalance represents a core pathological hub leading to cognitive dysfunction: on one hand, m6A dysregulation directly mediates the polarization of microglia towards a pro-inflammatory M1 phenotype, amplifying neuroinflammatory responses; on the other hand, it exacerbates neuronal injury by affecting the metabolism of pathological proteins such as Tau and α -synuclein. Targeting m6A regulatory enzymes (e.g., FTO inhibitors, METTL3 activators) has shown potential for improving cognitive function in preclinical models. Additionally, biomarkers like m6A in peripheral blood exosomes hold value for non-invasive diagnosis. In summary, m6A methylation serves as a critical link connecting common pathological mechanisms across various neurodegenerative diseases, offering broad translational application prospects in disease diagnosis and targeted therapy.

Keywords: m6A methylation; Neurodegenerative diseases; Cognitive impairment; Neuroimmune inflammation; Epitranscriptomics; Biomarkers; Targeted therapy

Online publication: August 12, 2026

1. Introduction

Neurodegenerative cognitive disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD),

represent major global public health challenges. Their core pathological features include pathological protein aggregation, progressive neuronal loss, and the increasingly recognized neuroimmune inflammatory response^[1,2]. Despite ongoing research, their pathogenesis remains incompletely elucidated, and effective disease-modifying therapies are lacking. Therefore, identifying common, modifiable early pathological nodes in these diseases is an urgent research priority.

Among numerous potential mechanisms, RNA epigenetics, particularly N6-methyladenosine (m6A) methylation, has rapidly emerged as a frontier topic in neuroscience^[4]. As the most prevalent and abundant internal chemical modification in eukaryotic mRNAs, m6A is not a static mark but a dynamic, reversible process co-regulated by methyltransferases (e.g., the METTL3/METTL14 complex, “writers”), demethylases (e.g., FTO, ALKBH5, “erasers”), and binding proteins (e.g., the YTHDF family, “readers”)^[14]. This “chemical modification–functional decoding” rapid regulatory mode endows m6A with unique spatiotemporal advantages in mediating neurobiological processes such as rapid synaptic remodeling, neurogenesis, and stress responses^[16,17].

Crucially, mounting evidence indicates that homeostatic imbalance of m6A methylation serves as a common key node driving neurodegenerative pathology. Studies have found characteristic alterations in m6A modification levels in disease models such as AD and PD, which directly participate in the onset and progression of cognitive dysfunction through multiple mechanisms, including regulating neuroimmune inflammatory pathways, affecting pathological protein metabolism, and impairing synaptic plasticity^[11,19]. For instance, m6A modification can specifically regulate the activation phenotype of microglia, driving their polarization towards a neurotoxic M1 state, thereby amplifying inflammatory cascades and accelerating disease progression^[25].

This review aims to systematically synthesize the latest research advances, providing an in-depth exposition, from the perspective of an “epitranscriptomic hub,” of the common pathological mechanisms of m6A methylation across various neurodegenerative cognitive disorders, with a particular focus on its core regulatory role in neuroimmune inflammation. Furthermore, we will discuss the development of diagnostic biomarkers and targeted therapeutic strategies centered on m6A, offering new insights and theoretical bases for early intervention in these diseases^[3].

2. Molecular mechanisms of m6A methylation and its neuroregulatory functions

2.1. Molecular regulatory network of m6A methylation

2.1.1. Enzymatic regulatory system of m6A modification

The formation of m6A modification is mediated by “writers”, such as the METTL3-METTL14 complex, where methyltransferase-like protein 3 (METTL3) and methyltransferase-like protein 14 (METTL14) form a heterodimer with catalytic function, specifically recognizing the RRACH sequence (a purine-rich conserved sequence) to catalyze methylation at the N6 position of adenosine^[7,8]. Demethylases (“erasers”), such as fat mass and obesity-associated protein (FTO) and AlkB homolog 5 (ALKBH5), maintain dynamic modification balance through oxidative demethylation. Notably, FTO exhibits an aberrant expression pattern in the brain tissue of AD patients, and changes in its activity significantly correlate with the efficiency of m6A demethylation and the degree of cognitive impairment^[9,10]. Animal model studies have found that in diabetes-related cognitive impairment, downregulation of ALKBH5 expression leads to abnormal Tau phosphorylation, suggesting that m6A homeostasis imbalance may be a significant mechanism through

which metabolic abnormalities trigger neurodegeneration^[11]. Additionally, environmental stressors such as hypoxia and neuroinflammation can reshape the m6A epitranscriptomic features of neurons by regulating the expression profiles of writers and erasers^[12,13].

The regulation of m6A modification is not limited to mRNA but also includes non-coding RNAs (e.g., lncRNA, circRNA) and pri-miRNA. m6A modifications on these non-coding RNAs also possess important regulatory functions in the nervous system, such as influencing miRNA maturation or circRNA stability, further expanding the breadth and depth of m6A regulation in epitranscriptomics.

2.1.2. Functional diversity of m6A reader proteins

YTH domain-containing family proteins (YTHDF proteins), as classical reader molecules, specifically recognize m6A modifications and regulate target RNAs. Among them, YTHDF1 promotes the translation efficiency of modified mRNAs, while YTHDF2 accelerates their degradation. This selective regulation exhibits spatiotemporal specificity in the nervous system^[14]. Particularly, YTHDF1 and YTHDF2 participate in the molecular regulatory network of synaptic plasticity and memory consolidation by regulating the expression programs of synapse-related genes. In AD models, dysfunction of the m6A-reader system has been observed, potentially affecting β -amyloid metabolic pathways and offering new intervention targets for therapy^[15]. Furthermore, studies have found that m6A-dependent translational regulation directly participates in the synthesis of memory-related proteins (e.g., ARC), solidifying its central role in higher cognitive functions^[16].

2.1.3. Precise regulation of gene expression mediated by m6A

m6A regulates gene expression through three primary mechanisms: (1) Structure-dependent degradation, altering RNA secondary structure to enhance nuclease sensitivity, e.g., YTHDF2-targeted degradation^[14]; (2) Translation activation, recruiting YTHDF1 to promote ribosome binding^[16]; and (3) Splicing regulation, affecting the binding sites of splicing factors, e.g., via hnRNP-dependent pathways^[16]. In cognitive function studies, overexpression of METTL3 upregulates the expression of neuroplasticity-related genes (such as BDNF and PSD95) and significantly improves cognitive behavioral performance in model animals. This epigenetic regulatory mechanism is also involved in neurogenesis, influencing the construction of neuronal networks by affecting the proliferation and differentiation of neural precursor cells.

2.2. Functions of m6A in the nervous system

2.2.1. Epigenetic regulatory programs in neural development

m6A modification participates in the regulation of neurogenesis in a spatiotemporally specific manner, dynamically and coordinately regulated by writers, erasers, and readers. In neural stem cells, METTL3-mediated m6A modification has been confirmed as a key determinant of the balance between proliferation and differentiation. Gene knockout experiments demonstrate that m6A deficiency leads to neural developmental arrest and cortical abnormalities^[17]. Mechanistically, METTL3-mediated m6A modification regulates histone methyltransferase Ezh2, influencing the eventual fate decision of neural stem cells towards neuronal or glial differentiation^[17]. Studies on environmental stimulation confirm that housing in an enriched environment promotes the proliferation of neural precursor cells by upregulating m6A modification levels (e.g., Sox2 mRNA methylation)^[18]. At the synaptic development level, the abundance of m6A modification in hippocampal neurons positively correlates with the expression of synaptic marker proteins like synapsin,

indicating its involvement in the molecular regulation of synaptic maturation. Notably, activity-dependent m6A modification rapidly responds to neuronal electrical activity, regulating the translation of immediate early genes (e.g., ARC), thereby providing an instantaneous regulatory mechanism for synaptic plasticity ^[18]. Experiments confirm that housing in an enriched environment significantly elevates m6A modification levels in neural stem cells, and this epigenetic reprogramming may be the molecular basis for environment-enhanced cognitive function.

2.2.2. Epigenetic regulatory mechanisms of synaptic plasticity

Synaptic plasticity, the molecular foundation of learning and memory, is exquisitely and precisely regulated by the m6A network. Electrophysiological studies confirm a correlation between the level of m6A modification and the strength of long-term potentiation (LTP) in the hippocampus ^[18]. Mechanistically, activation of N-methyl-D-aspartate receptors (NMDARs) induces remodeling of synaptic local m6A modifications, affecting synaptic strength by regulating the translational efficiency of key signaling molecules like calcium/calmodulin-dependent protein kinase II (CaMKII). Activity-dependent m6A modification, via YTHDF1-mediated translation activation, rapidly upregulates the expression of synaptic proteins (such as ARC, BDNF, PSD95), directly enhancing synaptic transmission efficacy ^[16,18]. In AD models, overexpression of METTL3 improves synaptic plasticity by restoring m6A-dependent translation of PSD95 ^[26]. Further studies indicate that METTL3 overexpression not only enhances PSD95 translation efficiency but also increases the density and maturity of dendritic spines, as validated by electron microscopy and confocal imaging. Additionally, METTL3-mediated m6A modification influences the expression of AMPA receptor subunits like GluA1, affecting excitatory synaptic transmission efficiency, thereby promoting synaptic functional recovery at both molecular and structural levels. In pathological states, brain tissue from AD patients exhibits global m6A modification dysregulation, and this abnormality significantly correlates with synaptic protein synthesis deficits and the extent of memory impairment ^[19].

3. Mechanisms of m6A methylation in cognitive dysfunction

3.1. Pathological features of m6A methylation in cognitive dysfunction

Within the molecular regulatory network, m6A dysregulation disrupts neural homeostasis, driving cognitive impairment, and its pathological features exhibit commonalities across related diseases.

Table 1. Pathological features of m6A in common cognitive disorders

Type	Core Pathology	m6A-Related Dysregulation Features
AD	A β Deposition & Tau Phosphorylation	Downregulated METTL3, Upregulated FTO ^[24]
Parkinson's Disease Dementia	α -synuclein Aggregation	Downregulated ALKBH5 leading to autophagy flux impairment ^[31]
Postoperative Cognitive Dysfunction (POCD)	Neuroinflammation	Decreased global transcriptomic m6A levels ^[5]
Diabetes-Related Cognitive Impairment	Tau Hyperphosphorylation	ALKBH5-DGKH pathway dysregulation ^[11]

3.2. Molecular mechanisms of m6A methylation mediating cognitive dysfunction

m6A dysregulation mediates cognitive impairment primarily through the following pathways:

- (1) Synaptic protein synthesis impairment, where YTHDF1 dysfunction hinders the translation of ARC and BDNF, leading to decreased synaptic plasticity ^[16,21];
- (2) Amplified neuroinflammation, as microglial m6A modification promotes M1 polarization, exacerbating A β -induced neurotoxicity ^[25];
- (3) Pathological protein accumulation, involving downregulated ALKBH5 leading to Tau hyperphosphorylation and METTL3 deficiency impairing α -synuclein clearance ^[11,31];
- (4) Metabolic-epigenetic interactions, e.g., hyperglycemia inhibits ALKBH5, blocking DGKH demethylation and ultimately leading to Tau pathology ^[11]. Additionally, dynamic changes in hippocampal m6A in aged animal models further confirm its role in cognitive decline ^[22].
- (5) Mitochondrial dysfunction and energy metabolism impairment: m6A modification affects mitochondrial dynamics and energy supply by regulating the expression of mitochondrial-related genes (e.g., Mfn2, OPA1). In AD models, METTL3 deficiency leads to reduced m6A modification and stability of Mfn2 mRNA, inducing mitochondrial fragmentation and decreased ATP synthesis, exacerbating neuronal energy crises and apoptosis.

4. Pathological roles of m6A methylation in major neurodegenerative cognitive disorders

4.1. Association of m6A methylation with Alzheimer's disease

In AD patients and transgenic animal models, m6A modification levels show significant changes in brain regions (e.g., hippocampus and cortex). Studies in amyloid precursor protein (APP)/presenilin 1 (PS1) transgenic mice confirm that global m6A methylation levels are significantly elevated in AD-related brain regions and positively correlate with pathological features such as A β deposition and neuronal loss ^[23]. Multiple core pathways are involved:

- (1) Synaptic dysfunction pathway: Reduced YTHDF1 expression in the hippocampus leads to dendritic spine loss ^[21];
- (2) Inflammation amplification pathway: Microglial m6A modification promotes IL-1 β release via Notch1 signaling, accelerating A β deposition ^[25];
- (3) Tau pathology pathway: FTO-mediated impairment of Tau mRNA demethylation exacerbates neurofibrillary tangle formation ^[9], collectively leading to cognitive decline ^[24]. Aberrant m6A modification also impairs neuronal survival by regulating the expression of synaptic plasticity-related genes (e.g., BDNF, SYP) and increases neuronal sensitivity to oxidative stress and inflammation. Recent studies also indicate that m6A modification affects A β -induced mitochondrial damage clearance by regulating the expression of mitophagy-related genes (e.g., PINK1, Parkin). METTL3 deficiency reduces m6A modification of PINK1 mRNA, decreasing its stability, thereby weakening mitophagy and accelerating neuronal senescence and death. The dynamic balance of m6A modification is co-regulated by writers (METTL3, METTL14) and erasers (FTO, ALKBH5). In AD, METTL3 expression is significantly downregulated, while FTO expression is upregulated ^[24]. Studies have found that altered m6A modifications can drive microglial polarization towards a pro-inflammatory phenotype (M1), exacerbating A β -induced neurotoxicity and worsening AD pathology ^[25].

Consequently, intervention strategies targeting m6A modification have shown promising therapeutic

prospects. For example, in AD mouse models, modulating METTL3 expression via pharmacological or genetic means has been shown to restore m6A homeostasis and improve synaptic plasticity and cognitive function based on the mechanisms described above (e.g., enhancing PSD95 translation, increasing dendritic spine maturity) ^[26].

4.2. Role of m6A methylation in other neurodegenerative diseases

Beyond AD, aberrant m6A modification is also associated with the pathogenesis of PD, amyotrophic lateral sclerosis (ALS), and other neurodegenerative diseases.

In PD, m6A dysregulation is closely linked to the apoptosis and dysfunction of dopaminergic neurons. Autophagy regulatory mechanism: METTL3 deficiency reduces m6A modification of LC3-II and Beclin-1, impairing α -synuclein clearance ^[31]. Oxidative stress pathway: FTO overexpression activates antioxidant pathways by demethylating Nrf2 mRNA, alleviating neuronal damage ^[10]. Additionally, m6A modification participates in regulating the expression of dopaminergic neuron-specific genes (e.g., TH, DAT). In PD models, METTL3-mediated m6A modification of TH mRNA enhances its translation efficiency, while aberrant FTO overexpression reduces TH protein synthesis, further exacerbating dopamine synthesis deficits and motor symptoms. Studies show elevated m6A methylation levels in the substantia nigra of PD patients, correlating with abnormal α -synuclein aggregation. METTL3 deficiency increases neuronal sensitivity to oxidative stress, while FTO overexpression can alleviate neuronal damage by reducing m6A levels.

In ALS, aberrant m6A modification is closely related to motor neuron degeneration. Studies confirm significantly elevated m6A methylation levels in the spinal cord and motor cortex of ALS patients, correlating with TDP-43 protein aggregation ^[27]. m6A modification influences neuronal survival through regulating autophagy and RNA metabolism. For example, increased m6A methylation inhibits the expression of autophagy-related genes, leading to abnormal protein accumulation and accelerated motor neuron death.

Furthermore, m6A modification plays a significant role in psychiatric disorders like depression. Altered m6A modification in the prefrontal cortex of depression patients may affect the expression of synaptic plasticity and neurotransmitter-related genes, contributing to cognitive dysfunction.

In summary, m6A methylation plays a crucial role in various neurodegenerative diseases, participating in disease initiation and progression through regulation of RNA metabolism, neuronal function, autophagy, and neuroinflammatory pathways.

5. Future research directions and clinical applications

5.1 Clinical application prospects of m6A methylation

5.1.1 Diagnostic and predictive value as biomarkers

Recent studies reveal characteristic changes in m6A-related molecules in neurodegenerative diseases, showing significant clinical translational potential. Non-invasive diagnosis: Serum exosomal m6A modification levels in AD patients are highly consistent with cerebrospinal fluid (CSF) findings ($r = 0.81$, $p < 0.001$), with exosomal YTHDF2 protein effectively distinguishing mild cognitive impairment (MCI) from AD stages ($AUC = 0.83$) ^[28,31]. Disease progression prediction: The co-expression pattern of m6A regulatory factors SNRPG/SNRPD2 can predict the risk of MCI-to-AD conversion ($HR = 3.21$) ^[4], while CSF m6A-modified circular RNAs (circRNAs) show 92% sensitivity for early AD detection ^[24], significantly outperforming the traditional A β 42/Tau ratio.

Technological innovations enabling precise subtyping: Single-cell nanopore sequencing technologies can now resolve m6A site maps of neuronal subpopulations, e.g., specific modifications of ARC mRNA in the hippocampal CA1 region ^[6,30]; microfluidic chips combined with electrochemical sensor platforms allow dynamic monitoring of m6A fluctuations in living CSF with a detection limit of 0.1 fM ^[32]. These advances lay the groundwork for establishing a “plasma exosome m6A profile - A β - PET - cognitive scale” multimodal diagnostic model, potentially enabling precise staging of AD.

In addition to peripheral blood and CSF, m6A markers in saliva and urine also show preliminary diagnostic potential. For example, m6A-modified circRNAs in salivary exosomes are significantly elevated in AD patients, offering convenient, non-invasive detection suitable for elderly population screening. Moreover, combining artificial intelligence-assisted imaging with m6A models could enable higher precision prediction of disease progression, providing a time window for individualized intervention.

5.2. Progress in targeted therapeutic strategies

Breakthroughs in targeting modification enzymes: The FTO inhibitor FB23-2 blocks pathological demethylation, improving spatial memory in manganese exposure models (35% increase in Y-maze correct rate) ^[10]; liposome-delivered METTL3 activators (SAM-Liposomes) can restore synaptic protein PSD95 translation (2.1-fold increase in expression) ^[26]; AAV-mediated ALKBH5 overexpression reverses high glucose-induced Tau hyperphosphorylation (60% reduction in p-Tau Ser396) ^[11].

Indirect regulatory strategies also show potential: The antioxidant N-acetylcysteine (NAC) can regulate the m6A-ATF4 axis, improving cognitive deficits in PTSD models (42% reduction in Morris water maze escape latency) ^[29]; multi-target synergistic interventions, e.g., combining an FTO inhibitor with a METTL3 activator, can both suppress A β production and enhance synaptic plasticity, increasing the novel object recognition index by 50% in APP/PS1 mice ^[26]. Recently, gene editing and epigenetic editing technologies (e.g., the dCas13b-m6A editing system) have been used to regulate the m6A modification level of specific mRNAs precisely. In PD models, the AAV-delivered dCas13b-ALKBH5 system successfully reduced m6A levels on α -synuclein mRNA, significantly alleviating its abnormal aggregation and neurotoxicity.

5.3. Challenges and strategies for clinical translation

Current translational research faces three core challenges:

- (1) Lack of detection standardization: Coefficient of variation (CV) >30% for m6A sequencing data across different platforms may lead to increased misdiagnosis rates.
- (2) Low blood-brain barrier (BBB) penetration: Small molecule inhibitor concentrations in the brain are typically less than 5% of peripheral doses, potentially causing peripheral toxicity.
- (3) Cell-specific effect conflicts: METTL3 activation protects synapses in neurons but promotes inflammation in astrocytes, potentially offsetting efficacy ^[26].

Furthermore, individual differences and disease heterogeneity represent significant issues in clinical translation. Genetic backgrounds, epigenetic states, and environmental exposures related to the m6A regulatory network vary considerably among patients, potentially leading to markedly different responses to the same intervention. Future efforts should integrate multi-omics data and real-world studies to establish personalized efficacy prediction models.

Counter-strategies focus on technological innovation and system optimization:

- (1) Establishing cross-center calibration protocols (e.g., using synthetic m6A-RNA standards) to promote detection standardization.
- (2) Developing exosome-encapsulated brain-targeted delivery systems (e.g., 8-fold increase in brain delivery efficiency for ALKBH5 inhibitors) ^[32].
- (3) Integrating multi-omics data (epitranscriptomics, proteomics, radiomics) to construct disease subtyping models, coupled with long-term safety assessments in non-human primates.
- (4) Utilizing artificial intelligence (e.g., AlphaFold-Epigen module) to predict associations between m6A modifications and phenotypes, ultimately achieving an individualized treatment model of “precise diagnosis – stage-based intervention.”

6. Conclusions and perspectives

m6A methylation serves as a key node in epitranscriptional regulation, participating in neural development, synaptic remodeling, and neuroinflammatory processes through dynamic regulation of RNA metabolism. Its homeostatic imbalance has been confirmed as a core pathological mechanism in neurodegenerative cognitive disorders. This review systematically integrates recent groundbreaking findings, for the first time revealing the common pathological hub of m6A dysregulation across AD, PD, POCD, and metabolic disease-related cognitive deficits. These discoveries deepen our understanding of the essence of cognitive impairment and also generate new strategies with translational potential.

In the diagnostic domain, non-invasive detection systems utilizing exosomal m6A profiles and CSF circRNA-m6A, combined with single-cell nanopore sequencing to resolve specific modification sites in hippocampal neurons, may achieve “pre-symptomatic staging”. Therapeutically, strategies targeting the m6A regulatory network, such as cross-target synergistic therapy, have shown groundbreaking advances. Clinical translation still faces challenges regarding molecular detection standardization, BBB penetration, and cell-specific effects, as discussed.

It is important to note that current m6A-targeted therapy research is primarily confined to animal models, and its safety and efficacy in humans require clinical validation. Future research should focus on constructing spatial epitranscriptomic maps, developing exosome-based drug delivery technologies, and AI-assisted design. Multi-center collaboration, integration of multi-omics data (epitranscriptomics, proteomics, radiomics), and long-term safety evaluations in primates are needed to advance m6A-targeted therapy from concept to clinical application, providing new avenues for the prevention and treatment of neurodegenerative cognitive disorders.

Disclosure statement

The authors declare no conflict of interest.

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Research Progress on the Role of PKM2 in Perioperative Neurocognitive Disorders in Elderly Patients

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Abstract: Perioperative Neurocognitive Disorders (PND) are common postoperative complications affecting cognitive and behavioral function in elderly patients, significantly impacting recovery quality. Its pathogenesis is closely linked to an imbalance in cerebral metabolic-immune homeostasis. Recent studies have identified the key metabolic and immune enzyme, Pyruvate Kinase M2 (PKM2), as a participant in the initiation and progression of PND. Surgical trauma and anesthesia can upregulate PKM2 via signaling pathways such as HMGB1, TLR4, and HIF-1 α , promoting its acetylation, dimerization, and nuclear translocation. Subsequently, activated PKM2 enhances oxidative stress and recruits NLRPs to drive neuroinflammation and synaptic plasticity decline, leading to postoperative cognitive dysfunction. These cascading changes, to a certain extent, affect postoperative neurological recovery. Mouse model studies have revealed therapeutic strategies targeting PKM2. This review summarizes the mechanisms by which PKM2 influences cognitive and behavioral function through the regulation of neuroinflammation and energy metabolism disorders, and discusses precise brain protection strategies targeting PKM2, offering new insights for the prevention and treatment of PND.

Keywords: Perioperative neurocognitive disorders; Elderly; Pyruvate kinase M2; Metabolic reprogramming; Targeted therapy

Online publication: August 12, 2026

1. Introduction

Perioperative neurocognitive disorder (PND) is a common complication in elderly surgical patients. The prevention and treatment of PND has become a major challenge affecting the postoperative survival and recovery of the elderly ^[1]. Aging reduces brain reserve capacity and systemic chronic inflammation (inflammaging) ^[2], increasing susceptibility to PND. Surgical and anesthetic stress acts as a “second hit”, activating the HMGB1-TLR4 pathway and exacerbating the PKM2-induced neuroinflammatory cascade

^[4,12]. This indicates that PKM2 connects the physiological characteristics of aging with perioperative neuroinflammatory responses. Currently, neuroinflammation and metabolic reprogramming are widely recognized as key processes in PND ^[3]. Accumulating evidence suggests that PKM2, possessing unique “metabolic-immune” features, plays a pivotal role in PND. Notably, anesthetics themselves can modulate PKM2: inhalational anesthetics like sevoflurane induce PKM2 activation and nuclear migration, while intravenous anesthetics like propofol help maintain its metabolic enzyme activity and suppress inflammation. This review aims to summarize recent basic research advances on the role of PKM2 in PND, focusing on its clinical translation pathways and precision intervention strategies, to provide new perspectives for perioperative brain protection.

2. Molecular properties of PKM2 and its functional hub role in the nervous system

PKM2 is a rate-limiting enzyme in the glycolytic pathway, catalyzing pyruvate metabolism. Its conformational plasticity and functional changes associated with different forms render it a critical nodal molecule integrating cellular energy and inflammatory responses ^[5]. Thus, PKM2 possesses both metabolic and immune functions, playing a significant role in PND development by transducing signals such as surgical trauma and anesthetic stress into downstream neuroinflammation and metabolic disorders, thereby triggering disease onset and progression.

2.1. Conformational plasticity of PKM2: Functional switching between tetramer and dimer

Under oxidative stress, inflammation, and other signaling stimuli, PKM2 undergoes various important post-translational modifications (including Ser37 phosphorylation, Tyr105 phosphorylation, and Lys433 acetylation) ^[6], promoting its dissociation from tetramers to dimers. The dimeric form can act as a co-activator, directly recruiting and stimulating transcription factors such as NF- κ B and STAT3, thereby amplifying inflammatory responses ^[5].

2.2. Cell-specific functions of PKM2 in the central nervous system

The expression and function of PKM2 in neural cells exhibit high specificity, constituting the cellular basis for its complex role in PND.

- (1) Microglia: Microglia highly express PKM2, which is a major determinant of their metabolic reprogramming and functional transformation. Polarization of microglia towards a pro-inflammatory M1 phenotype requires PKM2 activation, leading to the release of disease-promoting inflammatory molecules such as IL-1 β and TNF- α , which contribute to neuronal death and ignite a burst of microglia-mediated dynamic pro-inflammatory damage ^[8]. In experimental studies, knocking out PKM2 in microglia almost eliminated or prevented the production of other types of pro-inflammatory factors, significantly attenuating neuroinflammation by approximately 62%. ^[9]
- (2) Neurons: Under stress, neurons activate intracellular PKM2 dimers to carry out aerobic respiration, disrupting their reliance on glucose for energy supply and leading to the accumulation of large amounts of lactate, which contributes to cognitive deficits. This can result in reduced or even phagocytosed lactate at synapses and swelling (up to 55%) of postsynaptic densities due to lactate accumulation and disrupted synaptic calcium influx ^[10].

- (3) Blood-Brain Barrier (BBB): PKM2 dysregulation-mediated lactate accumulation can activate matrix metalloproteinase-9 (MMP-9), degrading tight junction proteins and compromising BBB integrity. Clinical studies show a significant positive correlation ($r=0.81$) between cerebrospinal fluid lactate levels and the BBB damage marker S100 β in PND patients¹¹¹¹, highlighting the central role of the “PKM2-lactate-MMP-9” axis in PND-associated BBB disruption.

3. Core mechanisms of perioperative stress regulating PKM2 to drive PND

Surgical trauma and anesthetic drugs, as the core perioperative stressors, converge on the key node PKM2 via different upstream signaling pathways, regulating its expression, conformation, and subcellular localization, thereby initiating the pathological cascade of PND.

3.1. Specific regulatory pathways of surgical trauma and anesthetics

3.1.1 Surgical trauma: The HMGB1-TLR4-NF- κ B axis upregulates PKM2 expression

Tissue injury releases high-mobility group box 1 (HMGB1), which promotes PKM2 transcription via the TLR4/MyD88/NF- κ B axis. Studies show that serum HMGB1 increases 3.3-fold, and hippocampal PKM2 mRNA and protein increase 2.1-fold and 1.8-fold, respectively, at 24 hours post-surgery, correlating with the timing of delirium onset ($r=0.73$, $P < 0.01$)¹¹³.

3.1.2. Inhalational anesthetic (Sevoflurane): The ROS-HIF-1 α axis promotes PKM2 nuclear translocation

Sevoflurane inhibits mitochondrial complex I (IC₅₀=0.34 mM), inducing a mitochondrial ROS burst (2.8-fold increase in DCF fluorescence intensity)¹¹⁴. ROS inhibits PHD activity, stabilizing HIF-1 α , which in turn enhances PKM2 transcription and recruits p300 for acetylation at the K433 site, promoting nuclear translocation (approximately 3.5-fold increase in nuclear PKM2, $P < 0.001$)¹¹⁵.

3.1.3. Intravenous anesthetic (Propofol): Antioxidant effects maintain PKM2 metabolic homeostasis

Compared to sevoflurane, propofol, possessing more phenolic hydroxyl groups, significantly reduces ROS (78% $\pm$ 6% 2-OH clearance level), inhibiting the initiation of the ROS-HIF-1 axis at its source.¹¹⁶ Concurrently, propofol inhibits the Raf-1/ERK signaling pathway, reducing Ser37 phosphorylation of PKM2 by 59%¹¹⁷. The combined effect promotes the retention of PKM2 in its fully active, soluble tetrameric form, ensuring normal glycolysis and thereby reducing hippocampal lactate accumulation by 55%, mitigating hippocampal neuronal death or neuroinflammation-associated brain edema, and exerting neuroprotective effects¹¹⁸. This may partially explain why sevoflurane anesthesia is more likely to induce postoperative delirium (POD) compared to propofol (34.5% vs. 19.2% incidence, $P=0.003$)¹¹³.

3.2. Spatiotemporal-specific regulation of PKM2 and PND subtype evolution

PKM2's role in the PND process exhibits a clear temporal sequence, driving the progression of different clinical subtypes. In the early postoperative period (0-6 hours), NF- κ B binds to the PKM2 promoter, rapidly upregulating its expression and initiating inflammatory pathways like TLR4/MyD88, marking the inflammatory initiation phase¹³¹³. Subsequently (6-24 hours), PKM2 undergoes nuclear translocation,

acting as a co-activator for STAT3 and NF- κ B, forming a positive feedback loop that significantly promotes the release of pro-inflammatory cytokines like IL-1 β and M1 polarization of microglia. This phase is closely associated with the development of postoperative delirium (POD)^[8, 26]. From 24 to 72 hours post-surgery, cytosolic PKM2 dimers dominate, leading to aberrant glycolytic flux and lactate accumulation, which activates MMP-9, disrupts the BBB, and causes cognitive fluctuations, corresponding to delayed neurocognitive recovery (DNCR)^[11, 21]. If metabolic disturbances persist (>72 hours), severe lactic acidosis and mitochondrial damage ensue, leading to a sharp decline in ATP synthesis and ultimately impairing synaptic plasticity, potentially progressing to postoperative neurocognitive disorder (PNCD)^[7, 18].

4. Specific mechanisms of PKM2 in PND: In-depth differentiation from AD and therapeutic implications

Although both PND and Alzheimer's disease (AD) involve cognitive impairment, the roles of PKM2 differ fundamentally, offering unique insights for targeted PND therapy. First, the triggers differ: PND is acutely induced by perioperative stress (surgery/anesthesia), whereas AD is driven by chronic A β /tau accumulation. Second, the kinetics of PKM2 activation differ markedly: PKM2 activation in PND is transient and self-limiting (peaking within 72 hours post-surgery), while in AD, it is persistent and progressive^[9]. Third, the core molecular modifications and cellular targets differ: PND is characterized by transient phosphorylation/acetylation primarily driving acute neuroinflammation via M1 polarization of microglia^[8, 20]; AD favors sustained A β -induced K433 acetylation, affecting both neurons (e.g., tau phosphorylation) and microglia. Finally, and most importantly, is the "reversibility" of the condition: the pathological state in PND, especially PKM2-mediated synaptic damage and inflammation, is essentially reversible, with functional recovery possible after drug withdrawal or intervention; AD pathology is largely irreversible^[9, 22]. These fundamental differences dictate distinct therapeutic strategies: PND focuses on "corrective" intervention during the acute window to break the PKM2-driven pathological cascade, whereas AD requires long-term "maintenance" therapy. Therefore, targeting PKM2 represents a more promising and time-sensitive strategy for PND.

4.1. Core pathway of neuronal injury: PKM2-mediated metabolic-synaptic axis dysregulation

In PND, neuronal injury does not arise from protein aggregation akin to AD but from acute functional disruption of the "metabolic-synaptic axis" due to PKM2 dysregulation.

- (1) Metabolic Initiation: Perioperative stress promotes PKM2 dimerization, disrupting glycolytic homeostasis in hippocampal neurons and leading to abnormal lactate accumulation.
- (2) Cascade Amplification: Accumulated lactate activates MMP-9, disrupting the BBB (forming a "metabolic-barrier disruption" positive feedback) and causing intracellular acidosis, inhibiting mitochondrial respiratory chain complex function and severely reducing ATP synthesis (by 72%)^[18, 21].
- (3) Functional Impairment: ATP depletion impairs synaptic structure and function, reducing PSD-95 protein cluster density and the success rate of LTP induction^[7].

4.2. Theoretical innovation and clinical implications: Why PKM2 is a more promising target for PND

Based on the aforementioned mechanistic differences between PND and AD, PKM2 emerges as a more

promising intervention target for PND due to three key advantages:

(1) Its acute, transient activation provides a clear intervention window (e.g., the 72-hour postoperative golden period), more amenable to short-term, potent intervention to break the acute pathological cascade compared to the chronic, persistent, and difficult-to-target processes in AD.

(2) The synaptic damage and inflammation in PND are inherently reversible, meaning that interventions targeting PKM2 can potentially “reset” the system and restore metabolic and synaptic function, whereas AD’s pathological accumulation and epigenetic changes are largely irreversible, limiting treatment efficacy.

(3) These characteristics underscore the fundamental difference in therapeutic strategies: PND focuses on “corrective” treatment to break the acute cascade, while AD necessitates long-term, safe “maintenance” therapy, making the drug development path for targeting PKM2 clearer and more feasible. Leveraging these advantages, utilizing biomarkers of PKM2 activation status (such as lactate, HMGB1, imaging markers) as entry points for precision medicine holds promise for accurate diagnosis, stratification, and individualized intervention in high-risk PND patients.

5. PKM2-targeted intervention strategies: From molecular mechanisms to clinical translation prospects

Currently, intervention strategies targeting PKM2 are primarily categorized as follows. They show promise in preclinical studies but face distinct challenges.

- (1) Small Molecule Inhibitors (e.g., Shikonin and its derivative SK-102): These covalently bind to the Cys424 site of PKM2, effectively inhibiting its dimerization and nuclear translocation. Studies show these strategies can significantly improve cognitive function, increasing the Neurological Recovery Index (NRI) by 35% and reducing escape latency in the Morris water maze by 31-38% ^[23]. They offer potent, rapid action but face challenges such as hepatotoxicity (e.g., a 200% increase in ALT) and low brain penetration (only 0.28). Recent research focuses on prodrug modifications (e.g., CPP-ACP) to reduce systemic toxicity ^[24].
- (2) Allosteric Activators (e.g., TEPP-46): These adopt a different approach by stabilizing the tetrameric form of PKM2 to maintain metabolic homeostasis. This strategy effectively restores hippocampal synaptic plasticity (75%±8% LTP recovery) and reduces the microglial activation marker Iba-1 by 55% ^[22]. These agents exhibit a better safety profile but are limited by ineffectiveness in AD models and limited efficacy against already nuclear-translocated PKM2. Combination strategies with anti-inflammatory drugs are currently being explored.
- (3) Gene Silencing Techniques (e.g., LNP-based siRNA Delivery Systems): These aim to address the root cause by specifically degrading PKM2 mRNA to reduce protein expression. Preclinical models show a silencing efficiency of up to 80% in the hippocampus ^[25]. Advantages include high target specificity and durable effect, but delivery efficiency and long-term safety remain unknown. Future strategies involve antibody-modified LNPs to enhance targeting towards microglia ^[25].
- (4) Natural Products and Derivatives (e.g., Shikonin derivatives): These are also attracting interest due to their multi-target effects and wide availability, improving neuroinflammation and oxidative stress. However, their precise mechanisms of action are often unclear, and their pharmacokinetics are complex, necessitating structural optimization for enhanced selectivity and bioavailability.

5.1. Clinical translation pathways and precision treatment

Translating the above strategies from bench to bedside requires addressing three key issues:

- (1) Biomarker-Guided Patient Stratification: Utilizing genetic markers (ApoE ϵ 4), other fluid biomarkers (PKM2 dimer, HMGB1, lactate) ^[19], or imaging markers (PET) for screening and stratifying high-risk groups.
- (2) Clinical Endpoints and Efficacy Evaluation: Phase II clinical trials could employ composite endpoints, including changes in 30-day postoperative MoCA scores, 90-day dementia conversion rate, 7-day delirium incidence, and changes in imaging biomarkers.
- (3) Combination Therapeutic Strategies: Combining PKM2 inhibitors with anti-inflammatory agents (e.g., IL-1 receptor antagonists), or PKM2 activators with neurotrophic agents (e.g., BDNF enhancers), presents potential for synergy.

5.2. Challenges and perspectives of PKM2-targeted intervention in the elderly

Due to age-related declines in liver and kidney function in elderly patients, adverse effects related to drug metabolism and tolerance are expected to increase. For instance, compounds with known hepatotoxicity (e.g., shikonin) require significant attention; developing novel prodrugs or delivery vector systems to reduce systemic exposure will be a major focus. Furthermore, comorbidities like diabetes can directly impact PKM2-targeted therapy. Hyperglycemia can directly modify PKM2 (e.g., O-GlcNAcylation) and may render patients less sensitive to certain inhibitors, necessitating patient stratification strategies ^[28]. Finally, the high heterogeneity among the elderly underscores the importance of utilizing key clinical biomarkers (e.g., preoperative HMGB1, lactate, ApoE ϵ 4 genotype) for individualized intervention. In the future, integrating PKM2-related biomarkers into comprehensive geriatric assessment frameworks may facilitate preoperative identification of high-risk PND patients and synergize with other perioperative geriatric management strategies (e.g., prehabilitation, multimodal analgesia) to achieve personalized brain protection.

6. Future research challenges and directions: Towards precision perioperative brain protection

6.1. Intelligent delivery systems

Utilizing engineered exosomes (e.g., CD47 ligand-modified) ^[19] or bacterial vectors for microglia-targeted delivery

6.2. Real-time intraoperative dynamic monitoring: From biomarkers to precision anesthesia management

Developing PKM2 activity monitoring methods based on Surface-Enhanced Raman Scattering (SERS) technology, offering pM-level sensitivity and conformational discrimination capabilities ^[26]. This requires advancing SERS probe clinical translation, developing portable devices, and establishing real-time warning-intervention closed-loop systems. Preliminary studies suggest this strategy could reduce delirium risk by 40% ^[27].

6.3. Precision medicine: Building risk prediction models and individualized dosing

Individual genetic backgrounds and underlying diseases significantly influence the efficacy and safety of PKM2-targeted interventions.

- (1) Integrating Multi-Omics: Integrating multi-omics data (e.g., genomics, proteomics, inflammatory cytokine profiles) with clinical parameters (e.g., age, medical history, blood glucose) using machine learning algorithms to build PND risk prediction models.
- (2) “Modification-Insensitive” Drug Development: Hyperglycemia in diabetic patients may induce O-GlcNAc modification of PKM2, leading to insensitivity to inhibitors ^[12]. Novel PKM2 modulators that can overcome or are unaffected by common pathological modifications need to be developed to suit complex clinical metabolic environments.

7. Conclusions and perspectives

This review explored the role of PKM2 in PND in the elderly, elucidated the “surgical trauma-anesthetic stress-PKM2-neuroinflammation” axis, and analyzed the similarities and differences between PND in the elderly and AD. As a metabolic-immune hub, PKM2 offers a potential precision strategy for perioperative brain protection in elderly surgical patients. Future research can leverage new-generation medical technologies, utilizing detection methods such as exosomes and SERS probes, to establish intraoperative monitoring-treatment optimization closed-loop feedback cycles. This will propel the precision development of perioperative medicine for elderly patients, ultimately achieving precise perioperative brain protection and improving postoperative cognitive and behavioral outcomes.

Disclosure statement

The authors declare no conflict of interest.

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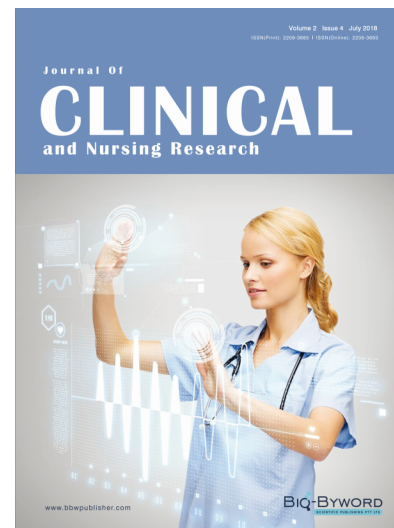
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