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Serum proteomics analysis of feline mammary carcinoma based on label-free and PRM techniques

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ABSTRACT

Background: Feline mammary carcinoma is the third most common cancer that affects female cats.
Objectives: The purpose of this study was to screen differential serum proteins in feline and clarify the relationship between them and the occurrence of feline mammary carcinoma.
Methods: Chinese pastoral cats were used as experimental animals. Six serum samples from cats with mammary carcinoma (group T) and six serum samples from healthy cats (group C) were selected. Differential protein analysis was performed using a Label-free technique, while parallel reaction monitoring (PRM) was performed to verify the screened differential proteins.
Results: A total of 82 differential proteins were detected between group T and group C, of which 55 proteins were down regulated and 27 proteins were up regulated. Apolipoprotein A-I, Apolipoprotein A-II (ApoA-II), Apolipoprotein B (ApoB), Apolipoprotein C-III (ApoC-III), coagulation factor V, coagulation factor X, C1q, albumen (ALB) were all associated with the occurrence of feline mammary carcinoma. Differential proteins were involved in a total of 40 signaling pathways, among which the metabolic pathways associated with feline mammary carcinoma were the complement and coagulation cascade and cholesterol metabolism. According to the Label-free results, ApoB, ApoC-III, ApoA-II, FN1, an uncharacterized protein, and ALB were selected for PRM target verification. The results were consistent with the trend of the label-free.
Conclusions: This experimen is the first to confirm ApoA-II and ApoB maybe new feline mammary carcinoma biomarkers and to analyze their mechanisms in the development of such carcinoma in feline.

Keywords: Feline mammary carcinoma; label-free; PRM; proteomics

INTRODUCTION

Feline mammary carcinoma (FMC) is the third most common tumor in cats. Mammary tumors are most common in cats between the ages of 10 and 12 years. The occurrence of feline mammary carcinoma is closely related to age, breed, hormone level and neutering [1]. FMC is highly invasive and histopathological observation after surgical resection of the tumor shows that 27% of the cases have local lymph node metastasis [2]. Despite advances in surgery,

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Conflict of Interest

The authors have no conflicts of interest to declare.

Author Contributions

Conceptualization: Zheng JS, Wei RY. Data curation: Wei RY, Zheng JS, Ruan HR. Formal analysis: Wei RY, Zheng JS. Investigation: Wei RY, Zheng JS, Wang Z. Methodology: Wei RY, Zheng JS, Zhu TT, Hou KW. Project administration: Wu R. Resources: Wu R. Software: Wei RY, Zheng JS, Wei X. Supervision: Wu R. Validation: Wei RY, Zheng JS. Visualization: Wei RY, Zheng JS. Writing - original draft: Wei RY, Zheng JS, Wu R. Writing - review & editing: Wei RY, Zheng JS, Wu R.

radiation and chemotherapy, the survival rate for mammary tumors in cats remains low, because tumors are already advanced when they are discovered, and therefore, are not suitable for radical resection. In order to improve the survival rate of cats with malignant mammary tumors, screening and identification of early biomarkers would be particularly important.

Proteomics have been widely used in tumor marker screening and other fields [3]. Compared with the proteome quantitation based on labeling approaches, the label free quantitation (LFQ) has the advantages of small sample sizes and the ability to detect an increasing number of low abundant proteins [4]. At the same time, LFQ allow a simultaneous detection of proteome without preparing experimental samples by introducing stable isotopes [5]. Moreover, LFQ is capable of a large number of samples from different sources [6,7]. These advantages make it the most commonly employed proteome quantification technology [8].

PRM is a proteomic technology of quantification performed using high-resolution hybrid mass spectrometers [9,10]. Compared with traditional validation methods such as western blot and enzyme-linked immunosorbent assay, parallel reaction monitoring (PRM) can detect multiple target proteins at the same time. It yields quantitative data over a wide dynamic range [11]. It has both qualitative and quantitative advantages, and does not require antibodies. PRM also has certain advantages compared with selected reaction monitoring (SRM), such as it is relatively easier to build the data acquisition method and provides high specificity. The results from PRM analysis showed at least 10-fold improvements in specificity and sensitivity and consumed less volume of serum samples compared with SRM. In addition, PRM acquired a full MS/MS spectra but in SRM, only three to five transitions are monitored [12]. These advantages make it an ideal method for proteomics validation.

In this study, label-free technology combined with PRM target validation was used to conduct proteomic analysis and research on serum samples from affected cats. This study also aimed to lay a foundation for the screening of biomarkers for the early diagnosis and study of feline mammary carcinoma.

MATERIALS AND METHODS

Ethics

The animal study was approved by the Institutional Animal Care and Use Committee (IACUC). All experimental procedures were performed in accordance with the regulations for the Administration of Affairs Concerning Experimental Animals approved by the school Council of Heilongjiang Bayi Agricultural University of China Daqing. The study protocol was approved by the Ethics Committee on the Use and Care of Animals of Heilongjiang Bayi Agricultural University (Daqing, China).

Materials

The disease material came from the feline mammary carcinoma cases received in the animal hospital of Heilongjiang Bayi Agricultural University. Six Chinese pastoral cats with an average age of 6.33 years with mammary carcinoma confirmed by histopathology were marked as the experimental group (group T). None of the cats had been treated with chemotherapy or radiotherapy. Another six serum samples from healthy cats of the same breed and an average age of 6.5 years were taken and labeled as the control group (group C). This experimental group information is shown in **Table 1**. Before performing a

Table 1. Experimental sample basic information

Sample	Variety	Age	Gender	Spaying status	Tumor type	Disease stage
T-1	Chinese pastoral cat	5	Female	Unspayed	Breast carcinoma	Without metastasis
T-2	Chinese pastoral cat	8	Female	Unspayed	Breast carcinoma	Without metastasis
T-3	Chinese pastoral cat	7	Female	Unspayed	Breast carcinoma	Without metastasis
T-4	Chinese pastoral cat	5	Female	Unspayed	Breast carcinoma	Without metastasis
T-5	Chinese pastoral cat	9	Female	Unspayed	Breast carcinoma	Without metastasis
T-6	Chinese pastoral cat	4	Female	Unspayed	Breast carcinoma	Without metastasis
C-1	Chinese pastoral cat	6	Female	Unspayed	-	-
C-2	Chinese pastoral cat	7	Female	Unspayed	-	-
C-3	Chinese pastoral cat	9	Female	Unspayed	-	-
C-4	Chinese pastoral cat	5	Female	Unspayed	-	-
C-5	Chinese pastoral cat	8	Female	Unspayed	-	-
C-6	Chinese pastoral cat	4	Female	Unspayed	-	-

tumor resection, five to eight mL blood was collected from the jugular vein and placed in a centrifugal tube without anticoagulant. The samples from the two groups were placed in refrigeration at 4°C for two hours, centrifuged at 4°C and 3,000 rpm for 10 min and 100 μL/tube of the serum was stored at −80°C.

Protein quantification and enzyme digestion

The collected serum was centrifuged for 30 min at 14,000 rpm. The supernatant was collected and quantified using 5 μL. The serum total protein concentration has been adjusted to be consistent prior to sampling. The total protein concentration was 50 μg/mL. The rest was stored at −80°C. The protein concentration was determined by the Bradford method. Protein samples were used for SDS-PAGE analyses. Cut the strip and add the decolorizing solution until the decolorization is completed. Next 200 μL of 75% acetonitrile (ACN) was added to each tube for five min and repeated three times. Pure water (200 μL) was then added for 5 min and repeated three times. Then, 200 μL 50 mM ammonium bicarbonate (ABC) was added for five min and repeated three times. After adding 30 μL 50 mM ABC, trypsin was added in the ratio of protein: trypsin of 50:1, and kept overnight at 37°C. The next day, add 100% ACN and oscillate for five min. Add 30 μL 0.1% formic acid to the precipitate. Add 200 μL 100% ACN, oscillate for five min, and take the supernatant to freeze dried.

Mass spectrometry detection

The products after enzymatic hydrolysis were analyzed by LC-MS/MS (Thermo Scientific, USA). Separation was carried out using a nanoliter flow HPLC liquid phase system, EASY-nLC1000 which was directly interfaced with the Thermo Orbitrap QE mass spectrometer. The liquid phase A was 0.1% acetonitrile formate aqueous solution (2% acetonitrile). B solution was 0.1% acetonitrile formate aqueous solution (84% acetonitrile). The chromatographic column, Thermo EASY column SC200 150 μm × 100 mm (RP-C18) (Thermo Scientific, USA) was balanced with 100% liquid A. Samples were collected from the automatic sampler to Thermo EASY column SC001 traps 150 μm × 20 mm (RP-C18) (Thermo Scientific, USA), then separated by the chromatographic column. The flow rate was 300 nL/min. The correlated liquid phase gradients were as follows:

- 0 to 105 min, the linear gradient of liquid B was from 0% to 45%.
- 105 to 110 min, the linear gradient of liquid B was from 45% to 100%.
- 110 to 120 min, Liquid B was maintained at 100%.

The enzymatic hydrolysate was separated by high performance liquid chromatography and analyzed by mass spectrometry using a Q-Exactive mass spectrometer (Thermo Scientific,

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USA). The analysis duration was 90 min, the detection method was positive ion mode and the scanning range of the parent ion was 300–2,500 m/z.

Protein identification and analysis

MaxQuant-software (Computational Systems Biochemistry under Prof. Jürgen Cox, DEU, Germany) was used for database identification and quantitative analysis of mass spectrometry data. The RAW file was submitted to the MaxQuant server when searched, an established database, UniProt Felinae.fasta (**Table 2** for parameters) was selected, and the database search was performed.

The strategy of match between runs was applied to serum protein samples from group T and group C. Perseus software (Computational Systems Biochemistry under Prof. Jürgen Cox, DEU, Germany) was used to supplement the missing values according to the low values of the normal distribution. After supplementation, the t-test was used to analyze the differential proteins. Differential proteins were screened according to a $p < 0.05$ and a fold-change > 1.5.

Verification of experimental results using PRM target metabolomics

Proteins were extracted and digested using trypsin, and all samples in the group were mixed in equal amounts to construct a protein mix, which was then built into a database. Each sample was separated by SDS-PAGE and analyzed by mass spectrometry using a Q-Exactive Fusion mass spectrometer (Thermo Scientific, USA).

Bioinformatics analysis

The Gene Ontology (GO) concept is intended to make possible, in a flexible and dynamic way, the annotation of homologous gene and protein sequences in multiple organisms using a common vocabulary that results in the ability to query and retrieve genes and proteins based on their shared biology. Three independent ontologies accessible on the World-Wide Web (http://www.geneontology.org) are being constructed: biological process, molecular function and cellular component [13]. Another use for GO ontologies that is gaining rapid adherence is the annotation of gene-expression data, especially after these have been clustered by similarities in pattern of gene expression [14]. Both Kyoto Encyclopedia of Genes and Genomes (KEGG) and GO parsing were performed using the UniProt annotation database of Felis catus. Protein interactions were analyzed using a String database. Gene products may be annotated to one or more GO nodes, and because of the structure of GO, a gene annotated to a given node is thus also annotated to all ancestral nodes of that specific node [15].

Table 2. Search parameters

Item	Values
Main search ppm	6
Missed cleavage	2
MS/MS tolerance ppm	20
De-isotopic	True
Enzyme	Trypsin
Database	Felinae
Fixed modification	Carbamidomethyl (C)
Variable modification	Oxidation (M), Acetyl (Protein N-term)
Decoy database pattern	Reverse
LFQ	True
LFQ min ratio count	1
Match between runs	2 min

The results filtering parameter was a peptide FDR ≤ 0.01.
LFQ, label-free quantification; FDR, false discovery rate.

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RESULTS

Determination of protein concentration

The protein concentration of each group of samples was measured after mixing. The protein concentration of each sample was calculated using the Bradford standard curve. The sample original concentration of group C was 22.27 mg/mL. The sample original concentration of group T was 26.29 mg/mL.

Differential protein screening

A total of 82 differentially expressed proteins were detected in group T, compared to group C (fold change >1.5, $p < 0.05$); of which 55 proteins were down regulated (including six unknown proteins) and 27 proteins were up regulated (including 12 unknown proteins) (**Table 3**).

Table 3. List of differential protein

Accession	Gene name	Ratio (T/C)	p value	Sig (T/C)
M5AXY1	*RBP4*	0.340154831	0.005468144	–1
P19707	*SAA1*	0.054343484	0.000288975	–1
M3VUN8		0.484964825	0.001937821	–1
Q6SA95	*F9*	0.508561675	0.011208751	–1
M3VV12	*CPN1*	0.433581397	0.000846272	–1
M3VYX8	*CKM*	0.054318521	0.002185755	–1
M3W922	*F5*	0.039875786	0.00162386	–1
M3W9M0	*CTSC*	0.623818382	0.000846481	–1
M3WAT6	*PLA2G7*	0.244947483	0.000547107	–1
M3WCX6	*SERPINA3*	0.472553687	0.006494813	–1
M3WFT4	*HSPA5*	0.254585428	0.003848397	–1
M3WG98	*FETUB*	0.338539837	0.000413087	–1
M3WKC7	*F10*	0.589201352	0.001317432	–1
M3WYZ8	*PGAM2*	0.444775417	0.022068764	–1
M3WN61	*ITIH4*	0.598273187	0.002881156	–1
M3WN87	*APOA2*	0.154237646	1.31441E-05	–1
M3WNX1	*APOC2*	0.265080498	0.001790853	–1
M3WZC2		0.119091719	0.000846624	–1
M3X116	*MAN2B1*	0.294465392	0.008850929	–1
M3X6U7		0.563686709	0.000238362	–1
M3XAB5	*QSOX1*	0.47045168	0.003733989	–1
M3W955	*APOA4*	0.645298212	0.000358232	–1
M3VWC6	*CD14*	0.584999947	0.007776608	–1
M3VWV2	*F11*	0.506921572	0.046079809	–1
M3VZ45	*PYGL*	0.30524973	0.022085741	–1
M3W9I5	*VTN*	0.655991477	0.020887897	–1
M3WAY1	*CPB2*	0.319637263	0.041152642	–1
M3WB06	*FN1*	0.360201549	5.61189E-05	–1
M3WCW9	*PPP4R4*	0.00420552	3.88075E-08	–1
M3WCX4	*SERPINA4*	0.645490262	0.007234816	–1
M3WEG4	*GPLD1*	0.629841982	0.000223236	–1
M3WEJ3	*LOC101097240*	0.52457714	0.015276461	–1
M3WFU5	*HABP2*	0.633298239	0.027278073	–1
M3WHB2	*HGFAC*	0.544596167	0.025876344	–1
M3WJK3	*C4BPA*	0.60350631	0.01046831	–1
M3XC93	*ACTA1*	0.597941906	0.009007362	–1
M3WKJ1	*TGFBI*	0.53966298	0.043554677	–1
M3WMF1	*CPN2*	0.583225783	0.017732428	–1
M3WNF1	*PON1*	0.614679704	0.000325913	–1
M3WNL1	*LCAT*	0.390329187	0.000912172	–1
M3WSC8	*APOC3*	0.045088855	0.042092242	–1
M3WZ75	*CRP*	0.618899644	0.000233478	–1

(continued to the next page)

Table 3. (Continued) List of differential protein

Accession	Gene name	Ratio (T/C)	p value	Sig (T/C)
M3X096	*CEL*	0.550054256	0.018068838	–1
M3X8P7	*APOD*	0.18635496	0.002091716	–1
M3X9R6	*APOB*	0.469251471	0.002458933	–1
M3XAV0	*PROS1*	0.589338131	0.01171963	–1
P07405	*HBA*	0.503946462	0.003003589	–1
P07412	*HBB*	0.119153625	0.012125021	–1
M3W3J4	*CA2*	0.304828166	0.002103913	–1
M3W949	*TIMP1*	0.445288076	0.020020294	–1
M3WF45	*THBS1*	0.252986799	0.038081664	–1
M3WGM9	*DNASE1*	0.229665329	0.007372795	–1
M3WND9	*LOC101091307*	0.478588155	0.000121836	–1
M3WT57	*PPBP*	0.12729283	0.000135858	–1
M3XFW7	*CLEC3B*	0.55652626	0.000805583	–1
M3WN28	*FGG*	8.446550951	5.44584E-05	1
P14450	*FGA*	24.00986865	8.8154E-06	1
A0A0A0MQ10	*B2M*	3.006122799	0.018135641	1
M3W022	*FGA*	52.60613479	2.79305E-06	1
M3W285	*IGFBP2*	1.805260304	0.000675015	1
M3W3E7	*PZP*	1.637796598	0.001506633	1
M3W3L1	*CDRT1*	45.511574	0.001091684	1
M3WCL4	*CD5L*	1.626557922	0.012816445	1
M3WF07	*AVPR2*	2.198814542	0.005486296	1
M3WS14	*CHID1*	16.90172405	0.000362214	1
M3WS63	*C1QA*	1.792088653	0.003957038	1
M3WUF8		1.683414605	0.02320278	1
M3X1D8	*POLM*	2.019029718	0.040168563	1
M3X2G1		1.657819485	0.002520486	1
M3X4B4		1.608082992	0.025560436	1
M3X4U4		1.71662824	0.035243888	1
M3X8D0	*LOC101098092*	2.120704462	0.003983756	1
M3XAZ9		3.66814103	0.003153746	1
M3XBL5	*JCHAIN*	1.718928362	0.002066203	1
M3XBM6	*IGHM*	1.559017816	0.03476085	1
M3XG21		1.791676147	0.00174948	1
M3XG22		2.983686145	0.001869249	1
M3VUY9	*C1QB*	1.662661959	0.005309183	1
M3WFB3	*TAF1C*	1.582380649	0.010907316	1
M3WII3	*FGB*	128.4162109	2.11552E-07	1
M3XES3		1.572430224	0.017941538	1
M3X833		1.629792726	0.003791816	1

Sig (T/C) = 1 means this protein was up-regulated in group T compared with group C; Sig (T/C) = –1 means this protein was reduce in group T compared with group C.

The differential proteins between the two sets of samples can be seen in **Fig. 1**, where each dot represents a protein. Green dots represent down-regulated proteins, and red dots represent up-regulated proteins. The ordinate is the negative logarithm of the *p* value and the abscissa is the logarithm of the ratio of the two groups. The larger the value, the more significant the difference in protein obtained and the greater the differential expression multiple of the protein between the two groups.

GO analysis

GO annotation results were divided into three categories: cellular component, molecular function and biological process annotations. The results of GO analysis are shown in **Table 4**. The results of cellular component annotation are shown in **Fig. 2A**. The results of molecular function annotation are shown in **Fig. 2B**. The results of biological process annotation are shown in **Fig. 2C**. The main proteins involved were ApoA-I, ApoA-II, ApoB, ApoC-III and blood coagulation factor V.

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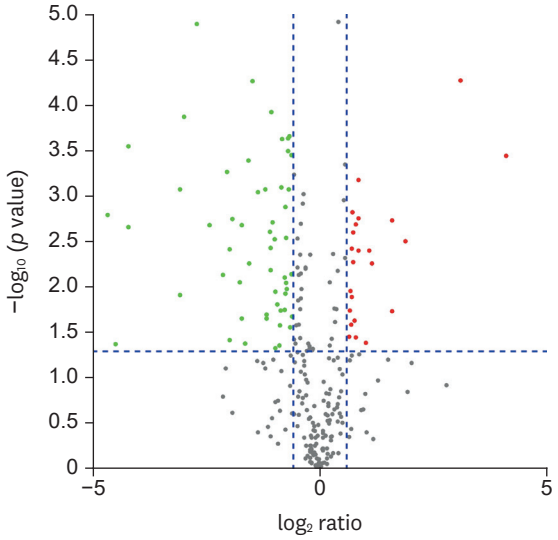

Fig. 1. Differential protein volcano map.

KEGG pathways of differentially expressed proteins

KEGG analysis showed that differential proteins were involved in 40 signaling pathways, with the up-regulated proteins concentrated in the proteasome. The down-regulated proteins were concentrated in complement and coagulation cascades, cholesterol metabolism, Staphylococcus aureus infection and the biosynthesis of antibiotics (**Fig. 3A and B**). The signaling pathways involved in additional differential proteins were complement and coagulation cascades and cholesterol metabolism. The main different proteins involved in these pathways were ApoA-IV, ApoA-II, ApoC-III, ApoB and C1q. Down regulated proteins included ApoA-IV, ApoA-II, ApoC-III and ApoB (**Table 5**).

Regulation analysis of protein interaction network

Compared with group C, the group T PPI network (https://string-db.org/) contained 45 proteins by cytoscape (Cytoscape Consortium, USA). There were nine up-regulated proteins and 36 down-regulated proteins (**Fig. 4**). Sixteen different protein annotations were obtained after removing unknown proteins. They were ApoA-II, HBB, ApoB, blood coagulation factor V, TIMP1, ApoC-III, CKM, SERPINA3, THBS1, HBA, FETUB, FGB, FGG, DNASE1, blood coagulation factor X and PPBP. Of those, ApoA-II, ApoB, and ApoC-III were differential proteins with high expression levels.

Table 4. GO analysis

Gene ontology	GO term	GO term ID	Proteins
Cellular component	Extracellular region	GO:0005576	*ApoA-IV*, *ApoM*, *ApoH*, *ApoA-II*, *ApoC-II*, *ApoA-I*, *ApoC-III*, *ApoD*, *ApoB*
	Nucleus	GO:0005634	*CLEC3B*, *DNASE1*, *SERPINB10*, *MGA*, *PSMB4*
	Cytoplasm	GO:0005737	*ApoA-I*, *ApoD*, *ApoB*, *HBA*, *blood coagulation factor V*
	Membrane	GO:0016020	*CPN1*, *CD14*
	Plasma membrane	GO:0005886	*ACTA1*
Molecular function	Receptor binding	GO:0005102	*ApoA-II*, *ApoA-I*, *ApoB*, *ApoC-III*
	Metal ion binding Categories	GO:0046872	*HBB*, *HBA*, *blood coagulation factor V*, *TIMP1*
Biological process	Immune system process	GO:0002376	*IGHE*, *IGLV2 33*
	Membrane organization	GO:0061024	*THBS1*, *IGHE*
	Signal transduction	GO:0007165	*ApoA-I*, *ApoC-II*
	Vesicle-mediated transport	GO:0016192	*CD14*, *THBS1*, *VTN*

GO, gene ontology.

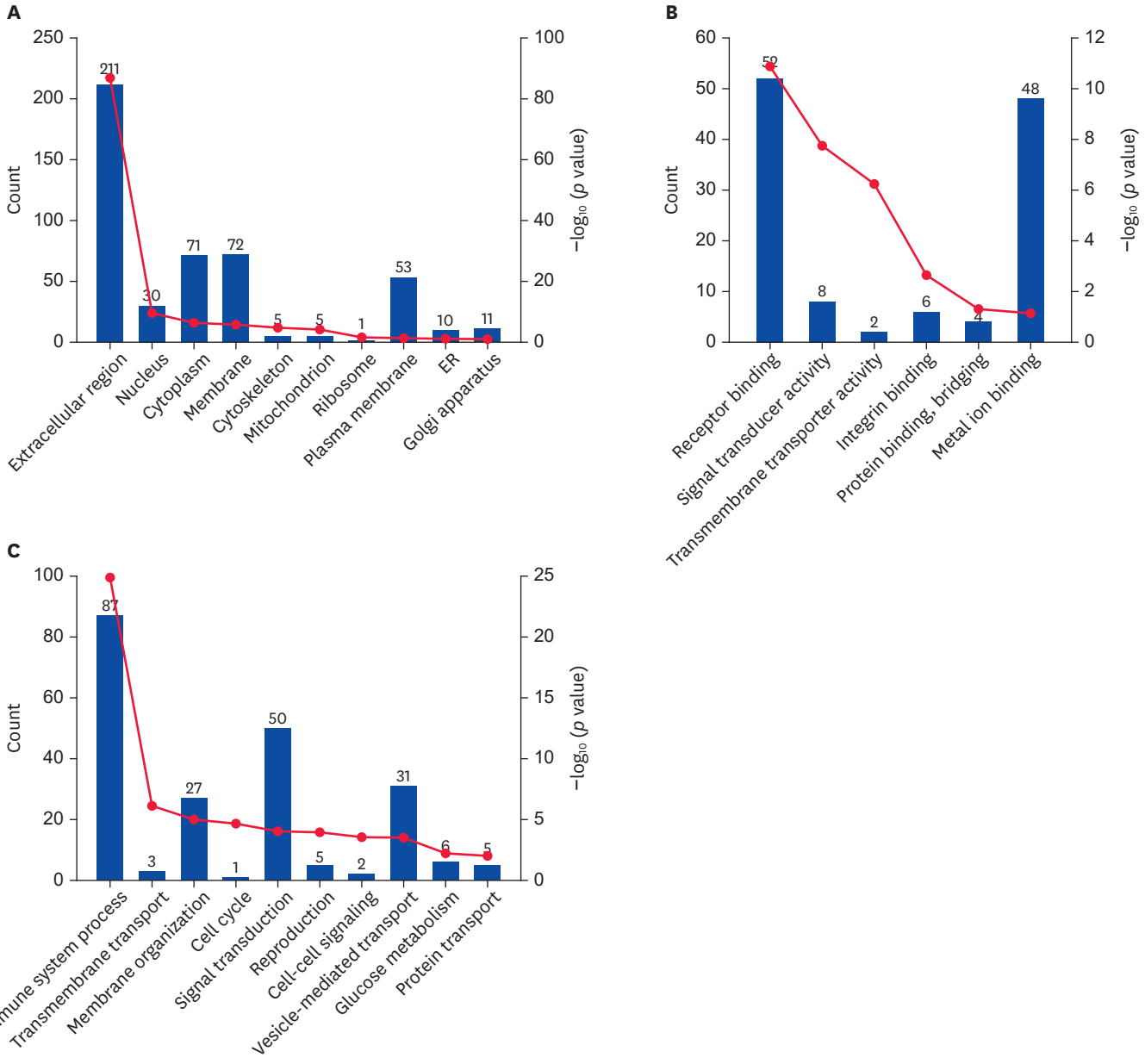

Fig. 2. Differential protein GO. The x-coordinate represents the cell components of GO Slim, the y-coordinate histogram is the number of proteins, and the red line is the enrichment degree of each cell component. (A) cellular component, (B) Molecular function, (C) Biological process.
GO, gene ontology.

Use the PRM target proteomics technique to verify the experimental results

Table 6 shows the quantitative results of PRM protein verification, including six in each of the T and C groups. The concentration was determined after mixing in the sample group. The results showed that the concentration of samples in group T and C met the experimental conditions and could be further tested.

Skyline software (MacCoss Lab, USA) was used to build a database, extract and analyze the mass spectrometry data and to calculate the ratio of each protein to each sample. Thirteen target proteins were selected for PRM validation. Because of the characteristics and abundances of some target proteins, six target proteins were quantified after the experiment

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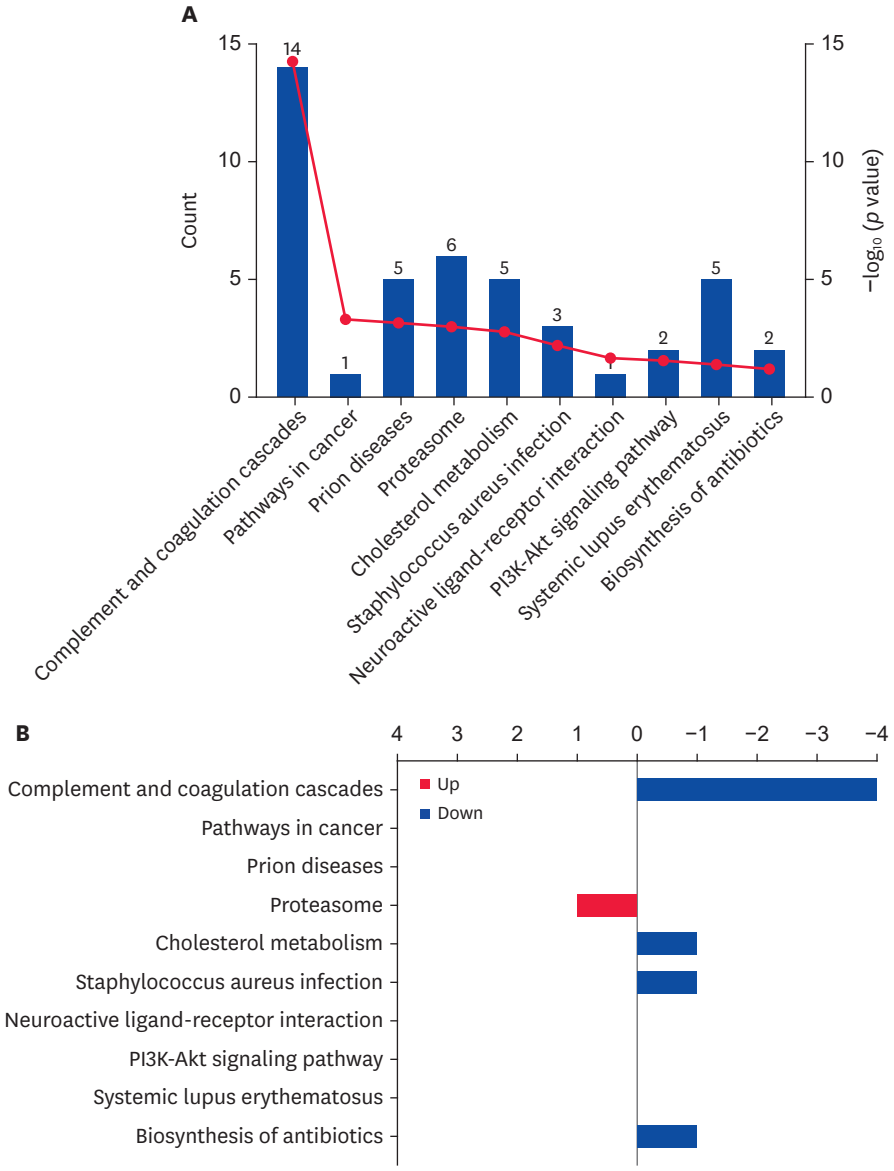

Fig. 3. KEGG analysis. (A) Analysis of KEGG pathways for differentially expressed proteins. (B) The number of up-regulated and down-regulated proteins. The abscissa represents KEGG entries, the ordinate histogram is the number of proteins, and the red line is the enrichment of each KEGG entry.
KEGG, Kyoto Encyclopedia of Genes and Genomes.

Table 5. List of differential proteins

Gene name	Description	Ratio (T/C)
ApoA-II	Apolipoprotein A-II	0.15
ApoC-III	Apolipoprotein C-III	0.045
ApoB	Apolipoprotein B	0.50
ApoA-IV	Apolipoprotein A-IV	0.65

was repeated three times. The relative expression was calculated and as can be seen from **Table 7**, the PRM results were consistent with the trend from the label-free results.

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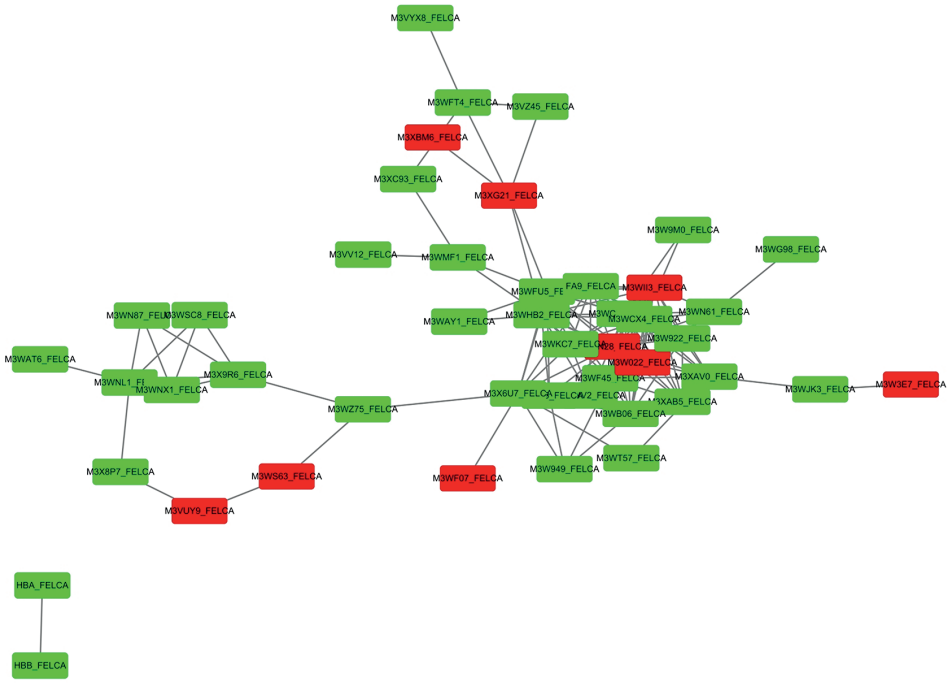

Fig. 4. Screening of protein-protein interaction network for differentially expressed proteins.

Table 6. Parallel reaction monitoring protein concentration

Sample No	Sample concentration (μg/uL)	Sample volume (μL)	Total protein (μg)	Whether it meets the experimental requirements
C	24.27	1,000	24,268.92	Meet
T	26.29	1,000	26,291.47	Meet

Table 7. Parallel reaction monitoring and label-free protein expression

Protein name	PRM protein expression ratio (T/C)	Label free protein expression ratio (T/C)
ApoB	0.384125116	0.502469215
ApoC III	0.389446911	0.045088854
ApoA II	0.351008361	0.154237646
FN1	0.041418351	0.360201549
Uncharacterized protein	0.720178648	0.635512035
ALB	0.886057883	0.851275633

DISCUSSION

The protein is early indicators or sensitive biomarkers for various diseases, but, there are few reports on the screening and identification of differential proteins in feline mammary carcinoma. The results showed that ApoA-I, ApoA-II, ApoC-III, ApoB, blood coagulation factor V, blood coagulation factor X, C1q and albumen (ALB) were the differential proteins related to the occurrence of feline mammary carcinoma.

ApoA-I is a primary structural and functional portion of high-density lipoprotein (HDL) and plays an indispensable role in cholesterol transportation and metabolism homeostasis. ApoA-II is a component of HDL where it has an important role in directing the fate of the metabolism of the lipid in the HDL. HDLs have been implicated in cholesterol delivery in breast cancer [16]. Cancer cells require cholesterol and other membrane components to optimize growth. Feline mammary carcinoma cells are also likely to consume a large amount

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of HDLs, which may have contributed to the significant decrease of ApoA-I and ApoA-II in our study. Also, there are reports that reduced serum ApoA-I levels correlate with the progression of human breast cancer and associated with the appearance of metastases [17,18]. In addition, relatively reduced serum HDL/ApoA-I levels have been found in patients with breast cancer [19]. In the clinic, the diagnostic value of APOA-I as a potential tumor biomarker was also reported in multiple malignancies, such as breast cancer, bladder cancer, ovarian cancer, lung cancer and cholangiocarcinoma [20]. Studies have shown the level of ApoA-II in serum was dramatically reduced in patients with gastric cancer and multiple myeloma [21,22]. Furthermore, the combination of ApoA-II and lipid significantly promoted the growth of pancreatic cancer cell lines and cell lines from lung, breast and prostate cancers [23]. At the same time, another study suggest that the expression of ApoA-II was significantly reduced in pancreatic cancer and ApoA-II might be used as an early diagnostic marker and risk factor for it [24]. In this experiment, ApoA-II was decrease most significantly in group T compared with group C, suggesting that Apo A-II could be used as a candidate biomarker for feline mammary carcinoma. However, further research is required to confirm this conclusion.

ApoB is the main apolipoprotein component of chyle particles and low density lipoprotein cholesterol (LDL-C). Research showed that lower levels of ApoB was associated with a 20% to 30% higher risk of developing estrogen receptor–positive breast cancer [25]. But another study showed that low expression of ApoB can reduce the risk of mammary and ovarian cancer [26]. In another syudy, ApoB was positively associated with cancer risk among men, female breast cancer risk was inversely associated with ApoB [27]. At the same time, another report examined the relationship of ApoA-I and ApoB with breast cancer risk and found no association [26]. So the relationship between ApoB and breast cancer is not well established. However, the observed lipid profile (higher HDL-C and ApoA-I, and lower non-HDL-C and ApoB) could reflect higher levels of, or greater response to, endogenous estrogens, which are associated with an increased risk of breast cancer, particularly estrogen receptor–positive disease [28]. In this experiment, the decreased expression of ApoB in group T may be related to the occurrence of feline mammary carcinoma. However, the mechanism of ApoB in feline mammary carcinoma still needs further study.

ApoC-III is a small soluble protein residing on the surface of ApoA and ApoB, and it can be found in HDL, very-low-density lipoprotein and chylomicron. Many studies have confirmed that ApoC-III can be used as a biomarker of cancer. ApoC-III gene polymorphisms are associated with cancer risk and studies have shown that ApoC-III expression levels in gastric cancer patients are reduced, making it a potential biomarker for gastric cancer [29]. At the same time, some studies showed that plasma ApoC-III is significantly decreased in patients with pancreatic cancer, ApoC-III may also be a biomarker of pancreatic cancer [30,31]. In another study, ApoC-III expression was significantly reduced in small cell lung cancer (SCLC) tissues, compared to non-small cell lung cancer and normal lung samples and indicating that ApoC-III could be used as a differentiating marker for SCLC [32]. The results of serum samples from patients with papillary thyroid carcinoma (PTC) identified that ApoC-III can be used as a biomarker for PTC and an indicator for PTC staging [33]. However, there are few reports on the relationship between ApoC-III and feline mammary carcinoma. In this experiment found that ApoC-III was reduced in group T compared to group C, but the role of ApoC-III in the occurrence of feline mammary carcinoma needs to be further studied.

Blood coagulation factor is an important part of the blood coagulation process and its physiological function is to repair the damaged blood vessels with platelets. The coagulation

process includes three stages, where activated blood coagulation factor X forms a complex with Ca^{2+}, phospholipid and blood coagulation factor V. Plasminogen is an inactive substance that needs to be converted into plasmase to initiate the next stage of coagulation. Blood coagulation factor V eventually activates thrombinogen to thrombin. In this experiment, due to the needs of the coagulation pathway, several blood coagulation factors participated in the coagulation process. Blood coagulation factor X and blood coagulation factor V are the key factors to activate thrombin, resulting in a decrease in blood coagulation factor X and blood coagulation factor V levels in group T.

Complement consists of nine components, and C1 has three subunits, C1q, C1r and C1s. Complement protein C1q is the first recognition subcomponent of the complement classical pathway that plays a vital role in the clearance of immune complexes, pathogens, and apoptotic cells [34,35]. In this experiment C1q was down regulated in group T. The reduced presence of this protein may have promoted immune evasion of the malignant cells, which leads to the development of malignant tumors. The role of C1q in the occurrence of feline mammary carcinoma needs to be further studied.

Albumina is the most important protein in plasma. It is synthesized in the liver and participates in many life activities such as pH regulation, transport function, and osmotic pressure maintenance. The CRP/ALB ratio has proved to be an important prognostic marker in patients with pancreatic and rectal cancers [36,37]. Some studies have shown that the combination of dNLR and ALB can improve the diagnostic accuracy of pancreatic cancer [38]. In addition, studies have shown that ALB is associated with a systemic inflammatory response and poor prognosis in patients with esophageal cancer after surgery [39]. In this experiment, the up-regulation of ALB may be due to the rapid proliferation of cancer cells, which accelerates energy exchange and material transport.

In conclusion, this study confirmed for the first time that ApoA-II and ApoB may be new biomarkers for feline mammary carcinoma. The mechanism of their action in the occurrence of feline mammary carcinoma was analyzed and provided a theoretical basis for further revealing the pathogenesis of feline mammary carcinoma.

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