Analysis of Differentially Expressed Proteins in Lymph Fluids Related to Lymphatic Metastasis in Breast Cancer Rabbit Model Guided by Contrast-Enhanced Ultrasound

Xu Jiachao[1], Zhang Xin[2], Yang Guangfei[2], Sun Wei[2], Wang Wen[2], and Mi Chengrong[2]

[1]Ningxia Medical University
[2]Affiliation not available

July 5, 2023

Abstract

Abstract: Objective: To identify differentially expressed proteins in the lymph fluid of rabbits with breast cancer lymphatic metastasis and normal rabbits, and to analyze and verify using proteomics technology. Materials and Methods: Rabbits with breast cancer lymph node metastasis and healthy rabbits were selected. Lymph fluids were precisely extracted under the guidance of percutaneous contrast-enhanced ultrasound (CEUS). Label-free quantitative proteomics (LFQ) was used to detect and compare differences, and differential protein expression was obtained. Bioinformatics analysis was performed using KEGG and GO analysis software, selecting the most significantly differentially expressed proteins. Finally, parallel reaction monitoring technology (PRM) was applied for validation. Results: A total of 547 significantly differentially expressed proteins were found in this study, including 371 upregulated proteins and 176 downregulated proteins. They were mainly involved in various cellular and metabolic pathways, including upregulated proteins such as BLVRA, IDH2, and downregulated proteins such as PDXK. The upregulated proteins PDIA3, PRKAR1A, and ABCC4 participated in immune regulation, endocrine regulation, and anti-tumor drug resistance regulation, respectively. Conclusion: These proteins are involved in the pathophysiological processes of tumor development and metastasis, and can be used as predictive indicators for breast cancer metastasis and as new targets for treatment.

Analysis of Differentially Expressed Proteins in Lymph Fluids Related to Lymphatic Metastasis in Breast Cancer Rabbit Model Guided by Contrast-Enhanced Ultrasound

Jiachao Xu[1,2+], Xin Zhang[1,2+], Guangfei Yang[2], Wei Sun[1,2], Wen Wang[2*], Chengrong Mi[2*]

[1]Ningxia Medical University, Yinchuan, China

[2]General Hospital of Ningxia Medical University, Yinchuan, China

[Grant Project] National Natural Science Foundation of China (82260351)

Abstract: Objective: To identify differentially expressed proteins in the lymph fluid of rabbits with breast cancer lymphatic metastasis and normal rabbits, and to analyze and verify using proteomics technology.

Materials and Methods: Rabbits with breast cancer lymph node metastasis and healthy rabbits were selected. Lymph fluids were precisely extracted under the guidance of percutaneous contrast-enhanced ultrasound (CEUS). Label-free quantitative proteomics (LFQ) was used to detect and compare differences, and differential protein expression was obtained. Bioinformatics analysis was performed using KEGG and GO analysis software, selecting the most significantly differentially expressed proteins. Finally, parallel reaction monitoring technology (PRM) was applied for validation.

Results: A total of 547 significantly differentially expressed proteins were found in this study, including 371 upregulated proteins and 176 downregulated proteins. They were mainly involved in various cellular and metabolic pathways, including upregulated proteins such as BLVRA, IDH2, and downregulated proteins such as PDXK. The upregulated proteins PDIA3, PRKAR1A, and ABCC4 participated in immune regulation, endocrine regulation, and anti-tumor drug resistance regulation, respectively.

Conclusion: Compared with normal rabbits, rabbits with breast cancer metastasis have various differentially expressed proteins in their lymph fluid. These proteins are involved in the pathophysiological processes of tumor development and metastasis, and can be used as predictive indicators for breast cancer metastasis and as new targets for treatment.

【Key words】 Breast cancer; Lymph node metastasis; Contrast-enhanced ultrasonography;Proteomics; Differentially expressed protein

1. Introduction

Breast cancer is one of the most common cancers among women worldwide, with more than 1.5 million women (accounting for 25% of all female cancers) diagnosed with breast cancer every year[1]. In China, the incidence and mortality of breast cancer rank first in the world[2]. Breast cancer is a metastatic cancer that initially spreads through the lymphatic system to various levels of lymph nodes, and finally metastasizes to distant organs such as bones, liver, lungs, and brain through the bloodstream, which is the main reason why it cannot be cured[3]. Accurate metastasis prediction is related to the formulation of treatment plans, prognosis, survival rate, and quality of life. However, breast cancer is a highly heterogeneous malignant disease with various functional phenotypes[4]. Proteomic, genomic and transcriptomic studies have achieved remarkable results in the exploration of cellular subtypes and the development of breast cancer lymphatic metastasis biomarkers[5]. Therefore, finding protein biomarkers related to breast cancer is important for predicting the progression of the disease, implementing early drug or intervention treatment, and thus reducing mortality caused by metastasis.

The process of lymphatic metastasis involves the lymphatic circulation, and tumor-related lymphatic vessels provide a direct route to the lymph nodes, enabling primary tumors to transmit concentrated signals, gradually reshaping and hijacking lymph node function from afar[6]. After a tumor develops, various aspects of the internal tumor environment will undergo complex biological effects[7]. Lymph fluid, as an essential component, is subject to these changes, with variations occurring in its cytokines and antibody components. However, due to the difficulty in obtaining lymph fluid, it has been under-researched. Percutaneous contrast-enhanced ultrasound (CEUS) technology can provide support in this area[8], enabling observation of the lymphatic vessels entering and leaving the sentinel lymph nodes and accurate extraction of lymph fluid. Therefore, in this study, we used CEUS technology to precisely extract lymph fluid from rabbits with breast cancer metastasis and healthy rabbits. Using LC-MS/MS analysis, we quantified proteins in the lymph fluid and performed bioinformatics analysis on differentially expressed proteins to identify protein biomarkers for breast cancer lymphatic metastasis and explore the function of differentially expressed proteins and the signaling pathways involved. This study lays the foundation for further exploration of predictive indicators and therapeutic targets for breast cancer metastasis in the future.

2.Materials and Methods

2.1. Precision extraction of lymph fluid

Ultrasound contrast agent is injected subcutaneously at the edge of the tumor in the breast cancer rabbit. The first (group) lymph node traced from the injection point along the lymphatic vessel is the sentinel lymph node (SLN), which is also known as SLN contrast-enhanced ultrasound (SLN CEUS). CEUS can clearly display the input lymphatic vessels of the SLN, which may reflect the state of the lymph fluid after tumor microenvironment formation. At the same time, CEUS is used to dynamically observe the progression of lymph node metastasis. Lymph fluid from the rabbit's sentinel lymph node at 8 weeks after tumor implantation was used to ensure that the metastatic lymph node was malignant. Accurate lymph

fluid extraction from normal rabbits under CEUS guidance was performed as a control.

2.2. Reagents and equipment Ammonium bicarbonate

Dithiothreitol (DTT), Iodoacetamide (IAA) and sodium carbonate (Sigma-Aldrich, USA); urea and sodium dodecyl sulfate (SDS) (Bio Rad, USA); trypsin (Promega, USA); BCA protein assay kit (Bio-Rad, USA). Q Exactive Plus mass spectrometer and EASY-nLC 1200 (Thermo Fisher Scientific, USA).

2.3. LFQ sample preparation

After freeze-drying the lymph fluid, each sample is mixed with 100˜200μL SDT lysis buffer based on the actual lymph fluid volume. Lysate is homogenized with a homogenizer and transferred to an EP tube. The lysate is then mixed with boiling water bath for 3 min, sonicated for 2 min, centrifuged at 16000g and 4°C for 20min, and the supernatant is collected. BCA method is used for protein quantification. A total of 100ug protein is taken from each sample for FASP digestion. The following steps are performed: add an appropriate amount of 1M DTT to a final concentration of 100mM to each sample, boiling water bath for 5 min, cool to room temperature. Add 200μL UA buffer (8M Urea, 150mM Tris-HCl, pH8.0) to mix, transfer to a 10KD ultrafiltration centrifuge tube, centrifuge at 12000g for 15min. Add 200μL UA buffer and centrifuge at 12000g for 15min, discard filtrate. Add 100μL IAA (50mM IAA in UA), shake at 600rpm for 1min, avoid light at room temperature for 30min, centrifuge at 12000g for 10min. Add 100μL UA buffer, and centrifuge at 12000g for 10min, repeat twice. Add 100μL NH4HCO3 buffer, centrifuge at 14000g for 10min, repeat twice. Add 40μL Trypsin buffer (6μg Trypsin in 40μL NH4HCO3 buffer), shake at 600rpm for 1min, incubate at 37°C for 16-18h. Change to a new collection tube, centrifuge at 12000g for 10min, collect filtrate, add an appropriate amount of 0.1% TFA solution, desalt the digested peptides using C18 Cartridge, and freeze-dry under vacuum. After drying, reconstitute the digested peptides with 0.1% TFA, measure peptide concentration, and prepare for LC-MS analysis.

2.4. LC-MS/MS analysis

Chromatographic separation of peptide samples is performed using a nanoflow Easy nLC 1200 chromatography system (Thermo Scientific). The buffer solution: A is a 0.1% formic acid aqueous solution, and B is a 0.1% formic acid, acetonitrile and water mixed solution (acetonitrile is 80%). The chromatographic column is equilibrated with 95% A solution. The sample is injected into a Trap Column (100μm*20μμ, 5μμ, ˜18, Δρ. Μαισϛη ΓμβΗ) ανδ σεπαρατεδ βψ α γραδιεντ τηρουγη τηε ϛηροματογραπηις αναλψσις ϛολυμν (75μμ* 150mm, 3μm, C18, Dr. Maisch GmbH) at a flow rate of 300nl/min. The liquid phase separation gradient is as follows: 0min-2min, linear gradient of B solution from 5% to 8%; 2min-90min, linear gradient of B solution from 8% to 23%; 90min-100min, linear gradient of B solution from 23% to 40%; 100min-108min, linear gradient of B solution from 40% to 100%; 108min-120min, B solution is maintained at 100%. After peptide separation, a Q-Exactive HF-X mass spectrometer (Thermo Scientific) is used for DDA (data-dependent acquisition) mass spectrometry analysis. The analysis time is 120min, detection mode: positive ion, parent ion scan range: 300-1800m/z, primary mass spectrometry resolution: 60,000 @m/z 200, AGC target: 3e6, primary mass spectrometry Maximum IT: 50 ms. Peptide secondary mass spectrometry is collected according to the following methods: after each full scan (full scan), 20 secondary mass spectra (MS2 scans) of the highest intensity parent ions are collected, with a secondary mass spectrometry resolution: 15,000 @ m/z 200, AGC target: 1e5, secondary mass spectrometry Maximum IT: 50 ms, MS2 Activation Type: HCD, Isolation window: 1.6 m/z, Normalized collision energy: 28.

2.5. Database retrieval

The resulting LC-MS/MS raw RAW files are imported into the Proteome Discoverer software (version 2.4, Thermo Scientific) and the search engine Sequest HT for database retrieval. The database used for searching is uniprot-Oryctolagus cuniculus (Rabbit) [9986]-43526-20211222.fasta, which is sourced from the website https://www.uniprot.org/taxonomy/9986 protein database, with a protein entry of 43526; and a download date of 2021.12.22.

2.6. PRM sample preparation

Take an appropriate amount of the sample and add 8M Urea, ice bath sonication, centrifugation at 4°C and 16000xg for 20min to collect the supernatant. BCA quantification is performed on the supernatant, and 15μg of each sample is run on gel. A total of 200μg of each sample is taken, DTT is added to a final concentration of 10mM, and incubated at 37°C for 1 h. IAA is added to a final concentration of 50mM and incubated in darkness for 30min. Each sample is added with trypsin (1:50) and incubated at 37°C overnight. After quantitative desalting, mix the samples in equal amounts to create a mix sample for testing.

2.7. LC-PRM/MS analysis

Take 2ug of peptide from each sample for LC-PRM/MS analysis. After sample loading, perform chromatographic separation using a nanoflow Easy nLC1200 chromatography system (Thermo Scientific). The buffer solutions are the same as described in section 2.4. The liquid phase separation gradient is as follows: 0 min — 5 min, linear gradient of B solution from 2% to 5%; 5 min — 45 min, the linear gradient of B solution from 5% to 23%; 45 min — 50 min, linear gradient of B solution from 23% to 40%; 50 min — 52 min, linear gradient of B solution from 40% to 100%; 52 minutes —-60 minutes, B solution is maintained at 100%. The Q Exactive HF-X mass spectrometer (Thermo Scientific) is used for targeted PRM mass spectrometry analysis. The original RAW files of the mass spectra obtained are analyzed using Skyline 4.1 software for PRM data.

2.8. Bioinformatics analysis

Proteins with FC > 1.5 or < 0.667 and $p < 0.05$ are considered as significantly differentially expressed proteins. Bioinformatics data are analyzed using Perseus software, Microsoft Excel, and R statistical computing software. Sequence annotations are extracted from UniProtKB/Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Ontology (GO). GO and KEGG enrichment analyses are performed using Fisher's exact test and FDR adjustment for multiple testing. GO terms are divided into three categories: biological processes (BP), molecular functions (MF), and cellular components (CC). Enriched GO and KEGG pathways are statistically significant at $p < 0.05$ level.

2.9. Statistical analysis

Data are analyzed using SPSS 20.0 software. Measurement data are presented as x±s and t-test is used for inter-group comparison. Count data are presented as percentages, and inter-group differences are compared using the $\chi 2$ test. P<0.05 indicates that the difference is statistically significant.

3. Results

3.1. Observation of breast cancer lymph node metastasis under CEUS guidance

We used contrast-enhanced ultrasound to observe the changes in sentinel lymph nodes during the metastasis process of the breast cancer lymphatic metastasis group. Percutaneous superficial ultrasound with Sonozoid (SNZ) revealed four contrast patterns in sentinel lymph nodes during breast cancer progression (Figure 1):

1. Uniform enhancement; 2. Non-uniform enhancement: a. non-enhanced area <25% (1-2 weeks), b. non-enhanced area 25%-50% (3 weeks), c. non-enhanced area 50%-75% (4 weeks), d. non-enhanced area >75%/completely non-enhanced (4-8 weeks); 3. Peripheral ring enhancement with no internal enhancement; 4. Complete absence of enhancement in the lymph node with the presence of surrounding lymphatic vessels bypassing.

3.2 Analysis of Lymphatic Fluid Differential Proteins

A total of 2647 proteins were detected in 6 samples by label-free quantification (LFQ) method. Using a 1.2-fold increase or decrease in protein expression as the criterion for significant changes, 547 differential proteins were identified. Compared with the normal group (CG), the metastasis group (LNPG) contained 371 upregulated proteins and 176 downregulated proteins. The heatmap shows the hierarchical clustering of 547 differential proteins (FC > 1.2 or < 0.833, p-value < 0.05) (Figure 2).

3.3. Differential Protein GO Function Enrichment Analysis

A Gene Ontology (GO) function enrichment analysis was conducted on the differentially expressed proteins in the lymphatic fluid of breast cancer rabbits and normal rabbits (Figure 3A). The main biological processes (BP) include: cellular macromolecule catabolic process, protein catabolic process, etc. (Figure 3B). The main cellular components (CC) include: proteasome core complex, endopeptidase complex, catalytic complex, etc. (Figure 3C). The main molecular functions (MF) include: molecular function modulator, serine endopeptidase inhibitor activity, threonine endopeptidase activity, etc. (Figure 3D).

3.4. Differential Protein KEGG Enrichment Analysis

Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, the differentially expressed proteins were subjected to pathway enrichment analysis. It was found that these differential proteins are involved in 20 main pathways (Figure 4A), including: proteasome pathway, complement and coagulation cascades pathway, and pentose phosphate pathway, etc. The upregulated proteins are mainly involved in pathways such as the proteasome pathway, carbon metabolism pathway, and cysteine and methionine metabolism pathway. The downregulated proteins are mainly involved in pathways such as complement and coagulation cascades pathway, cholesterol metabolism pathway, and Staphylococcus aureus infection pathway (Figure 4B).

3.5. PRM Validation

Parallel reaction monitoring (PRM) was conducted on the differentially expressed proteins in the lymphatic fluid of metastasis group and normal group rabbits. Ten differential proteins were found to have consistent trends with the results from the LFQ proteomics technology (Table 1). The upregulated proteins include: PDIA3, BLVRA, IDH2, PRKAR1A, RAB1A, ABCC4, and MAP4; while the downregulated proteins include: ANXA8, PDXK, and GC. The protein abundance results related to the PRM detection can be seen in Figure 5.

4.Discussion

Lymphatic metastasis is a major factor affecting the prognosis of various cancers, including breast cancer. Although cancer cells spread through various ways, the structure of the lymphatic system makes it the prime cause of cancer metastasis. Despite the current understanding of the pathophysiology of breast cancer, the molecular mechanisms, especially those related to lymphatic metastasis, are still unclear. This may be due to the difficulty in obtaining lymphatic fluid. Identifying differentially expressed proteins and signaling pathways involved in breast cancer metastasis is critical to understanding its mechanism and exploring biomarkers related to metastasis. In this study, we used CEUS technology to extract lymphatic fluid for comparative analysis, quantitative evaluation, and functional identification of LFQ proteomics, and validated differentially expressed proteins with PRM technology.

In our study, differentially expressed proteins in lymphatic fluid of rabbits with metastatic breast cancer were mainly involved in various cellular and metabolic processes, with upregulated proteins participating in these pathways. As breast cancer develops, the tumor microenvironment inevitably changes, as does the body's metabolic environment. For example, amino acids are essential nutrients for all living cells and are vital for the proliferation and maintenance of tumor cells. Since tumors grow faster, their cells have a higher demand for amino acids. Recent studies have shown that some amino acid metabolic pathways, such as glutamine, serine, glycine, and proline, are altered in breast cancer, suggesting that amino acid transport may be crucial for the proliferation and progression of breast cancer. At the same time, mechanisms of hypoxia-adaptive metabolic responses, including increased glycolysis and decreased tricarboxylic acid cycle, serve to reduce the production of mitochondrial reactive oxygen species.

We identified upregulated proteins PDIA3, PRKAR1A, and ABCC4 in lymphatic fluid of metastatic breast cancer rabbits using LC-MS/MS. PDIA3, also known as ERp57, is a 58 kD glucose-regulated protein that also acts as a chaperone, modifying and folding proteins and having de-basic and redox functions. PDIA3 plays a role in the quality control of newly synthesized glycoproteins, participates in the assembly of major histocompatibility complex (MHC) class I molecules, and regulates immune responses and immunogenic

cell death. Despite no literature reporting a direct link between PDIA3 and breast cancer development, it has been shown to be upregulated in various cancers and involved in cancer initiation, progression, and chemosensitivity, suggesting its potential as a cancer biomarker and therapeutic target. PRKAR1A is a gene that directs the synthesis of protein kinase A (PKA) regulatory subunits and is one of the critical components of the PKA tetramer. It is the primary mediator of cAMP function in various mammalian cellular processes, including cell differentiation, proliferation, and apoptosis. Its inactivating mutations can lead to Carney syndrome, characterized by cardiac myxoma and multiple endocrine tumors. Therefore, PRKAR1A mutations may serve as a predictor of breast cancer metastasis. ABCC4 is the fourth member of the ATP-binding cassette (ABC) protein C subfamily, also known as multidrug resistance-associated protein 4 (MRP4). ABCC4 was discovered due to its role in mediating drug resistance in various tumor types. High expression of ABCC4 is detected in drug-resistant tumors, including neuroblastoma, prostate cancer, pancreatic cancer, and acute myeloid leukemia, where MRP4 expression is associated with poor prognosis. Thus, the increased drug resistance in breast cancer may be linked to the high expression of ABCC4. The upregulation of these proteins in breast cancer lymphatic fluid suggests their potential as risk factors for metastasis.

However, the technical difficulty in accurately extracting lymphatic fluid using CEUS limits its application to animal research. Additionally, proteomics technology has high economic costs, making it challenging to promote in clinical practice.

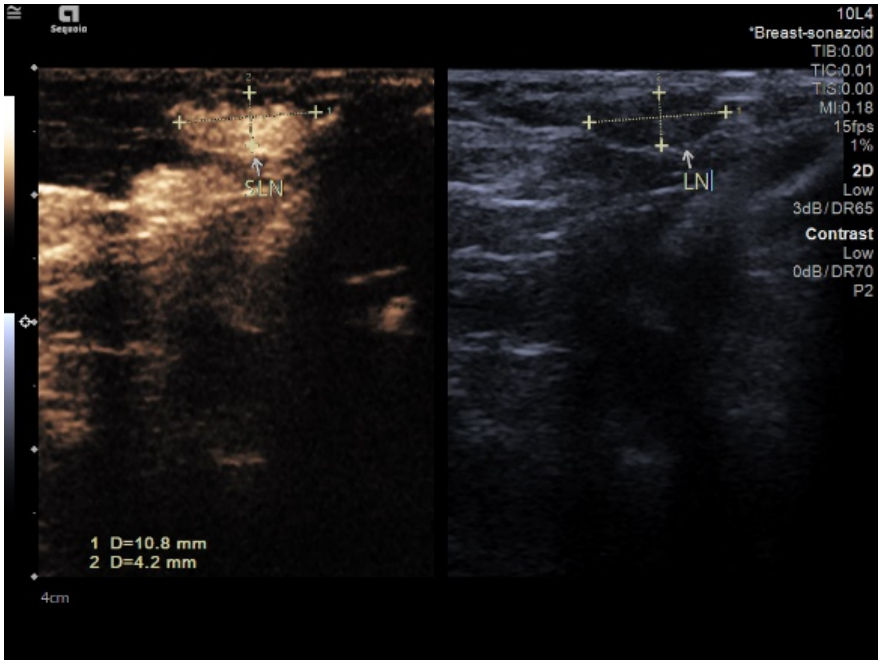
In conclusion, this study innovatively employed CEUS technology for lymphatic fluid extraction, as well as proteomic and mass spectrometry analysis techniques to investigate differentially expressed proteins and related functions in metastatic breast cancer rabbits. The upregulated or downregulated proteins and various enriched pathways identified in this study are associated with the pathophysiological processes of breast cancer onset and progression. Their expression in lymphatic fluid suggests they could serve as predictors of breast cancer metastasis, and provide new insights for future research on novel targets for metastatic breast cancer.

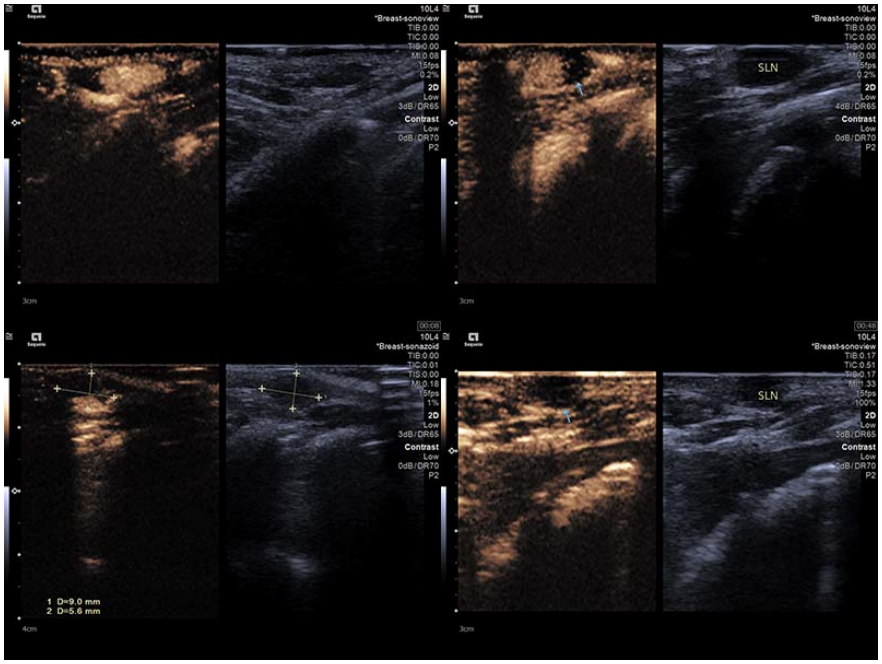
Reference

1. McGuire S. World Cancer Report 2014. Geneva, Switzerland: World Health Organization, International Agency for Research on Cancer, WHO Press, 2015. Adv Nutr. 2016 Mar 15;7(2):418-9.
2. Sun YS, Zhao Z, Yang ZN, Xu F, Lu HJ, Zhu ZY, Shi W, Jiang J, Yao PP, Zhu HP. Risk Factors and Preventions of Breast Cancer. Int J Biol Sci. 2017 Nov 1;13(11):1387-1397.
3. Elzamly S, Badri N, Padilla O, Dwivedi AK, Alvarado LA, Hamilton M, Diab N, Rock C, Elfar A, Teleb M, Sanchez L, Nahleh Z. Epithelial-Mesenchymal Transition Markers in Breast Cancer and Pathological Responseafter Neoadjuvant Chemotherapy. Breast Cancer (Auckl). 2018 Aug 1;12:1178223418788074.
4. Yeo SK, Guan JL. Breast Cancer: Multiple Subtypes within a Tumor? Trends Cancer. 2017 Nov;3(11):753-760.
5. Mani DR, Krug K, Zhang B, Satpathy S, Clauser KR, Ding L, Ellis M, Gillette MA, Carr SA. Cancer proteogenomics: current impact and future prospects. Nat Rev Cancer. 2022 May;22(5):298-313.
6. du Bois H, Heim TA, Lund AW. Tumor-draining lymph nodes: At the crossroads of metastasis and immunity. Sci Immunol. 2021 Sep 10;6(63):eabg3551.
7. Arneth B. Tumor Microenvironment. Medicina (Kaunas). 2019 Dec 30;56(1):15.
8. Cui Q, Dai L, Li J, Xue J. Accuracy of CEUS-guided sentinel lymph node biopsy in early-stage breast cancer: a study review and meta-analysis. World J Surg Oncol. 2020 May 29;18(1):112.
9. Cha YJ, Kim ES, Koo JS. Amino Acid Transporters and Glutamine Metabolism in Breast Cancer. Int J Mol Sci. 2018 Mar 19;19(3):907.
10. El Ansari R, McIntyre A, Craze ML, Ellis IO, Rakha EA, Green AR. Altered glutamine metabolism in breast cancer; subtype dependencies and alternative adaptations. Histopathology. 2018 Jan;72(2):183-190.
11. Kim S, Kim DH, Jung WH, Koo JS. Expression of glutamine metabolism-related proteins according to molecular subtype of breast cancer. Endocr Relat Cancer. 2013 May 21;20(3):339-48.

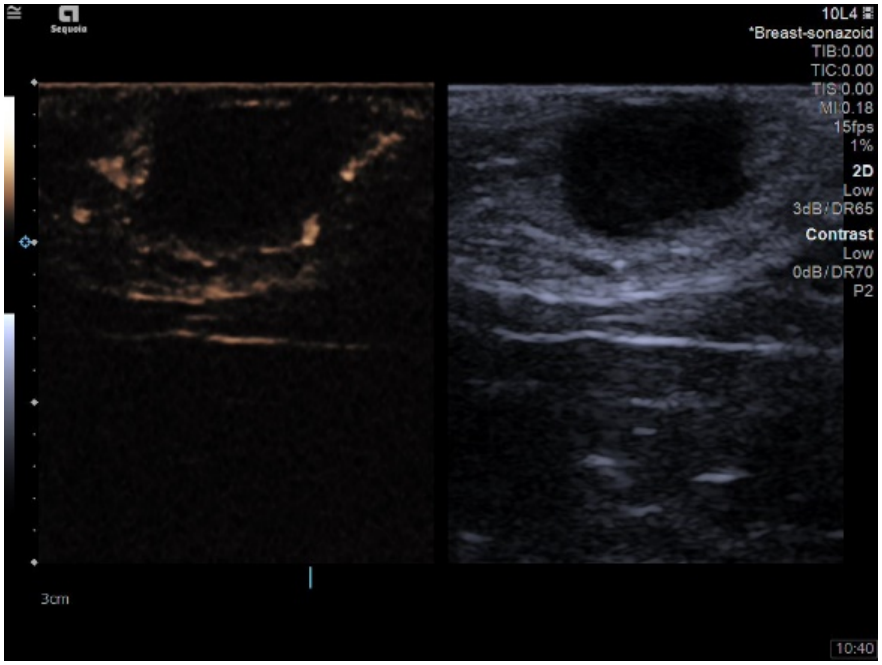
12. Craze ML, Cheung H, Jewa N, Coimbra NDM, Soria D, El-Ansari R, Aleskandarany MA, Wai Cheng K, Diez-Rodriguez M, Nolan CC, Ellis IO, Rakha EA, Green AR. MYC regulation of glutamine-proline regulatory axis is key in luminal B breast cancer. Br J Cancer. 2018 Jan;118(2):258-265.
13. Semenza GL. Hypoxia-inducible factors: coupling glucose metabolism and redox regulation with induction of the breast cancer stem cell phenotype. EMBO J. 2017 Feb 1;36(3):252-259.
14. de Heer EC, Jalving M, Harris AL. HIFs, angiogenesis, and metabolism: elusive enemies in breast cancer. J Clin Invest. 2020 Oct 1;130(10):5074-5087.
15. Chichiarelli S, Altieri F, Paglia G, Rubini E, Minacori M, Eufemi M. ERp57/PDIA3: new insight. Cell Mol Biol Lett. 2022 Feb 2;27(1):12.
16. Hettinghouse A, Liu R, Liu CJ. Multifunctional molecule ERp57: From cancer to neurodegenerative diseases. Pharmacol Ther. 2018 Jan;181:34-48.
17. Song D, Liu H, Wu J, Gao X, Hao J, Fan D. Insights into the role of ERp57 in cancer. J Cancer. 2021 Mar 1;12(8):2456-2464.
18. Park S, Park JH, Jung HJ, Jang JH, Ahn S, Kim Y, Suh PG, Chae S, Yoon JH, Ryu SH, Hwang D. A secretome profile indicative of oleate-induced proliferation of HepG2 hepatocellular carcinoma cells. Exp Mol Med. 2018 Aug 3;50(8):1-14.
19. Chen Y, Lin MC, Wang H, Chan CY, Jiang L, Ngai SM, Yu J, He ML, Shaw PC, Yew DT, Sung JJ, Kung HF. Proteomic analysis of EZH2 downstream target proteins in hepatocellular carcinoma. Proteomics. 2007 Sep;7(17):3097-104.
20. Li S, Zhao X, Chang S, Li Y, Guo M, Guan Y. ERp57-small interfering RNA silencing can enhance the sensitivity of drug-resistant human ovarian cancer cells to paclitaxel. Int J Oncol. 2019 Jan;54(1):249-260.
21. Czarnecka AM, Synoradzki K, Firlej W, Bartnik E, Sobczuk P, Fiedorowicz M, Grieb P, Rutkowski P. Molecular Biology of Osteosarcoma. Cancers (Basel). 2020 Jul 31;12(8):2130.
22. Pitsava G, Stratakis CA, Faucz FR. *PRKAR1A* and Thyroid Tumors. Cancers (Basel). 2021 Jul 30;13(15):3834.
23. Kirschner LS. PRKAR1A and the evolution of pituitary tumors. Mol Cell Endocrinol. 2010 Sep 15;326(1-2):3-7.
24. Kochel TJ, Fulton AM. Multiple drug resistance-associated protein 4 (MRP4), prostaglandin transporter (PGT), and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) as determinants of PGE2 levels in cancer. Prostaglandins Other Lipid Mediat. 2015 Jan-Mar;116-117:99-103.
25. Huynh T, Norris MD, Haber M, Henderson MJ. ABCC4/MRP4: a MYCN-regulated transporter and potential therapeutic target in neuroblastoma. Front Oncol. 2012 Dec 19;2:178.
26. Rasmuson A, Kock A, Fuskevåg OM, Kruspig B, Simón-Santamaría J, Gogvadze V, Johnsen JI, Kogner P, Sveinbjörnsson B. Autocrine prostaglandin E2 signaling promotes tumor cell survival and proliferation in childhood neuroblastoma. PLoS One. 2012;7(1):e29331.
27. Montani M, Hermanns T, Müntener M, Wild P, Sulser T, Kristiansen G. Multidrug resistance protein 4 (MRP4) expression in prostate cancer is associated with androgen signaling and decreases with tumor progression. Virchows Arch. 2013 Apr;462(4):437-43.

A B





C D



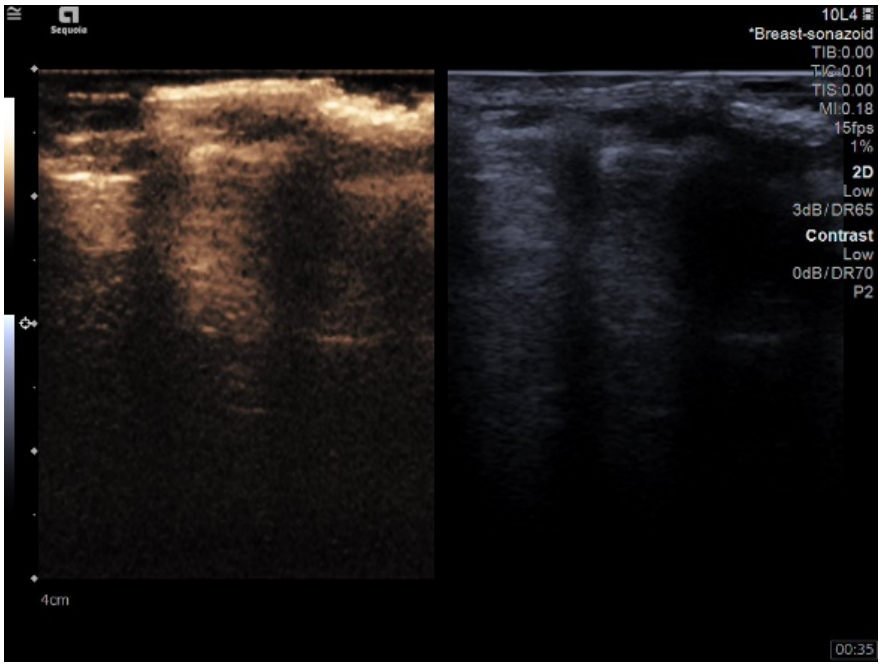
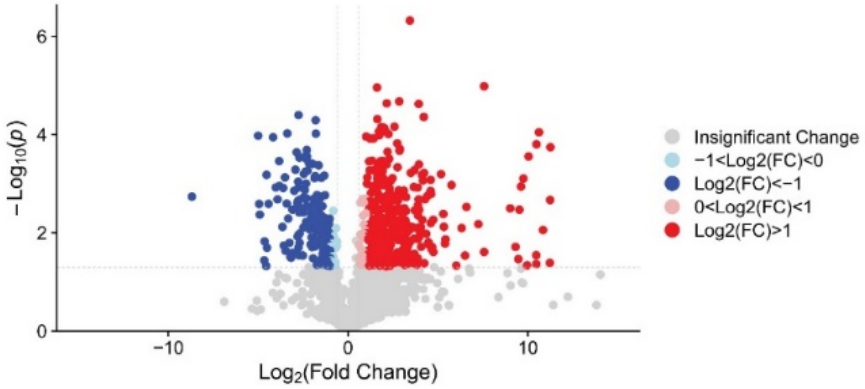
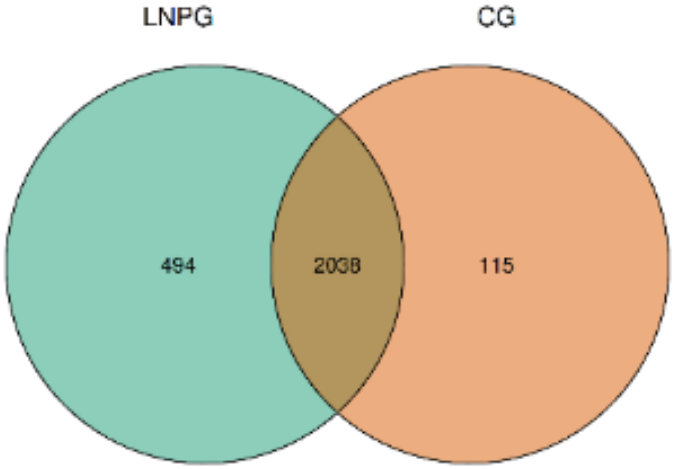


Figure 1: Ultrasound imaging patterns of sentinel lymph nodes. (A) Homogeneous enhancement, (B) Heterogeneous enhancement, (C) Peripheral ring enhancement with no internal enhancement, (D) No enhancement with lymphatic vessel bypass around the lymph node.

A B





C

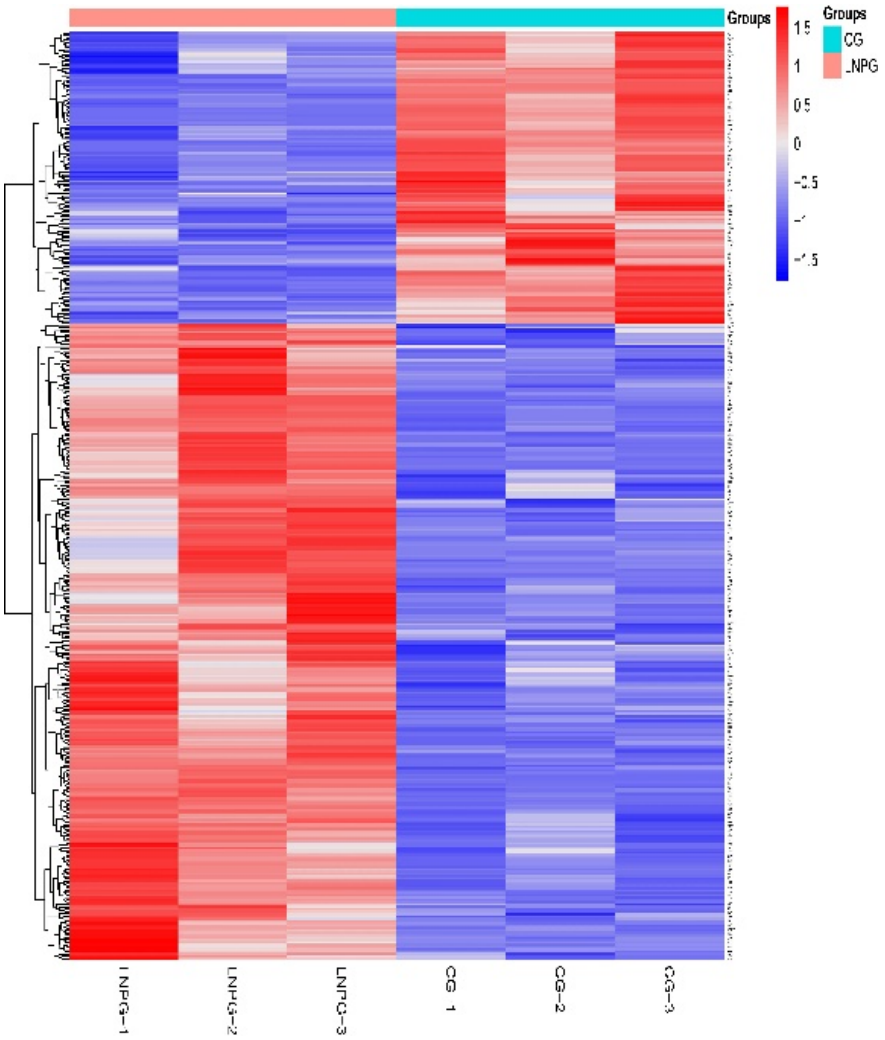
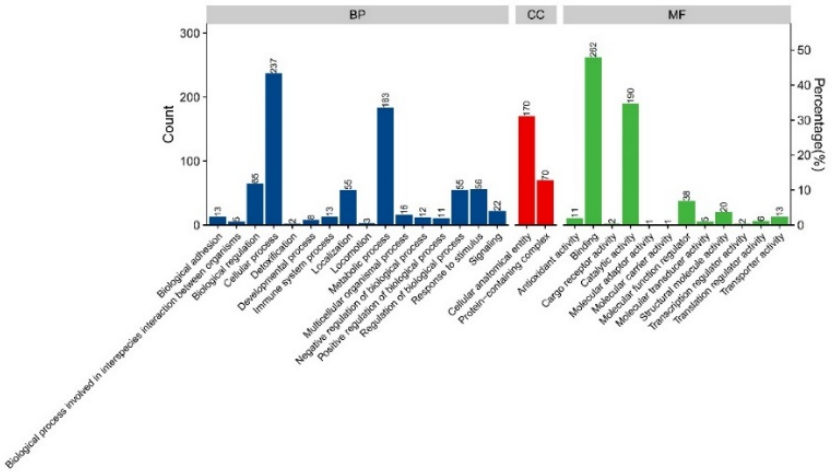
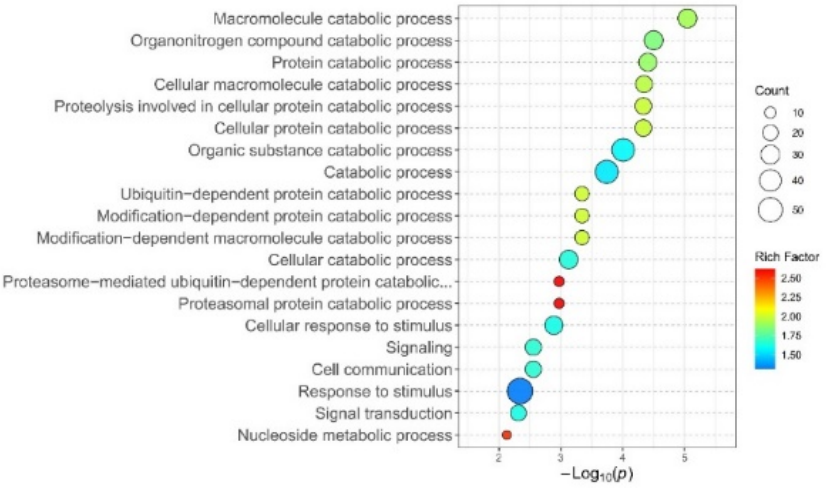


Figure 2: (A) Volcano plot of differential proteins, (B) Venn diagram of LNPG vs CG comparisons, (C) Heatmap of cluster analysis of differential proteins.

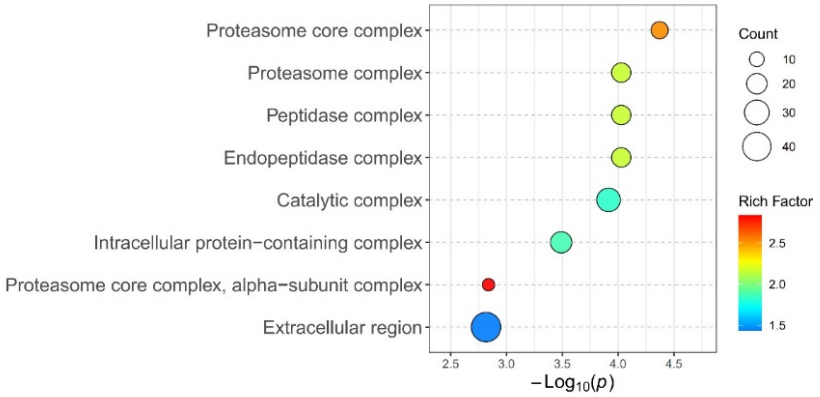
A



B



C



D

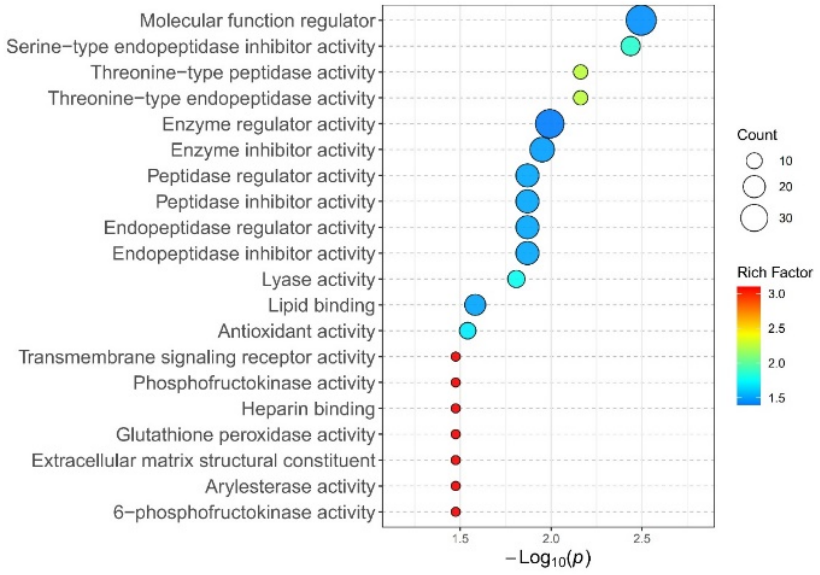
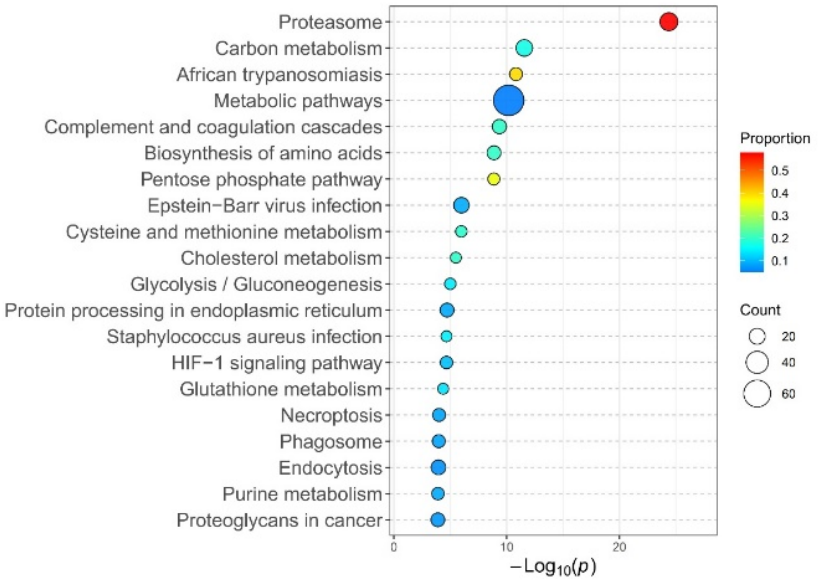


Figure 3: GO enrichment analysis of LNPG vs CG differential proteins (A) GO term annotation, (B) Top 20 BP term enrichment bubble chart, (C) Top 20 CC term enrichment bubble chart, (D) Top 20 MF term enrichment bubble chart.

A



B

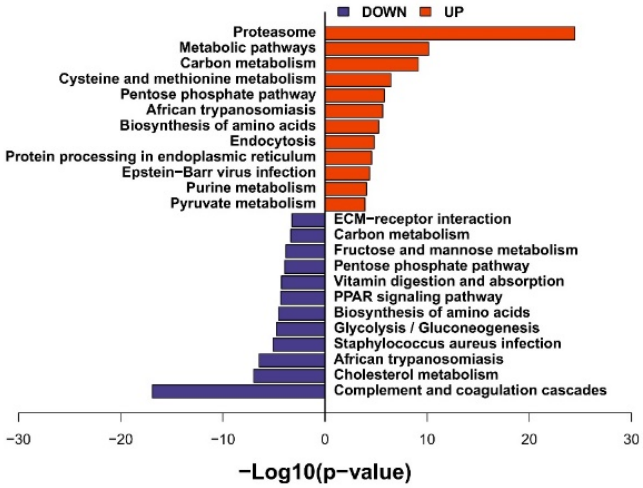
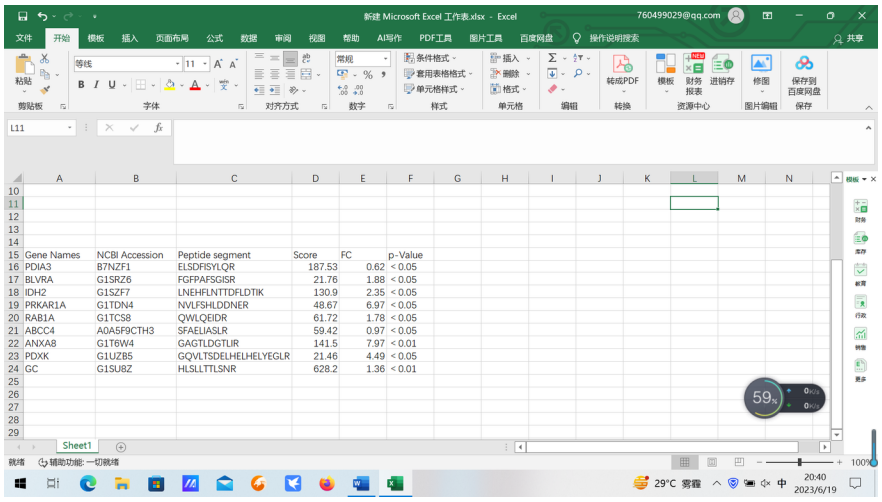


Figure 4: KEGG enrichment analysis of LNPG vs CG differential proteins (A) Top 20 KEGG pathway bubble chart, (B) Top 12 KEGG pathway enrichment butterfly chart.



Gene Names	NCBI Accession	Peptide segment	Score	FC	p-Value
PDIA3	B7NZF1	ELSDFISYLQR	187.53	0.62	< 0.05
BLVRA	G1SRZ6	FGFPAFSGISR	21.76	1.88	< 0.05
IDH2	G1SZF7	LNEHFLNTTDFLDTIK	130.9	2.35	< 0.05
PRKAR1A	G1TDN4	NVLFSHLDDNER	48.67	6.97	< 0.05
RAB1A	G1TCS8	QWLQEIDR	61.72	1.78	< 0.05
ABCC4	A0A5F9CTH3	SFAELIASLR	59.42	0.97	< 0.05
ANXA8	G1TBW4	GAGTLDGTLIR	141.5	7.97	< 0.01
PDXK	G1UZB5	GQVLTSDELHELHELYEGLR	21.46	4.49	< 0.05
GC	G1SU8Z	HLSLLTTLSNR	628.2	1.36	< 0.01

Table 1: Validation of differential proteins using LFQ proteomics.

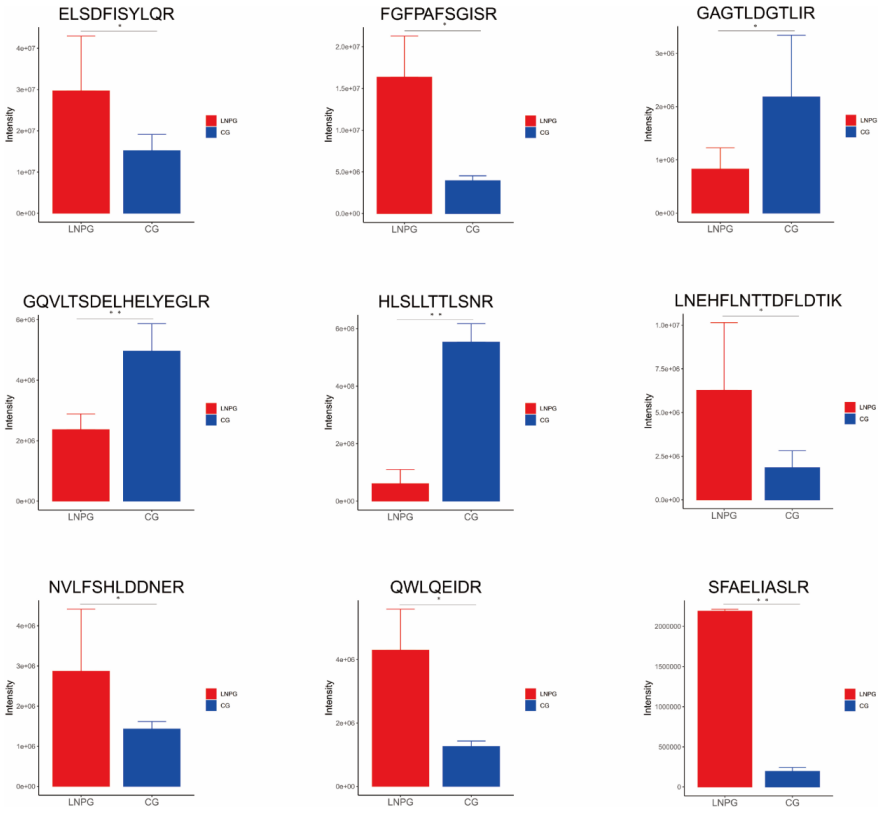


Figure 5: PRM validation results for differential proteins.

Author contributions

Jiachao Xu and Xin Zhang conceived the study and completed the paper, Chengrong Mi and Wen Wang reviewed the paper. All the authors revised and approved this paper.