



Exploring the significance of IgM expression in the lymphatic system during breast cancer progression

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Background: Breast cancer is a malignant tumor characterized by metastasis. It first spreads through the lymphatic system to lymph nodes of various levels and can subsequently metastasize to distant organs via the bloodstream. Therefore, identifying protein biomarkers associated with breast cancer is of great significance for predicting disease progression, implementing early drug therapy or interventions, and improving prognosis. This study aims to investigate the dynamic changes in immunoglobulin M (IgM) levels in lymphoid tissues and lymph fluid during the progression of breast cancer and to perform quantitative analysis using experimental methods.

Methods: Sixteen purebred female New Zealand White rabbits were selected from the Animal Experimentation Center of Ningxia Medical University to establish a breast cancer lymph node metastasis model. Lymph node implantation was performed on the experimental group, while a normal control group and a breast cancer control group were established. Lymph fluid was collected periodically from rabbits in each group, and breast tumor and sentinel lymph node (SLN) tissues were obtained at the end of the experiment. An enzyme-linked immunosorbent assay (ELISA) was used to analyze differences in IgM protein expression among different tissue groups, and immunohistochemical experiments were conducted to detect changes in Ki-67 expression. Proteomics techniques were employed to identify differentially expressed proteins in the lymphatic fluid of each group, and bioinformatics analysis was performed using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to screen for differentially expressed proteins and the relevant pathways in which they are involved.

Results: The expression of IgM proteins undergoes dynamic changes during the progression of breast cancer. ELISA results showed that IgM protein expression differed significantly among tissue groups ($P \leq 0.05$). Immunohistochemical analysis revealed significantly elevated Ki-67 expression in lymph node metastasis tissues ($P \leq 0.05$). Proteomic analysis identified a total of 270 differentially expressed proteins, including 4 significantly altered IgM-related proteins. These differentially expressed proteins are primarily involved in cellular metabolism and signaling pathways, such as the “NF- κ B signaling pathway”, “complement and coagulation cascade”, and “B-cell receptor signaling pathway”.

Conclusions: Through comparative analysis of lymphatic fluid and lymphoid tissue across different groups, this study identified multiple differentially expressed proteins in a rabbit model of breast cancer lymphatic metastasis. Currently, the specific associations of these proteins with breast cancer metastasis and proliferation remain unclear. The study suggests that IgM-related differentially expressed proteins warrant further attention, and their potential significance in breast cancer diagnosis awaits verification in subsequent studies.

Keywords: Breast cancer; immunoglobulin M protein (IgM protein); sentinel lymph node (SLN); lymph node metastasis; proteomics

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Introduction

Breast cancer accounts for approximately one-third of all malignant tumors in women, with a mortality rate of about 15% among confirmed cases (1,2). The global distribution of breast cancer is influenced by the complex interplay of multiple factors, including genetics, environment, and lifestyle. Despite decades of decline in mortality due to advances in early detection and treatment, breast cancer remains the second leading cause of cancer death among women (3). Breast carcinogenesis is a multistep process involving a series of genetic and environmental events that drive the transformation of normal cells through stages such as hyperplasia, precancerous lesions, and carcinoma *in situ* (4). For early-stage breast cancer, the 10-year

survival rate for breast-conserving surgery combined with radiotherapy is 80.9%, superior to mastectomy (67.2%). Targeted therapies and immunotherapies show promise in triple-negative breast cancer (TNBC), but challenges persist due to its immunosuppressive microenvironment. Despite these advances, metastasis—particularly to the lungs, bones, and liver—remains the leading cause of death, driven by epithelial-mesenchymal transition (EMT) and immune evasion mechanisms (1,5,6). Therefore, identifying breast cancer-associated biomarkers early and initiating timely treatment are crucial for reducing mortality from metastatic disease progression.

Lymphatic vessels serve not only as a transport system for fluids and cells but also as a vital component of the immune system (7,8). Lymph generation occurs primarily through two mechanisms: first, significant interstitial edema (i.e., fluid accumulation resulting from extravasation during normal capillary circulation); second, altered mechanical stress on the extracellular matrix, including pressure effects generated by physiological activities such as respiratory movements, arterial pulsations, and skeletal muscle contractions. In this process, anchoring filament microfibrils connecting lymphatic endothelium to the extracellular matrix play a crucial role; when the aforementioned mechanical stimuli induce matrix deformation, these specialized fibers promote the opening of primary lymphatic vessels through traction effects (9). Lymph nodes (along with other secondary lymphoid organs like the spleen) serve as the foremost sites where immune cells exchange information, reside, proliferate, and initiate adaptive immune responses. By draining interstitial fluid and transporting any potential antigens to lymph nodes, the lymphatic system optimizes immune responses. This allows circulating antigen-specific lymphocytes to patrol homologous antigens across all lymph nodes without requiring direct sampling of peripheral tissues. Therefore, lymphatic vessels can also be regarded as the “sensory” organs of the immune system, with their drainage function primarily serving as a pathway for sampling tissue fluid from peripheral and central tissues, along with all antigens and related proteins contained therein (10,11). The lymphatic system acts as an

Highlight box

Key findings

- In this study, using a rabbit model of lymph node metastasis in breast cancer, we found that immunoglobulin M (IgM) protein expression undergoes dynamic changes as the disease progresses and exhibits significant differences across different tissue groups ($P \leq 0.05$). Ki-67 expression was significantly elevated in metastatic lymph node tissues ($P \leq 0.05$). Proteomics analysis identified a total of 270 differentially expressed proteins, including 4 significantly altered IgM-related proteins, which were primarily enriched in signaling pathways such as NF- κ B, the complement and coagulation cascades, and the B-cell receptor pathway.

What is known and what is new?

- Breast cancer primarily metastasizes through the lymphatic system; therefore, identifying relevant protein biomarkers is crucial for predicting disease progression and improving prognosis.
- In a rabbit model, we conducted a systematic analysis of the protein expression profiles in lymphatic fluid and lymphoid tissues, quantitatively confirmed the dynamic changes in IgM levels, and identified several relevant signaling pathways.

What is the implication, and what should change now?

- This study suggests that IgM-associated differential proteins may be involved in lymphatic metastasis in breast cancer and hold promise as new diagnostic markers or therapeutic targets. Further validation using clinical samples and functional mechanism studies are needed to clarify their specific roles and provide a basis for early intervention.

interface between innate and adaptive immunity, actively communicating and sensing inflammatory stimuli from the periphery. Lymphatic metastasis is the primary route of metastasis in breast cancer, and lymph node metastasis is closely associated with lymphatic circulation (12). Tumor-associated lymphatic vessels serve as direct conduits from the primary tumor to lymph nodes, enabling the primary tumor to transmit cytokine signals that progressively remodel and hijack lymph node function from a distance (13). Lymph fluid, as a key component of the lymph node microenvironment, undergoes corresponding alterations in its composition—including cytokines and antibodies—in response to changes within the tumor microenvironment (TME) (14).

Immunoglobulin M (IgM) protein is the first antibody to appear during embryonic development and humoral immune responses, it forms the body's first line of defense, responsible for identifying and eliminating infectious particles while also clearing transformed cells (15,16). Most breast cancer research has focused on developing IgG-like molecules as biomarkers or for late-stage cancer treatment, but autoantibodies (IgM) and tumor-associated antigens (abnormally structured proteins or carbohydrates) are yet to be examined as early diagnostic tools for breast cancer. IgM exists in two forms: native IgM, present in organisms without prior antigen exposure and constituting part of the first line of defense; and adaptive IgM, which emerges after immune challenge. Native IgM plays significant roles in maintaining tissue homeostasis, promoting phagocytic clearance of apoptotic cells, and preventing infectious and autoimmune diseases. It also exhibits notable functions in recognizing and eliminating precancerous and cancerous cells (17). Adaptive IgM proteins are the first antibodies to appear after immune challenge, characterized by monoreactivity and high affinity (17,18). Adaptive immunity serves as the primary protective mechanism against most infections and enveloped bacteria, and provides the host with protective immunity against malignant tumors (19). IgM is initially produced and then undergoes isotype switching to IgG. Therefore, the aim of this study was to explore dynamic changes in IgM expression within lymphatic fluid and lymphoid tissues during breast cancer progression using a VX2 rabbit model, and to preliminarily characterize IgM-related proteins through proteomic analysis.

The VX2 Breast Cancer Lymph Node Metastasis Rabbit Model is easy to operate, minimally invasive, and

characterized by rapid tumor growth. It effectively mimics the postoperative conditions of human breast cancer, providing an experimental animal model for exploring new therapeutic approaches for patients with lymphatic metastasis or recurrent metastatic breast cancer. We present this article in accordance with the ARRIVE reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-2025-1-2755/rc>).

Methods

Experimental animals and grouping

Common-grade purebred female New Zealand White rabbits (female, $n=16$; 6 months old), weighing 2.0–2.5 kg were purchased from the Ningxia Medical University Laboratory Animal Center (hereinafter referred to as rabbits).

The rearing conditions for rabbits are as follows: temperature: 18–29 °C (64–84 °F); relative humidity: 40–70%; noise level: ≤ 60 decibels; animal lighting: 100–500 lx; adequate food and water supply to meet the rabbits' physiological needs.

The experimental groups were primarily divided into four categories: the normal rabbit group (Control group, CG), the breast cancer positive control group (BCCG), the breast cancer lymph node metastasis persistently positive group (PPG), and the breast cancer lymph node metastasis positive to negative group (PTNG). The CG served as the normal control group, the BCCG as the experimental control group, and the remaining groups were collectively designated as experimental groups.

Sixteen rabbits were assigned to groups using a completely randomized design: All rabbits were numbered first, then random numbers were generated using a random number table. These random numbers were sorted in ascending order, and rabbits were assigned to groups sequentially. Numbers one to three constituted the normal rabbit group, numbers four to six formed the breast cancer positive control group, and numbers seven to 16 comprised the experimental group; then randomly distribute the 16 rabbits among different cages. No one other than the experimenters knew the experimental group assignments.

Sixteen rabbits were randomly assigned to three groups: the normal rabbit group comprised three rabbits, the breast cancer positive control group comprised three rabbits, and the experimental group comprised a total of 10 rabbits.

Establishment of the VX2 breast cancer lymph node metastasis rabbit model

The tissue block implantation method for establishing the VX2 breast cancer lymph node metastasis model features minimal trauma, high success rates, and a high incidence of lymph node metastasis.

Select two male tumor-bearing rabbits (purchased from Shanghai Yinxi Biotechnology Co., Ltd., Shanghai, China). Euthanize the rabbits via intravenous injection of sodium pentobarbital (100 mg/kg). Under sterile conditions, dissect the tumor tissue and mince it with ophthalmic scissors to prepare a 0.5–1 mm³ tumor tissue suspension for modeling.

Female rabbits in the experimental group were placed in a supine position using restraints and anesthetized with inhaled isoflurane (induction concentration: 2%; maintenance concentration: 1.5%) for two minutes. Injection was performed once corneal reflexes diminished, muscle relaxation occurred, or respiration slowed. At the site of the third left mammary gland, a 50-mL syringe needle was used to withdraw 10 mL of VX2 tumor-bearing tissue suspension. The needle tip was positioned beneath the mammary gland for subcutaneous injection. After injection, the needle was slowly withdrawn while applying pressure to the injection site for five to 10 seconds. Tumor implantation was completed once the skin wheal subsided. Tumor development and lymph node changes were observed daily. Humanitarian endpoints: Immediate euthanasia is performed if uncontrollable pain or other critical conditions arise during the experiment. Tumor size is monitored throughout the study; if tumor volume exceeds 10% of the experimental rabbit's body weight, the experiment is terminated promptly. The *in vivo* experiments in this study were performed under a project license (approval No. KYLL-2022-0173) granted by Medical Research Ethics Review Committee of General Hospital of Ningxia Medical University, in compliance with the Experimental Animal Center of Ningxia Medical University guidelines for the care and use of animals.

Fine-needle aspiration pathology slide staining

At weeks five and 10, the experimental group and BCCG patients were positioned supine to fully expose the left axilla and left third nipple. Following thorough disinfection, a five milliliters syringe was used to perform real-time ultrasound-guided multiple puncture aspirations in different directions within the lesion tissue. The aspirated tissue was smeared

and examined under a microscope.

Ultrasound contrast and image acquisition

- (I) Inject the ultrasound contrast agent Sonazoid (perfluoropropane microspheres for injection, GE Healthcare) at the tumor margin of female rabbits. Real-time observation reveals the first lymph node enlargement along the lymphatic vessels from the injection site, indicating sentinel lymph node (SLN) metastasis and confirming successful model establishment.
- (II) Following model establishment, measure the size of mammary tumors and lymph nodes in the experimental group and acquire images weekly until the conclusion of the experiment. The same researcher measured the sizes of mammary tumor tissues and SLN tissues in rabbits from each group during ultrasound measurements, without employing a blinded approach.

Lymphatic fluid collection

Lymph node intervention therapy commenced two weeks after establishing the VX2 breast cancer model and continued for eight consecutive weeks. Guided by contrast-enhanced ultrasound (CEUS) technology (commonly referred to as ultrasound contrast imaging in China), SLN lymphatic fluid was extracted according to group assignments and stored at –80 °C.

ELISA experiment

After 10 weeks, collect breast tumor tissue and SLN tissue from rabbits in each group. Thoroughly homogenize the mammary tumor tissue and lymph nodes in PBS (pH 7.4). Centrifuge the homogenate at 5,000 ×g for 10 minutes, collect the supernatant, and detect using a rabbit IgM Enzyme-linked immunosorbent assays (ELISA) detection kit (MiaoNuo Biological Reagent Sales Department). In the Excel worksheet, plot the standard curve with the standard concentration on the x-axis and the corresponding OD values on the y-axis, using the blank well as well 0. Calculate the concentration values for each sample based on the curve equation. Each sample is tested in triplicate. Quantitative analysis during ELISA testing was performed by the same researcher using a blinded method (i.e., the operator was unaware of sample grouping information).

Immunohistochemistry experiment

Ten weeks later, tissue samples of mammary tumors and SLN were collected from rabbits in each group. Specimens were fixed in 4% paraformaldehyde and routinely embedded in paraffin.

After routine dewaxing of sections to water, perform antigen retrieval: place sections in citrate retrieval solution (pH 6.0) and conduct heat-induced antigen retrieval in an autoclave. Continue retrieval for 10 minutes after the pressure valve opens, then allow to cool naturally to room temperature. Rinse with distilled water for five minutes. To block endogenous peroxidase activity, sections were incubated at room temperature in three percent methanol hydrogen peroxide solution (protected from light) for 20 minutes. After washing with distilled water for five minutes, sections were immersed in PBS buffer (pH 7.4) for 1 minute. Subsequently, add five percent bovine serum albumin (Wuhan BST, Wuhan, China; Cat. No. AR0004) and block at room temperature for 30 minutes to block non-specific binding sites.

After blocking, discard the blocking solution and add the primary antibody working solution. The primary antibody used is the Ki-67 monoclonal antibody (catalog number ab16667, clone SP6, Abcam, Shanghai, China), which exhibits broad species cross-reactivity (including rabbit). Incubate overnight at four degrees Celsius in a humidified chamber at a dilution of one: 150. Wash three times with PBS buffer for 5 minutes each. Subsequently, add the secondary antibody, horseradish peroxidase-labeled goat anti-mouse/rabbit IgG polymer (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China; catalog No. PV-6000), and incubate at 37 °C for 20 minutes. Wash again three times with PBS buffer for five minutes each. Finally, develop color at room temperature using the DAB (dihydroxybenzidine) color development kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Cat. No. ZLI-9018). Control the development time under a microscope for approximately two to three minutes until a brownish-yellow positive signal appears with no nonspecific staining in the background. Terminate staining with tap water. Counterstain sections with hematoxylin solution (Zhuhai Bixin Biotechnology Co., Ltd., Zhuhai, China; Cat. No. BA-4041), differentiate with hydrochloric acid-ethanol, blue with tap water, dehydrate with graded ethanol, clear with xylene, and mount with neutral resin.

Examine each slide under low magnification, avoiding areas of tissue necrosis, folding, and non-specific staining

at the edges. Subsequently, using K-Viewer image viewing software, randomly select six non-overlapping high-power fields (×20) per slide. Simultaneously capture images at ×10, ×20, and ×40 magnifications for verification. Quantitative analysis is based on the ×20 field images. Semi-automated quantification is performed using the immunohistochemistry (IHC) Profiler plugin within the ImageJ image analysis system (NIH, USA). The system automatically identified brown-yellow positive cell nuclei and calculated the average optical density (AOD) value. The average positive rate across the six fields per slide constituted the final Ki-67 proliferation index for each sample. To minimize subjective bias, image acquisition and quantitative analysis were performed blinded by the same investigator throughout the process (i.e., the operator was unaware of sample grouping information).

Proteomics analysis

Protein extraction: a total of 10 samples were processed. An appropriate volume of SDT lysis buffer was added to each sample, transferred to an EP tube, then subjected to boiling water bath treatment for three minutes, followed by ultrasonic disruption for two minutes. Centrifugation was performed at four degrees Celsius, 16,000 g for 20 minutes. The supernatant was collected and protein quantification was performed using the BCA method. Protein quantification data for each sample is detailed in the quality control report. For each sample group, an appropriate amount of protein was diluted five: one (v/v) with 5X loading buffer, boiled for five minutes, and subjected to eight to 16 percent SDS-PAGE electrophoresis.

Protein digestion: for each sample, an appropriate amount of protein was subjected to FASP digestion as follows: Add DTT to 100 mM in each sample, boil for 5 min, then cool to room temperature. Add 200 µL UA buffer (eight M Urea, 150 mM Tris-HCl, pH 8.0), mix thoroughly, transfer to a 10 kDa ultrafiltration centrifuge tube, and centrifuge at 12,000 g for 15 min. Add 200 µL UA buffer, centrifuge at 12,000 g for 15 min, and discard the filtrate. Add 100 µL IAA (50 mM IAA in UA), vortex at 600 rpm for 1 min, incubate at room temperature in the dark for 30 min, centrifuge at 12,000 g for 10 min. Add 100 µL UA buffer, centrifuge at 12,000 g for 10 min. Repeat twice. Add 100 µL NH₄HCO₃ buffer, centrifuge at 14,000 g for 10 min. Repeat twice. Add 40 µL Trypsin buffer (6 µg Trypsin in 40 µL NH₄HCO₃ buffer), vortex at 600 rpm for 1 min, incubate at 37 °C for 16–18 h. Transfer

to a fresh collection tube, centrifuge at 12,000 g for 10 min. Collect the supernatant. Desalt the enzymatically digested peptides using a C18 Cartridge, then freeze-dry under vacuum. Resuspend the desalted peptides in 0.1% formic acid (FA) after drying. Determine the peptide concentration for LC-MS analysis.

Data-Independent Acquisition (DIA) Mass Spectrometry Data Acquisition: an appropriate amount of peptides from each sample was subjected to chromatographic separation using a Vanquish Neo UHPLC system (Thermo Scientific). Buffer: Mobile phase A was 0.1% formic acid in water; mobile phase B was 0.1% formic acid in acetonitrile-water (acetonitrile 80%). The column was equilibrated with 96% Buffer A. Samples were injected into the Trap Column (PepMap Neo five μm C18, 300 μm $\times$ five mm, Thermo Scientific) followed by gradient separation on the analytical column (μPAC Neo High Throughput column, Thermo Scientific). The entire process was analyzed by DIA mass spectrometry using an Orbitrap Astral mass spectrometer (Thermo Scientific) following a 288 SPD program. Electrospray ionization voltage: 2.2 kV. Detection mode: positive ions. Parent ion scan range: 380–980 m/z. MS1 resolution: 240,000. AGC target: 500%. MS1 maximum IT: 3 ms. MS2 resolution: 80,000, AGC target: 500%, MS2 maximum injection time: 3 ms, RF lens: 40%, MS2 activation type: HCD, isolation window: two Th, normalized collision energy: 25%, cycle time: 0.6.

DIA mass spectrometry data analysis: DIA-NN 1.8. One software was used to analyze DIA mass spectrometry data. Mass spectrometry data were aligned against the curated UniProtKB (Swiss-Prot) database. Trypsin was selected as the digestion enzyme. Database searches were configured with a maximum of one uncut site, a precursor ion mass tolerance of 10 ppm, and a fragment ion mass tolerance of 10 ppm. Cysteine urea methylation was defined as a fixed modification, while N-terminal acetylation and methionine oxidation were treated as variable modifications for database searches. The maximum number of variable modifications was set to 1. Peptide length was restricted to seven to 30 amino acids, with charge range one to four. fragment ion m/z range was set to 150–2,000. Database search results were filtered and exported at <1% false discovery rate (FDR) at both the peptide spectrum matching level and the protein level. The proteomics dataset used in this study is available in an online repository. LC-MS/MS data have been uploaded to iProX (accession number: IPX0015814001).

Statistical analysis

Statistical analysis of ELISA and immunohistochemistry experiments was performed using GraphPad Prism 10 software. Multiple-group comparisons were conducted using one-way ANOVA, followed by Tukey's post hoc test for pairwise comparisons. Data are expressed as mean $\pm$ SD. $P < 0.05$ indicates statistically significant differences.

Proteomics bioinformatics analysis was performed using Microsoft Excel and R statistical software. Hierarchical clustering analysis and volcano plot generation were implemented via the R statistical language. Sequence annotation data were sourced from UniProtKB/Swiss-Prot, the Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Ontology (GO). GO and KEGG enrichment analyses employed Fisher's exact test with FDR correction for multiple testing. GO terms were categorized into three major groups: biological process (BP), molecular function (MF), and cellular component (CC). Enriched GO and KEGG pathways achieved statistical significance at $P < 0.01$ in the Fisher's exact test. Concurrently, a protein-protein interaction (PPI) network was constructed using Cytoscape software in conjunction with the STRING database. $P < 0.05$ indicates statistically significant differences.

Results

Successful establishment of a VX2 breast cancer lymph node metastasis rabbit model

On day five post-implantation, one female rabbit died, and one rabbit exhibited disappearance of breast tumor tissue on day 13. In the remaining 11 experimental group rabbits, a distinct mass appeared at the implantation site on day seven post-implantation. Ultrasound imaging revealed enlarged SLN, confirming successful model establishment with a tumor formation rate of 92.3% (Figure S1).

Fine-needle aspiration pathology slide staining results

The staining results of fine-needle aspiration pathological sections at Week five indicated successful establishment of the VX2 breast cancer lymph node metastasis rabbit model, with a tumor formation rate of 92.3%. Findings at Week 10 demonstrated lymph node metastasis in this model (Figure 1).

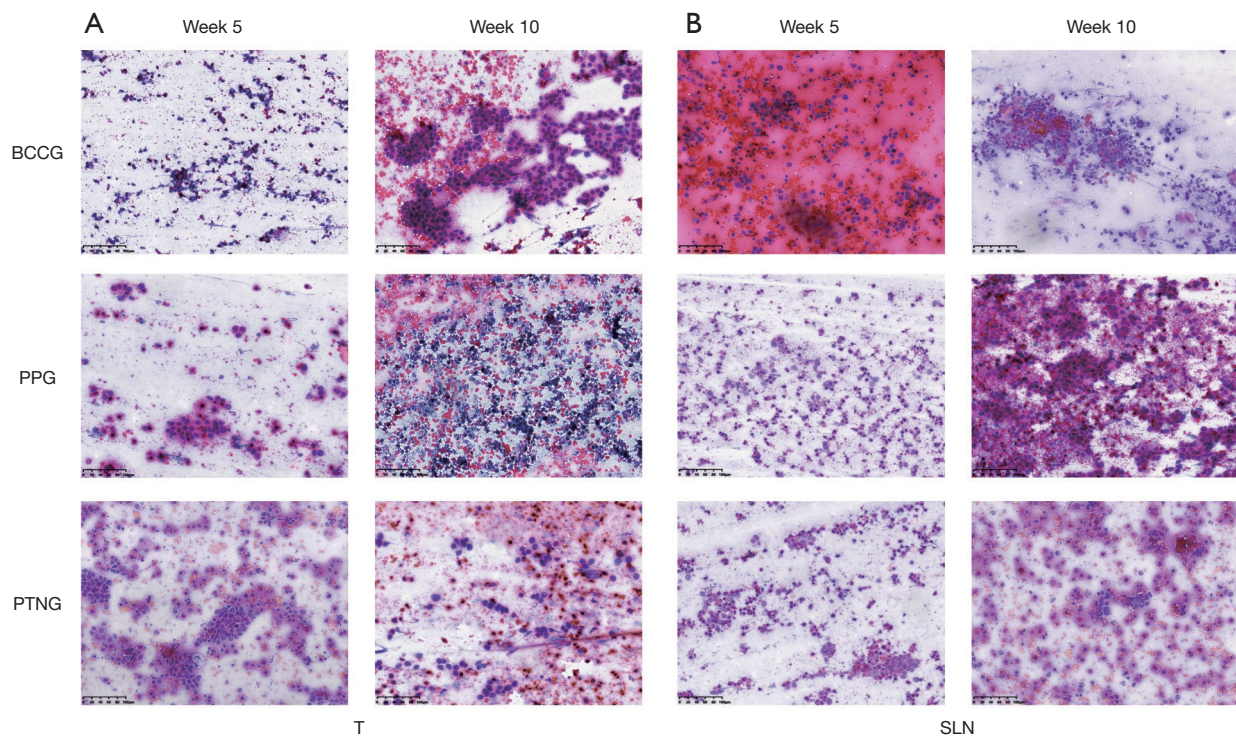


Figure 1 Standard H&E staining of fine needle aspiration biopsy specimens from sentinel lymph nodes and breast tumor tissues in each group. Positive cells appear purple or light purple. (A) Stain of fine needle aspiration biopsy specimens from breast tumor tissues in each group. (B) Stain of fine needle aspiration biopsy specimens from sentinel lymph node tissues. BCCG, breast cancer positive control group; H&E, hematoxylin and eosin; PPG, persistently positive lymph node metastasis group; PTNG, positive-to-negative lymph node metastasis group; SLN, sentinel lymph node.

Lymphatic fluid extraction and interventional therapy outcomes

Two weeks after tumor transplantation, the experimental group underwent interventional therapy involving periodic lymphatic fluid aspiration. Ultrasound imaging (Figure S2) revealed that in three rabbits from the experimental group, the volume of breast tumor tissue had decreased, while SLN either shrank or showed no significant changes. In the remaining four rabbits, no reduction in breast tumor tissue was observed, with SLN either enlarged or showing no significant change; one rabbit developed secondary lymph node metastasis. Tumor and SLN dimensions were measured throughout the experiment (Figure 2), and rabbits were grouped based on their response to the intervention. The PPG (n=4) comprised rabbits exhibiting increased breast tumor tissue volume and enlarged or unchanged SLN. The PTNG (n=3) included rabbits with reduced breast tumor tissue volume and decreased or unchanged SLN. The CG included three rabbits, and the BCCG included three rabbits. One rabbit exhibited secondary lymph node metastasis and

was therefore excluded (Tables 1,2). Due to the insufficient number of rabbits exhibiting secondary lymph node metastasis, the biological replication criteria in statistics were not met; therefore, this study was excluded. A flowchart was created to clarify the experimental steps (Figure S3).

ELISA experimental results

Differences in IgM protein levels were detected in SLN tissues and breast tumor tissues across all groups. Results showed that in SLN tissues, compared to the CG and BCCG, the experimental group exhibited significantly increased IgM protein levels in the PPG and relatively decreased levels in the PTNG; the BCCG had higher IgM protein levels than the CG. Among the experimental groups, the PPG exhibited the highest IgM protein content, while the PTNG showed the lowest. In breast tumor tissues, compared with the BCCG, the experimental PPG demonstrated a significant increase in IgM protein content, whereas the PTNG showed minimal change. Within the experimental groups, the PPG had the highest IgM protein

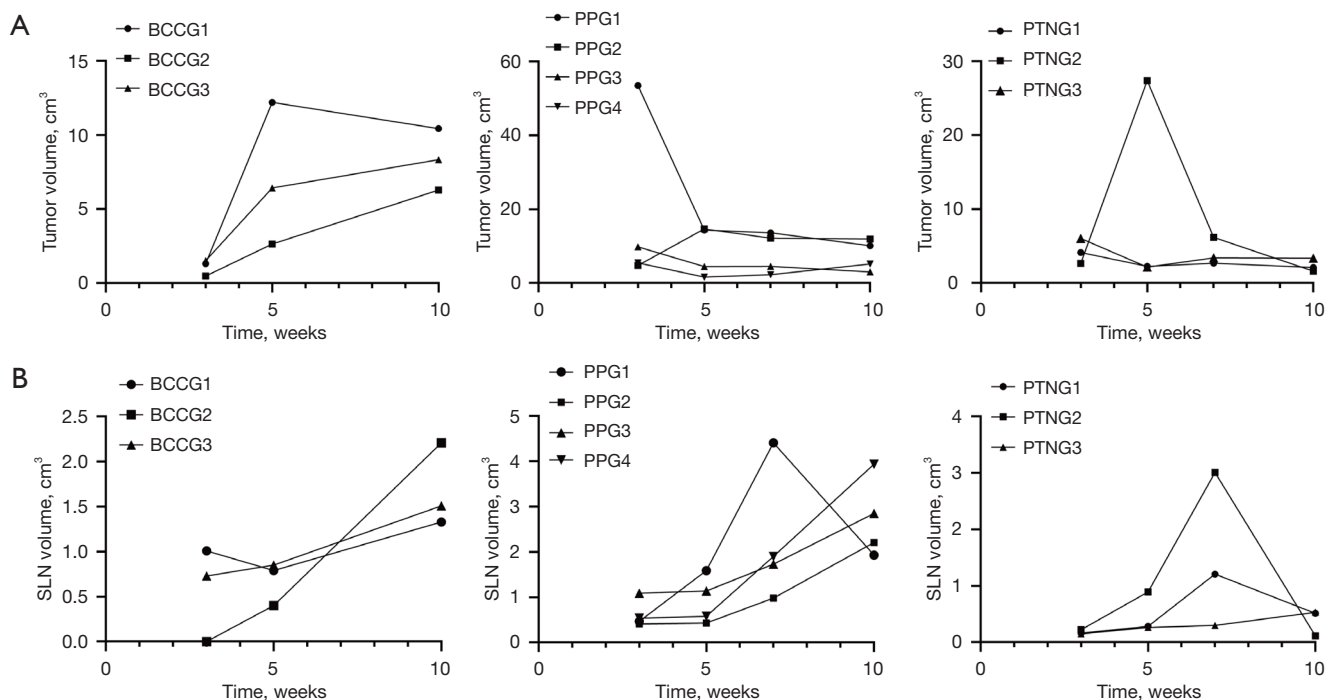


Figure 2 Tumor volume and sentinel lymph node volume in rabbits at weeks 3, 5, 7, and 10. (A) Tumor volume of rabbits in each group during weeks 3, 5, 7, and 10. (B) Volume of sentinel lymph nodes in rabbits at weeks 3, 5, 7, and 10 across each group. BCCG, breast cancer positive control group; PPG, persistently positive lymph node metastasis group; PTNG, positive-to-negative lymph node metastasis group; SLN, sentinel lymph node.

content, and the PTNG had the lowest (Figure 3).

Immunohistochemistry results

Ki-67 expression was detected in breast tumor tissues and SLN tissues across all groups (Figure 4). Results indicated that compared with the CG, the Ki-67 expression level in SLN tissue was higher in the BCCG. Among the three groups, the PPG exhibited the highest Ki-67 expression in SLN tissue, while the PTNG showed the lowest expression. However, there was no significant difference in Ki-67 expression levels among the three groups in breast tumor tissue (Figure 5).

Differentially expressed proteins in lymph fluid

Using the DIA (data-independent acquisition) analysis method, a total of 6,135 proteins were detected across 10 samples. With a fold change threshold of ≥ 1.5 as the criterion for significant expression, 270 differentially expressed proteins were identified through multi-group comparisons (Figure 6).

GO enrichment analysis of differentially expressed proteins

GO enrichment analysis was performed on differentially expressed proteins in lymph fluid across rabbit groups (Figure 7A). BPs primarily included “response to bacteria”, “complement activation”, and “humoral immune response” (Figure 7B). CCs primarily encompassed “extracellular region”, “extracellular space”, and “blood particles” (Figure 7C). MFs mainly included “endopeptidase inhibitor activity”, “endopeptidase-regulating enzyme activity”, and “molecular function inhibitor activity” (Figure 7D).

KEGG enrichment analysis of differentially expressed proteins

KEGG enrichment analysis was performed on differentially expressed proteins in lymph fluid across rabbit groups. Results revealed these proteins participated in 30 major pathways, including “Phagosome”, “NF- κ B Signaling Pathway”, “Rap1 Signaling Pathway”, and “Staphylococcus aureus Infection” (Figure 8A). The top 20 pathways included

Table 1 Size of breast tumors, sentinel lymph nodes in rabbits of different groups at different time periods

Group	Three-week size (cm ³)	Five-week size (cm ³)	Seven-week size (cm ³)	Ten-week size (cm ³)
BCCG1				
Tumor	1.32	12.22	–	10.45
SLN	1.01	0.79	–	1.33
BCCG2				
Tumor	0.48	2.65	–	6.29
SLN	0	0.40	–	2.21
BCCG3				
Tumor	1.52	6.43	–	8.34
SLN	0.73	0.85	–	1.51
PPG1				
Tumor	53.5	14.35	13.63	10.14
SLN	0.47	1.59	4.41	1.93
PPG2				
Tumor	4.75	14.66	12.2	11.91
SLN	0.41	0.43	0.98	2.21
PPG3				
Tumor	9.81	4.46	4.51	3.01
SLN	1.09	1.14	1.73	4.38
PPG4				
Tumor	5.49	1.64	2.27	5.15
SLN	0.54	0.58	1.9	3.94
PTNG1				
Tumor	4.14	2.25	2.68	2.12
SLN	0.16	0.28	1.21	0.51
PTNG2				
Tumor	2.66	27.4	6.19	1.60
SLN	0.22	0.89	3.01	0.11
PTNG3				
Tumor	6.04	2.16	3.40	3.35
SLN	0.15	0.26	0.3	0.53

BCCG, breast cancer positive control group; PPG, persistently positive lymph node metastasis group; PTNG, positive-to-negative lymph node metastasis group; SLN, sentinel lymph node.

Table 2 Experimental grouping

Cluster	CG	BCCG	PPG	PTNG	SLNMG
Quantities	3	3	4	3	1

BCCG, breast cancer positive control group; CG, normal rabbit group; PPG, persistently positive lymph node metastasis group; PTNG, positive-to-negative lymph node metastasis group; SLNMG, secondary lymph node metastasis group.

“Complement and Coagulation Cascades”, “Asthma”, “Viral Myocarditis”, and “B Cell Receptor Signaling Pathway” (Figure 8B). Sankey diagrams revealed that most proteins primarily functioned in the “Immune System”, “Immune Diseases”, “Infectious Diseases: Bacterial”, “Infectious Diseases: Parasitic”, and “Cardiovascular Diseases” pathways (Figure 8C).

Intergroup comparison differences in IgM protein

Through pairwise comparisons and multiple comparisons, four IgM proteins exhibiting differences across groups were identified (Figure 9). Among these, the A0A0C6G3N5 protein [IgM heavy chain VDJ region, Amino acids: 211, *Oryctolagus cuniculus* (Rabbit)] showed an upward trend, while the A0A0C6G3P3 protein [IgM light chain, Amino acids: 211, *Oryctolagus cuniculus* (Rabbit)], A0A1Y1B9M1 protein [IgM light chain, Amino acids: 214, *Oryctolagus cuniculus* (Rabbit)], and A0A1Y1B9K3 protein [IgM light chain, Amino acids: 216, *Oryctolagus cuniculus* (Rabbit)] exhibited downward trends.

Discussion

Breast cancer metastasis is the primary cause of patient mortality, with EMT being essential for its initiation (20,21). Additionally, proteins secreted by immune cells and the TME may also trigger the onset of breast cancer metastasis (22). Lymph node metastasis represents the primary route of breast cancer spread, with axillary lymph node involvement serving as the most significant predictor of overall recurrence and survival in breast cancer patients (23,24). Specific immune evasion events may signal the transition from *in situ* to invasive breast cancer, suggesting that detection based on autoimmune responses could enable effective early diagnosis (25). In particular, the IgG κ chain has been demonstrated to serve as a prognostic

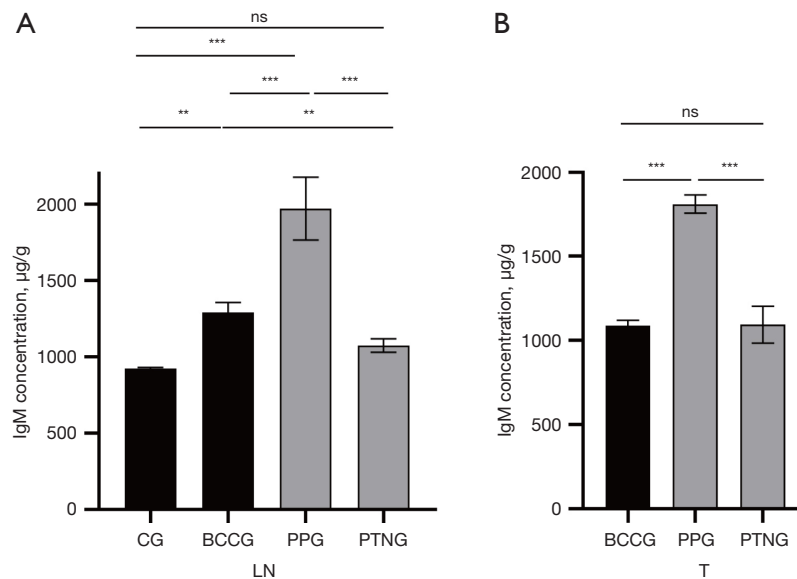


Figure 3 Changes in IgM protein content between groups. (A) Sentinel lymph node tissue IgM protein concentration in each group. (B) IgM protein concentration of breast tumor tissues in each group. ns, no significance; **, $P < 0.01$; ***, $P < 0.001$. BCCG, breast cancer positive control group; CG, normal rabbit group; IgM, immunoglobulin M; LN, lymph node; PPG, persistently positive lymph node metastasis group; PTNG, positive-to-negative lymph node metastasis group; T, tumor.

biomarker for breast cancer, enabling prediction of disease response (26). Lewis C (LeC) antigen, a Gal β 1-3GlcNAc disaccharide structure, serves as the precursor to the H1 blood group antigen and is abnormally expressed in various epithelial tumors. Anti-LeC antibodies are detectable in nearly all (~95%) healthy individuals. However, levels of this antibody in the blood of breast cancer patients are significantly lower than in healthy women, suggesting a potential role in antitumor defense (27). Continuous advancements in proteomics technology have made it possible to elucidate the molecular mechanisms of disease at the protein level (28). For example, proteomics and bioinformatics approaches applied to chemotherapy-resistant breast cancer cell lines revealed underlying resistance mechanisms to doxorubicin (DR), paclitaxel (PR), and tamoxifen (TAR), identifying potential prognostic proteins significantly impacting overall survival (29). As a crucial component of the lymph node microenvironment, lymph fluid analysis has identified key biological markers and potential therapeutic targets for early breast cancer metastasis by examining compositional changes across different lymph fluid groups, thereby contributing to both treatment strategies and prognosis.

IgM protein, as a polyreactive and low-affinity immunoglobulin secreted by B cells, serves as part of the

body's first line of defense. Native IgM protein recognizes cell surface neoantigens and enables antigen-presenting cells (APCs) to initiate adaptive immune responses (30). IgM expression also varies across different tumor progression stages. Large-scale surveys of patients with multiple non-hematopoietic tumors revealed alterations in serum immunoglobulins IgG, IgA, and IgM. Elevated serum IgG and IgA levels were observed in skin cancer and lung cancer patients, while only serum IgA levels increased in patients with gastrointestinal tumors and uterine tumors. In previous studies, PSA-IgM and iXip demonstrated favorable efficacy in prostate cancer diagnosis and management (31). Anti-Glycan IgG and IgM proteins also exhibited high sensitivity and specificity for diagnosis and staging in colorectal cancer (32). Further studies indicate that mannose is transported into cells via the same mechanism as glucose, but mannose disrupts intracellular glucose metabolism, reduces expression of Bcl-2 family proteins, and subsequently kills tumor cells. This effect is not observed when other monosaccharides like galactose, fructose, fucose, or glucose are added. Gastric cancer cells produce anti-mannose IgM proteins to mitigate this disruptive risk (33). In glioblastoma, native IgM protein exerts a protective effect (34). In breast cancer, experiments reveal that tumor development increases

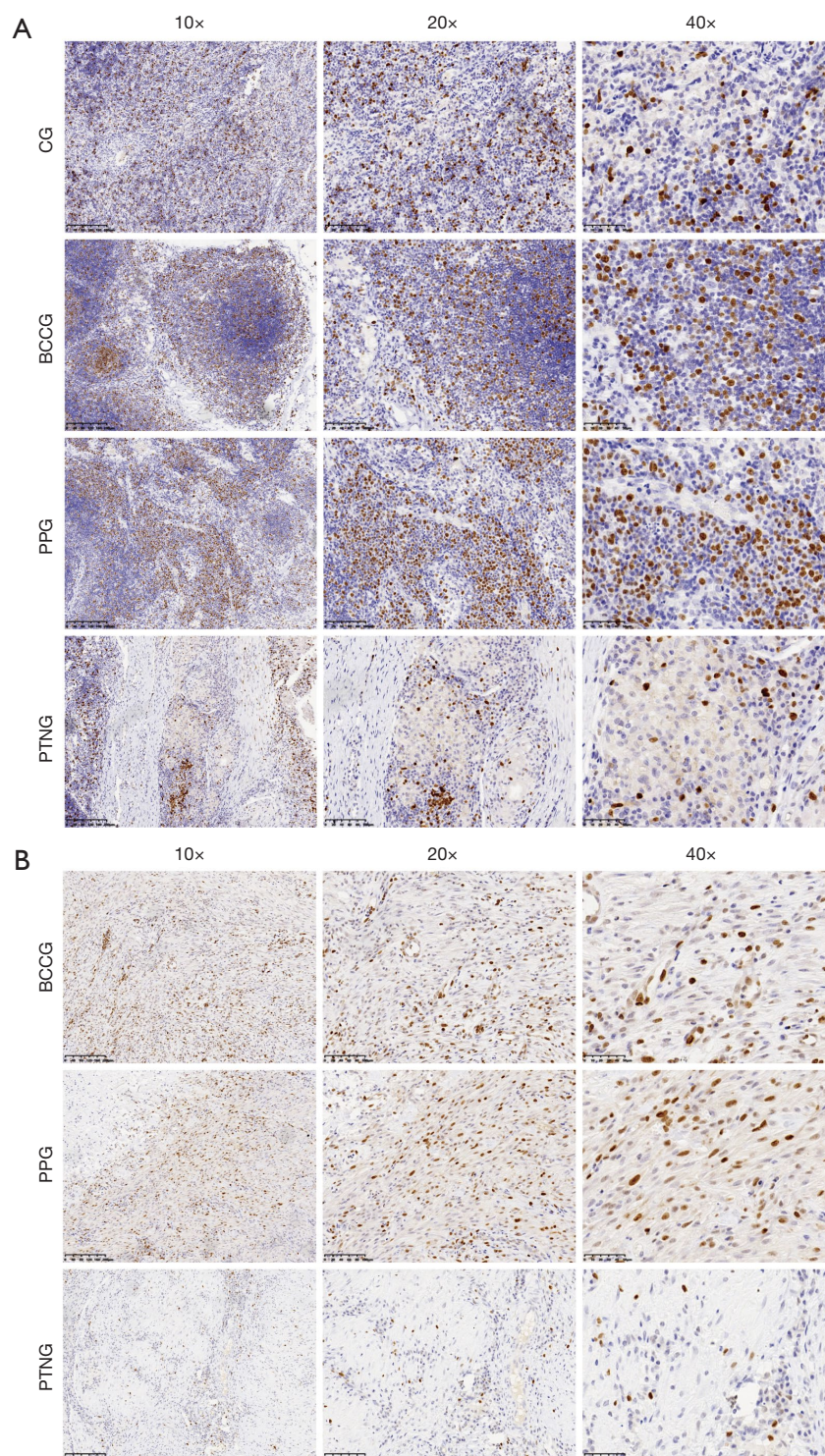


Figure 4 Ki-67 immunohistochemistry of sentinel lymph node and breast tumor tissues of each group. Nuclei were blue and positive results were brown or tan in color. (A) Immunohistochemistry of sentinel lymph node tissue at various magnifications. (B) Immunohistochemistry of breast tumour tissue from each group at different magnifications. BCCG, breast cancer positive control group; CG, normal rabbit group; PPG, persistently positive lymph node metastasis group; PTNG, positive-to-negative lymph node metastasis group.

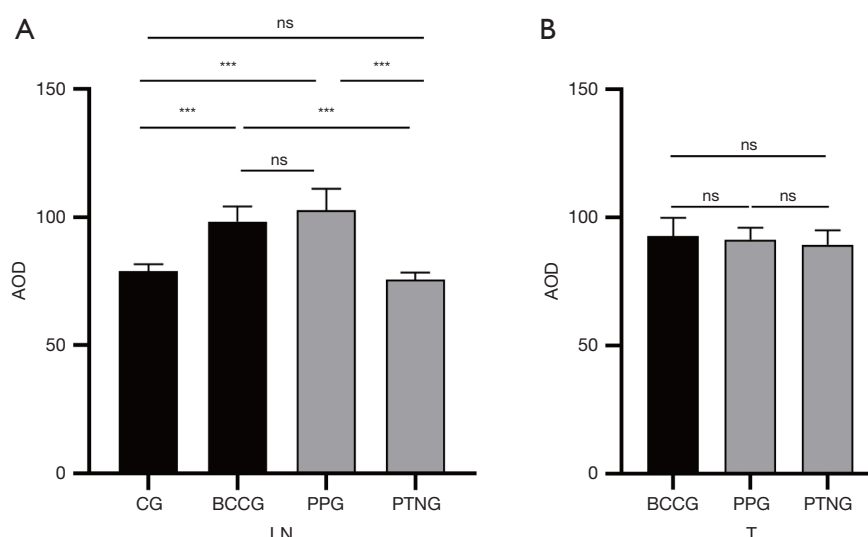


Figure 5 Standardised quantitative analysis of Ki-67 immunohistochemical staining in sentinel lymph nodes and breast tumour tissue across all groups. (A) Standardised quantitative immunohistochemical staining of sentinel lymph node tissue across all groups. (B) Standardised quantitative immunohistochemical staining of breast tumour tissue across all groups. ns, no significance; ***, $P < 0.001$. AOD, average optical density; BCCG, breast cancer positive control group; CG, normal rabbit group; LN, lymph node; PPG, persistently positive lymph node metastasis group; PTNG, positive-to-negative lymph node metastasis group; T, tumor.

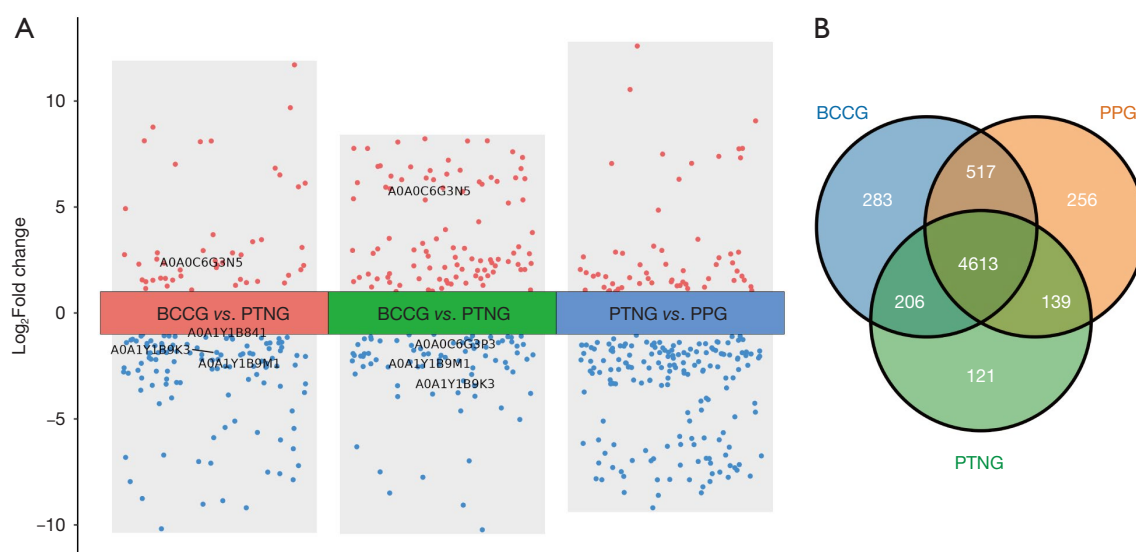


Figure 6 Analysis of lymph fluid protein expression. (A) Volcano plot comparing each pair of groups. (B) Venn diagram showing expression patterns across all groups. BCCG, breast cancer positive control group; PPG, persistently positive lymph node metastasis group; PTNG, positive-to-negative lymph node metastasis group.

IgM protein levels, and lymph node intervention therapy significantly elevates IgM protein content. Ki-67, a key prognostic indicator of breast cancer proliferation, shows that higher positivity rates indicate faster tumor growth

and poorer prognosis (35). Experimental findings revealed that the PTNG exhibited the lowest Ki-67 expression levels, indicating a more favorable prognosis compared to other groups. Proteomics analysis revealed dynamic

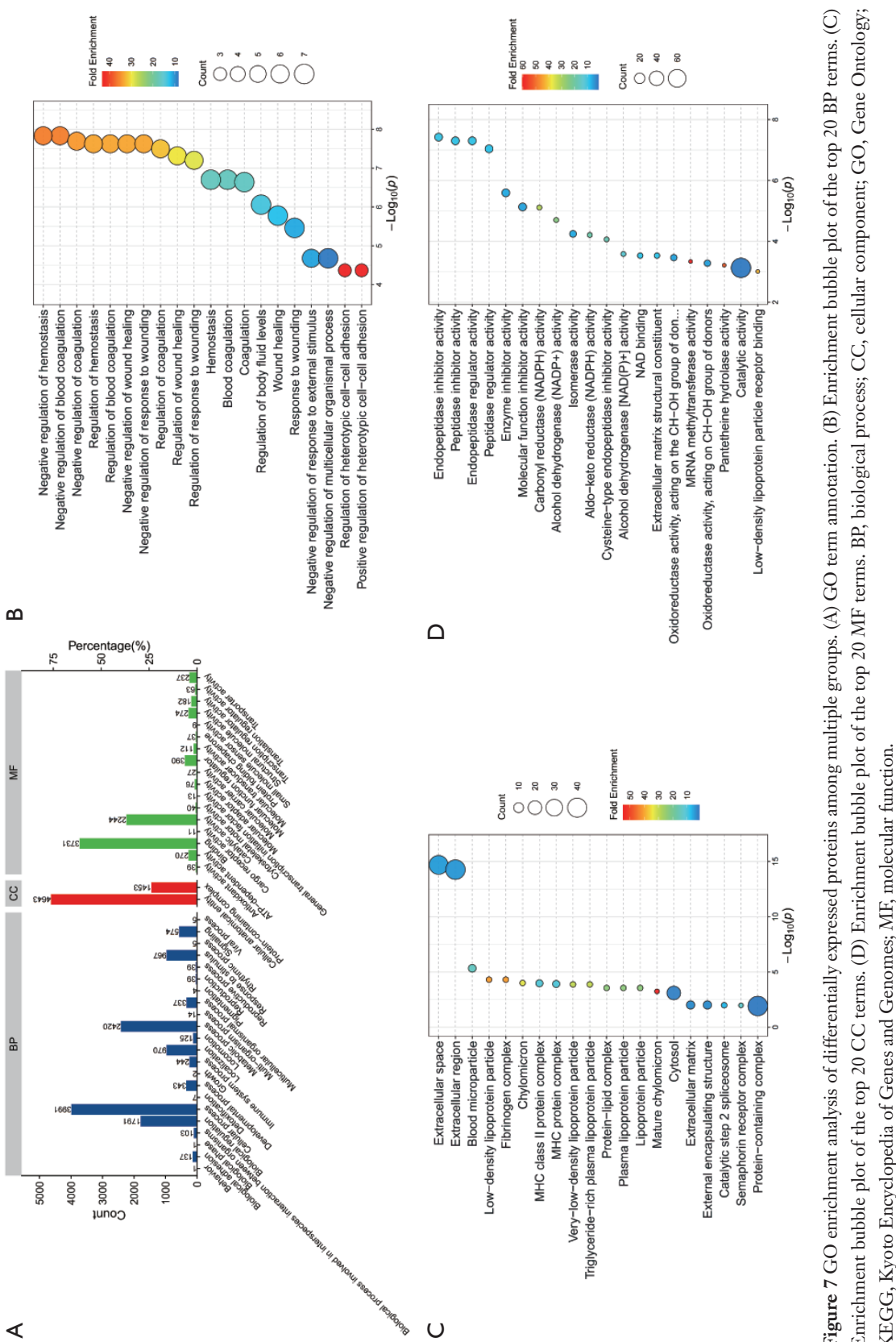


Figure 7 GO enrichment analysis of differentially expressed proteins among multiple groups. (A) GO term annotation. (B) Enrichment bubble plot of the top 20 BP terms. (C) Enrichment bubble plot of the top 20 CC terms. (D) Enrichment bubble plot of the top 20 MF terms. BP, biological process; CC, cellular component; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular function.

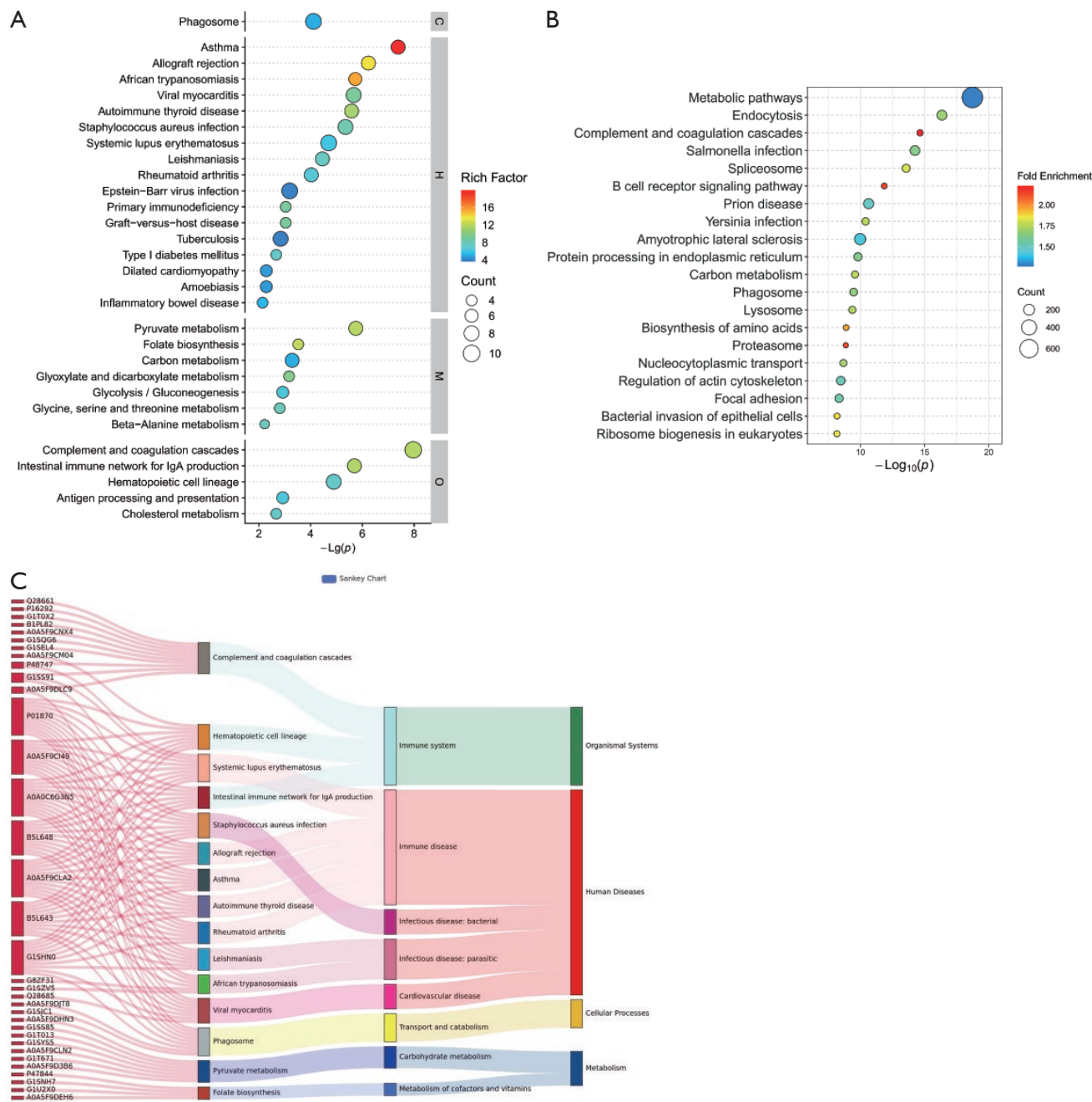


Figure 8 KEGG enrichment analysis of differentially expressed proteins in multi-group comparisons. (A) KEGG pathway bubble chart. (B) Top 20 KEGG pathway enrichment bubble chart. (C) KEGG pathway Sankey diagram. KEGG, Kyoto Encyclopedia of Genes and Genomes.

changes in IgM protein expression levels during breast cancer progression. However, as this study employed an observational design without functional experimental validation, the observed association does not imply that IgM possesses direct tumor-suppressing or causal

protective effects. Its potential biological functions require further confirmation through basic research. Future research should determine the expression changes of IgM protein across different stages of breast cancer, validate its feasibility as a breast cancer biomarker, and explore its

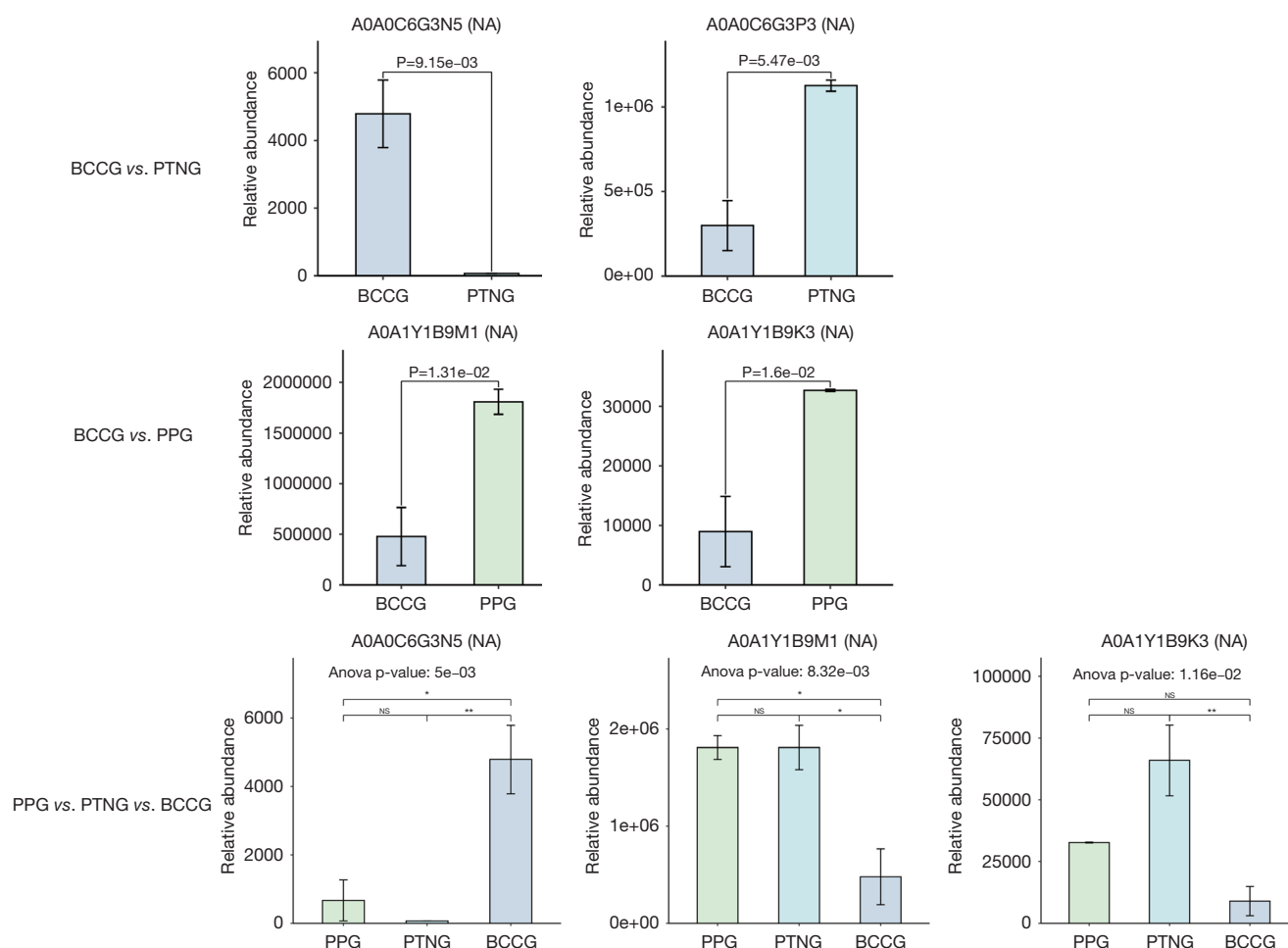


Figure 9 Relative expression levels of differential IgM proteins between groups. ns, no significance; *, $P < 0.05$; **, $P < 0.01$; NS, not significant. BCCG, breast cancer positive control group; IgM, immunoglobulin M; PPG, persistently positive lymph node metastasis group; PTNG, positive-to-negative lymph node metastasis group.

mechanisms in breast cancer screening, treatment, and prognosis. Furthermore, given the limitations of animal models and the potential for experimental bias due to inter-individual variability, experimental errors should be minimized through multiple replicate experiments and increased animal numbers per group. Compared to other cancer types, systematic research on the immunobiological functions of IgM in breast cancer remains limited. Previous studies have reported associations between intratumoral IgM proteins or serum IgM levels and disease progression in ovarian (36), lung (37,38), colorectal cancers (39), and metastatic urothelial carcinoma (40); but these findings exhibit significant heterogeneity. This study employed a VX2 breast cancer lymph node metastasis rabbit model to investigate rabbit lymph fluid proteomics,

revealing the expression of IgM proteins during tumor progression and identifying a potential association with lymph node metastasis. Unlike well-established breast cancer immunological markers such as IgG, tumor-infiltrating lymphocytes (TILs), and PD-L1, IgM—a key effector molecule of innate immune responses—may reflect earlier stages of the host's innate immune recognition of tumor-associated antigens. This finding expands the existing immunological research framework for breast cancer, suggesting that future studies should focus on the role of innate immunity in the early stages of tumor immunity.

In summary, this study identified differential changes in IgM protein expression during breast cancer progression, which exerted certain effects on the proliferation and

metastatic capacity of breast cancer. This suggests that IgM protein may serve as a potential therapeutic target for breast cancer, offering novel therapeutic approaches. Future studies could integrate proteomics to examine IgM protein expression changes across early, intermediate, and advanced breast cancer stages. Additionally, animal experiments involving IgM protein injection could be conducted to observe tumor size alterations, thereby validating IgM protein's effects on breast cancer proliferation and metastasis.

Conclusions

In this study, using a rabbit model, we systematically analyzed the protein expression profiles in lymphatic fluid and lymphoid tissues, quantitatively validated the dynamic changes in IgM levels, and identified several relevant signaling pathways.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The *in vivo* experiments in this study were performed under a project license (approval No. KYLL-2022-0173) granted by Medical Research Ethics Review Committee of General Hospital of Ningxia Medical University, in compliance with the Experimental Animal Center of Ningxia Medical University guidelines for the care and use of animals.

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