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Original Article

Protein profiling of papillary thyroid carcinoma with and without lymph node metastasis: a proteomic study

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Abstract: Papillary thyroid carcinoma (PTC) is the most common thyroid carcinoma and fortunately the prognosis is generally good. However, lymphatic metastasis of PTC increases mortality and need aggressive therapy. Thus, it is critical to distinguish PTC with lymph node metastasis (LNM) from PTC without LNM, and identification of biomarkers for PTC with LNM is a major research goal. In this study, we conducted a quantitative proteomics analysis to investigate differentially expressed proteins in PTC with LNM and without LNM. Bioinformatics analysis was performed to unveil physiologically relevant information about the altered proteins. We identified 153 proteins that were expressed differentially specific in PTC with LNM, and 85 proteins were differentially expressed only in PTC without LNM. Bioinformatics analysis revealed that extracellular matrix organization, TCA cycle and binding and uptake of ligands by scavenger receptors as the main categories, and these process may contribute to the lymphatic metastasis of PTC. Our study also suggested that ANXA5, ANXA6, ANXA7, S100A9 and S100A11 might be potential biomarkers for PTC with LNM. These results advanced our understanding of molecular mechanisms underpinning PTC with LNM, and may spare PTC patients without LNM from receiving unnecessary aggressive treatment.

Keywords: Papillary thyroid carcinoma, lymph node metastasis, proteomics, bioinformatics

Introduction

Papillary thyroid carcinoma (PTC) is a well-differentiated tumor that accounts for about 80% of thyroid carcinomas [1]. Over 78% of PTC patients are initially diagnosed with lymph node metastases (LNM) [2, 3]. Although the mortality of PTC is relatively low, PTC patients with LNM are unfortunately plagued with increased risk of recurrence, radioiodine-resistance and distant metastases, which require subsequent aggressive surgical and adjuvant treatments [4]. Moreover, the cancer mortality rate in PTC with LNM is reported to be almost ten-fold higher than PTC without LNM [5]. Unfortunately, the pathophysiological mechanisms of the PTC with LNM remain poorly understood. Therefore, to avoid performing unnecessary aggressive surgical and adjuvant treatments on PTC patients without LNM, it's essential to investigate molecular mechanisms of PTC with LNM and identify biomarkers for distinguishing PTC with LNM from PTC without LNM.

Proteomics approaches can provide a comprehensive analysis of all proteins in a sample and are increasingly applied in the field of identifying novel biomarkers in thyroid cancer [6, 7]. Using two-dimensional gel electrophoresis (2DE) based proteomics, Srisomsap *et al.* found that ATP synthase D chain and prohibitin are significantly upregulated in PTC compared to follicular thyroid carcinoma [8]. Using MALDI-TOF-MS, Brown *et al.* identified 15 proteins including S100A6, moesin, HSP70 and proteins associated with mitochondrial function, that are differentially expressed in PTC patients compared with normal thyroid tissue [9]. Meanwhile, Fan *et al.* demonstrated that haptoglobin alpha-I chain was upregulated, while apolipoprotein C-I and apolipoprotein C-III were downregulated, in serum of PTC patients using SELDI-TOF-MS [10]. A number of other studies

Table 1. Summary of clinical features of the patients included in proteomics study

Clinical characteristic	PTC with LNM	PTC with non-LNM	P
Number of subjects	6	6	-
Mean age (range, year)	32.8 (21-45)	36.2 (31-40)	0.40
Gender (male/female)	3/3	3/3	-
Mean tumor sizes (range, cm)	2.0 (1.2-3.2)	1.6 (1.2-2.0)	0.26
TNM classification			
T1N0		1	
T1N1a	1		
T1N1b	1		
T3N0		5	
T3N1a	1		
T3N1b	3		

The statistical significance of the difference (*p* value) between the two groups was determined using a Student's *t*-test.

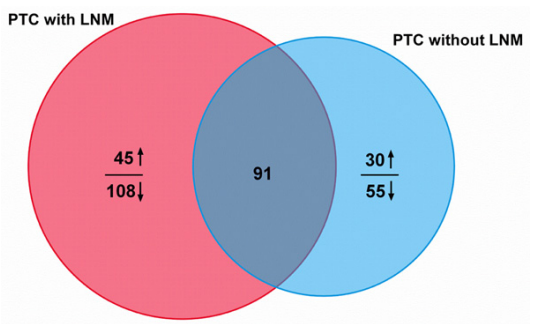

Figure 1. Venn chart for comparing differentially expressed proteins in PTC with and without LNM.

also used proteomics to identify differentially expressed proteins in thyroid cancer, and reported a diversity of proteins being involved in PTC [6, 11-14]. However, to date, few studies have explored the proteomic changes that occur during tumor progression in PTC with LNM. Jung *et al.* identified that ANXA3 decreased significantly in PTC using 2DE based proteomics, and this was more pronounced in subgroup with LNM [15]. Nipp *et al.* used MALDI-IMS to identify protein biomarkers for PTC with LNM, and found that thioredoxin, S100-A10 and S100-A6 were upregulated and significantly related to PTC with LNM. Moreover, they demonstrated that lymphatic metastasis of PTC was associated with TGF-β-dependent EMT pathway [16]. Furthermore, Park *et al.* found increased expression of vimentin in PTC with LNM by 2DE-based proteomics analysis, while the expression of HSP60 was upregulated in PTC without LNM [17]. Given the inconsistent results of the previous studies, reliable biomarkers for distinguishing PTC with LNM from PTC without LNM are still needed.

In the present study, we conducted TMT labeling-based quantitative proteomics analysis and performed bioinformatics analysis to compare proteins that are differentially expressed in PTC with LNM and PTC without LNM. Our results suggested that extracellular matrix (ECM) disorganization and citric acid (TCA) cycle played an essential role in distinguishing PTC with LNM from PTC without LNM. Moreover, we identified a number of novel potential biomarkers for PTC with LNM.

Materials and methods

Reagents

Iodoacetamide, dithiothreitol and urea were obtained from GE Healthcare (LC, UK). A BCA protein assay kit and TMT Mass Tagging Kits were purchased from Thermo Scientific (NJ, USA). Protease inhibitor cocktail and sequencing-grade trypsin were purchased from Roche (Basel, Switzerland). Sequencing-grade endoproteinase Lys-C was obtained from Promega (WI, USA).

Patients and samples

Thyroid samples from 12 PTC patients who underwent thyroidectomy from January 2014 to October 2014 were randomly collected from the department of head and neck surgery, Peking University Cancer Hospital & Institute. Informed consent was obtained from all patients. This study was approved by Ethical Committee of Peking University Cancer Hospital & Institute. Six PTC patients with LNM and six PTC patients without LNM were included in this study. Primary PTC tissue and normal noncancerous tissues were obtained from each patient by one surgeon during thyroidectomy procedure. Tissues were divided into four groups: normal noncancerous tissue from PTC with LNM (Group 1), tumor tissue from PTC with LNM (Group 2), normal noncancerous tissue from PTC without LNM (Group 3) and tumor tissue from PTC without LNM (Group 4). All sam-

Table 2. Representative altered proteins in PTC with LNM and PTC without LNM

Accession	Description	Score	Coverage	# Unique Peptides	127/126 Adjust	131/130 Adjust
P12429	Annexin A3 GN=ANXA3 SV=3 - [ANXA3_HUMAN]	66.446474	50.77	14	0.531	0.721
P08758	Annexin A5 GN=ANXA5 SV=2 - [ANXA5_HUMAN]	268.26004	63.75	17	0.599	0.893
P08133	Annexin A6 GN=ANXA6 SV=3 - [ANXA6_HUMAN]	227.42427	53.79	30	0.623	0.727
P20073	Annexin A7 GN=ANXA7 SV=3 - [ANXA7_HUMAN]	61.493749	27.25	10	0.676	0.832
P26447	Protein S100-A4 GN=S100A4 SV=1 - [S10A4_HUMAN]	36.15038	27.72	4	1.585	1.275
P06702	Protein S100-A9 GN=S100A9 SV=1 - [S10A9_HUMAN]	24.510287	47.37	5	0.447	0.853
P06703	Protein S100-A6 GN=S100A6 SV=1 - [S10A6_HUMAN]	23.014371	55.56	5	1.239	1.528
P31949	Protein S100-A11 GN=S100A11 SV=2 - [S10AB_HUMAN]	67.12176728	42.86	4	1.154	1.579

ples were snap-frozen in liquid nitrogen and then stored at -80°C until needed.

Protein extraction and TMT labeling

Tissue was ground to a powder in liquid nitrogen. Following addition of 5 μL lysis buffer (8 M urea in PBS, 1×cocktail, 1 mM PMSF) per mg of tissue, samples were incubated at 4°C for 30 min and centrifuged at 12,000 rpm for 15 min. Supernatant was collected and protein concentrations were determined using the BCA Protein Assay Kit. Proteins from each group were pooled and incubated with 10 mM dithiothreitol at 55°C for 1 h, and 100 μg of protein was incubated with 25 mM iodoacetamide for 2 h at room temperature in the dark. Proteins were digested with trypsin/Lys-C Mix at a protein/protease ratio of 25:1 overnight at 37 dithiothreitol. Subsequently, TMT isobaric labeling reagents (0.8 mg TMT dissolved in 40 μL of 99.9% acetonitrile) were used to label samples according to the manufacturer instructions as follows: TMT-126 for Group 1, TMT-127 for Group 2, TMT-130 for Group 3, and TMT-131 for Group 4. An equal amount of digested protein from each group was then pooled for subsequent proteomics analysis.

High performance liquid chromatography (HPLC)

Pooled TMT6-labeled protein digests were dissolved in 100 μL 0.1% trifluoroacetic acid (TFA) and loaded onto an Xbridge BEH300 C18 column (4.6×250 mm^2, 5 μm, 300 Å, Waters, USA) with the UltiMate 3000 HPLC workstation. Fractions were collected in 47 tubes from 2-72 min every 1.5 min, dried to 20 tubes in a vacuum concentrator and dissolved in 20 μL of 0.1% TFA for subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS

Samples were analyzed using a Q Exactive mass spectrometer with nano-LC for LC-MS/MS. Labeled digestion fractions were separated by a 60 min gradient elution at a flow rate of 0.30 μL/min on an Ultimate 3000 RSLCnano System (Thermo Scientific, USA) interfaced with a Thermo Q Exactive Benchtop mass spectrometer. Asilica capillary column (75 μm ID, 150 mm length; Upchurch, Oak Harbor, WA) packed with C18 resin (300 Å, 5 μm; Varian, Lexington, MA) was used. The Q Exactive mass spectrometer was operated in data-dependent acquisition mode using Xcalibur2.1.2 software. A single full-scan mass spectrum was acquired using an Orbitrap (400-1,800 m/z, 60,000 resolutions) and was followed by 10 data-dependent MS/MS scans at 27% normalized collision energy (HCD).

Data interpretation

Protein identification was performed using Thermo Scientific Proteome Discoverer software suite 1.4. MS raw data were searched against the reviewed Swiss-prot human FASTA database from UniProt (released on July 18th, 2015) using the SEQUEST search engine with the following parameters: static modifications = carbamidomethylation (C, +57.021 Da) and TMT6-plex (K and peptide N terminals), dynamic modification = oxidation (methionine, M), precursor mass tolerance = 20 ppm, fragment mass tolerance = 0.02 Da, and a maximum of two missed cleavages were allowed. Hits required identification of at least two statistically significant unique peptides ($P < 0.05$, with a false discovery rate of 5%) and a score ≥ 10. Quantification was performed using TMT6-plex with experimental bias normalized against median protein.

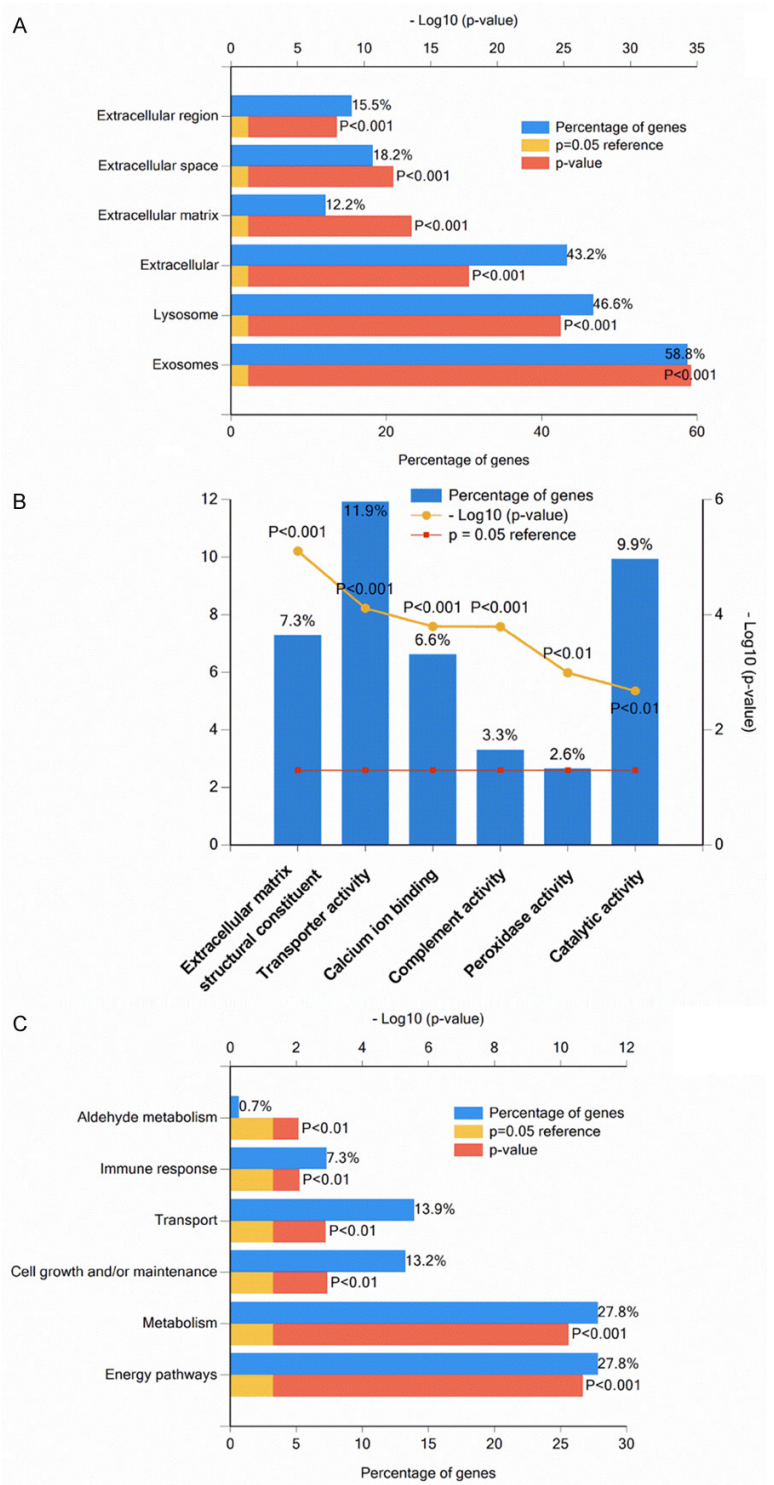

Figure 2. Enrichment analysis of proteins differentially expressed in PTC with LNM. A: Bar graph of cellular component enrichment analysis. B: Column graph of molecular function enrichment analysis. C: Bar graph of biological process enrichment analysis.

TMT6-127/TMT6-126 and TMT6-131/TMT6-130 ratios were obtained and protein abundance changes were adjusted using the mean ratio of ACTB (Accession Number: P60709, mean value = (TMT6-127/TMT6-126 + TMT6-131/TMT6-130)/2) as an internal control. A fold change in protein abundance of ≥ 1.5 was defined as differential expressed.

Enrichment analysis of proteins that were differentially expressed in either PTC with LNM or PTC without LNM was performed with FunRich software 2.1.2 (significant level was set at 0.001). Functional interaction networks of the differentially expressed proteins were analyzed using ClueGOCy to scape plugin version 2.1.7. The REACTOME ontology database (released on May 5th, 2015) was used, and the Benjamini-Hochberg correction method was set to minimizing the false discovery rate. All MS proteomic data had been deposited in the Proteome X change Consortium via the PRIDE partner repository with the dataset identifiers PXD003225 [18].

Results

Clinical characteristics of the subjects included

The clinical characteristics of the 12 patients included in this study (**Table 1**) were classified according to the TNM classification system [19]. Of the six PTC patients with LNM, two were at the T1 stage and four were at the T3 stage. Of the six PTC patients without LNM, one was at the T1 stage and five were at the T3 stage. No difference was found in age or tumor size between patients of PTC with LNM and PTC without LNM.

Protein profile difference between PTC patients with LNM and without LNM

To investigate potential differences in the protein profiles of PTC patients with LNM and without LNM, the complete proteomes of thyroid tumor tissues and normal noncancerous thyroid tissues were analyzed, with adjacent noncancerous thyroid tissue used as a control. We identified a total of 3035 proteins in thyroid tissue, of which two or more unique peptides were identified for 1492 proteins with a score ≥ 10. Tumor tissue data were compared with normal noncancerous tissue to identify differentially expressed proteins in PTC with LNM, and 257 proteins were differentially expressed (Supplemental File Sheet 1). Notably, 13 of these differentially expressed proteins were keratins, and these were excluded from further analysis. In total, 58 proteins were upregulated and 186 proteins were downregulated in PTC with LNM. The same proteomics analysis was then performed on tissue from PTC patients without LNM, and 176 proteins were found to be differentially expressed (excluding 7 keratins; Supplemental File Sheet 2). Of these, 43 proteins were upregulated and 133 proteins were downregulated. Finally, to identify proteins contributing to lymphatic metastasis, we compared differentially expressed proteins in PTC with and without LNM, and found that 91 proteins expressed differentially in both PTC with and without LNM, while 153 were differentially expressed specifically in PTC with LNM, and 85 were differentially expressed specifically in PTC without LNM (**Figure 1**).

Bioinformatics analysis of proteins differentially expressed in PTC with LNM

Since 153 proteins were differentially expressed only in PTC with LNM, we speculated these proteins may play a role in promoting lymphatic metastasis of PTC and could be potential. Further analysis revealed that ANXA3 was significantly downregulated in PTC with LNM but only slightly downregulated in PTC without LNM, consistent with previous findings [15]. ANXA5, ANXA6 and ANXA7 were also downregulated in PTC with LNM, but not PTC without LNM, suggesting these proteins could be potential biomarkers for PTC with LNM. Expression of S100A4 was reported to be upregulated in PTC with LNM [20], and we found S100A9 to be downregulated in PTC with LNM. To our knowledge, this is the first time that S100A9 has been associated with lymphatic metastasis of PTC (**Table 2**). These findings indicated that our data were reliable and could be used for subsequent analysis, and we subsequently performed bioinformatics analysis on the 153 proteins that were differentially expressed only in PTC with LNM.

Proteins were classified by enrichment analysis using FunRich analysis tool. In the cellular component category, exosomes (58.8%), lysosome (46.6%), extracellular (43.2%), ECM (12.2%), extracellular space (18.2%) and extracellular region (15.5%) were enriched (**Figure 2A**). Molecular function enrichment analysis revealed that many of the differentially expressed proteins fell into ECM structural constituent (7.3%), transporter activity (11.9%), calcium ion binding (6.6%) and complement activity (3.3%) subcategories (**Figure 2B**). Furthermore, energy pathway and metabolism were prominent biological process categories (**Figure 2C**). These findings suggest that exosomes, ECM and metabolism may be essential for the lymphatic metastasis of PTC.

To analyze the protein-protein interaction (PPI) networks of the differentially expressed proteins specific to PTC with LNM, ClueGO was used to perform PPI analysis. Of the 153 altered proteins, 152 were successfully matched with the REACTOME database. After excluding the interference of proteins highly enriched in blood, including proteins implicated in clotting cascade and O_2/CO_2 exchange in erythrocytes, our results demonstrated that the differentially expressed proteins were mainly implicated in ECM organization, the TCA cycle, lipid digestion, mobilization and transport, chylomicron-mediated lipid transport and terminal pathway of complement (**Figure 3**).

Since ECM and TCA cycle are reported to be critical for cancer progression [21, 22], special attention was paid on the differentially expressed proteins involved in ECM organization (CAPNS1, COL15A1, FGA, FGB, LAMA5, LAMB1, LAMC1, MFAP4, NID1 and TNXB) and the TCA cycle (IDH3A, MDH2 and NNT) (**Table 3**). Quantitative proteomics analysis identified 10 proteins associated with ECM organization were downregulated in PTC with LNM but were unaltered in PTC without LNM, indicating that downregulation of ECM organization may play a role in PTC with LNM. Three proteins associat-

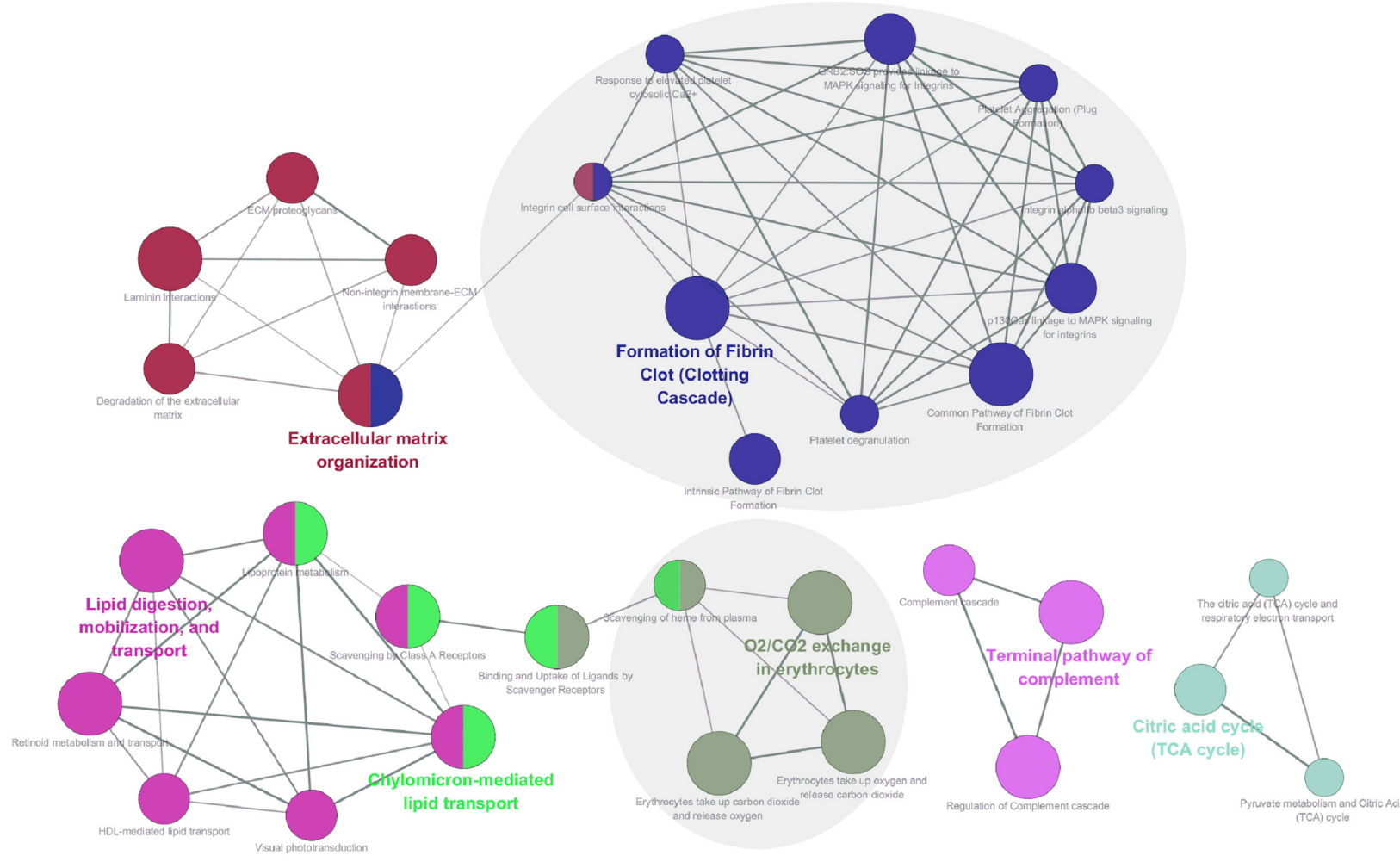

Figure 3. Protein-protein interaction network analysis of proteins differentially expressed in PTC with LNM. Proteins associated with the formation of fibrin clot (Clotting cascade) and O_2/CO_2 exchange in erythrocytes were excluded for subsequent analysis and are highlighted with grey.

Table 3. Representative altered proteins in PTC with LNM

Accession	Description	Score	Coverage	# Unique Peptides	127/126 Adjust	131/130 Adjust
P02671	Calpain small subunit 1 GN=CAPNS1 SV=1 - [CPNS1_HUMAN]	54.6704185	46.27	6	0.699	0.892
P02675	Collagen alpha-1 (XV) chain GN=COL15A1 SV=2 - [COFA1_HUMAN]	85.07380104	11.31	10	0.629	0.969
O15230	Fibrinogen alpha chain GN=FGA SV=2 - [FIBA_HUMAN]	160.7492447	30.02	20	0.591	0.936
P07942	Fibrinogen beta chain GN=FGB SV=2 - [FIBB_HUMAN]	271.9882531	47.45	16	0.656	0.914
P11047	Laminin subunit alpha-5 GN=LAMA5 SV=8 - [LAMA5_HUMAN]	978.6098063	38.05	96	0.680	1.035
P55083	Laminin subunit beta-1 GN=LAMB1 SV=2 - [LAMB1_HUMAN]	547.049089	34.55	49	0.689	1.125
P14543	Laminin subunit gamma-1 GN=LAMC1 SV=3 - [LAMC1_HUMAN]	666.0754437	44.81	59	0.697	1.146
P22105	Microfibril-associated glycoprotein 4 GN=MFAP4 SV=2 - [MFAP4_HUMAN]	59.10511756	20	4	0.670	1.192
P50213	Nidogen-1 GN=NID1 SV=3 - [NID1_HUMAN]	287.4234896	38.81	36	0.685	1.070
P40926	Tenascin-X GN=TNXB SV=4 - [TENX_HUMAN]	192.4550142	19.26	47	0.694	0.942
Q13423	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial GN=IDH3A SV=1 - [IDH3A_HUMAN]	35.39522576	20.49	6	1.695	1.264
P40926	Malate dehydrogenase, mitochondrial GN=MDH2SV=3 - [MDHM_HUMAN]	289.9215314	63.91	18	1.663	1.074
Q13423	NAD(P) transhydrogenase, mitochondrial GN=NNT SV=3 - [NNTM_HUMAN]	106.550061	23.85	22	1.590	0.872

ed with the TCA cycle were upregulated, indicating hyperactive metabolism in PTC with LNM.

Bioinformatics analysis of proteins differentially expressed in PTC without LNM

We found 85 proteins that were differentially expressed only in PTC without LNM that could be potential biomarkers for distinguishing PTC with and without LNM. Overexpression of S100A6 was observed, as previously described by Brown *et al*. [9], but in contrast to the findings of Nippet *al*. [16]. Since S100A6 was not dysregulated in PTC with LNM in our study. Moreover, we found that S100A11 was upregulated specifically in PTC without LNM (**Table 2**).

Given the inconclusive results discussed above, we performed a comprehensive bioinformatics analysis of the 85 altered proteins, and 83 were successfully mapped to the database, with only Ig gamma-1 chain and Ig lambda-2 chain unmapped. Similar to the differentially expressed proteins specific to PTC with LNM, cellular component enrichment analysis revealed that the most proteins were associated with exosomes (41.5%), ECM (12.2%), extracellular (37.8%) categories. Identification of proteins located in the extracellular region (19.5%), muscle myosin complex (4.9%) and I band (3.7%) suggests they may be potential biomarkers for PTC without LNM (**Figure 4A**). Further classification based on molecular function revealed primary associations with molecule activity (9.6%), cytoskeletal protein binding (7.2%), and ECM structural constituent (13.3%) categories, as observed in PTC with LNM (**Figure 4B**). Cell growth and/or maintenance (29.9%) were the most enriched biological process category, following exclusion of highly abundant muscle proteins (**Figure 4C**). Taken together, our findings suggest that proteins related to structural molecule activity, cytoskeletal protein binding and cell growth and/or maintenance could be used for distinguishing metastatic PTC from non-metastatic PTC.

Analysis of PPI networks involving proteins differentially expressed specifically in PTC without LNM was performed, and all 85 altered proteins were successfully matched with the database. Muscle contraction and platelet degranulation PPI networks enriched in muscle and blood were excluded for subsequent analysis (**Figure 5**). Interestingly, similar to the PTC with LNM data described above, 11 of the proteins altered only in PTC without LNM (ASPN, BGN, COL1A1, COL3A1, COL5A1, DMD, EFEMP2, LAMA2, LUM, TIMP1 and VTN) were involved in ECM organization. However, while these were all downregulated in PTC with LNM, 9 of the 11 proteins in PTC without LNM were upregulated. Moreover, the proteins (ALB, COL1A1 and COL3A1) associated with binding and uptake of

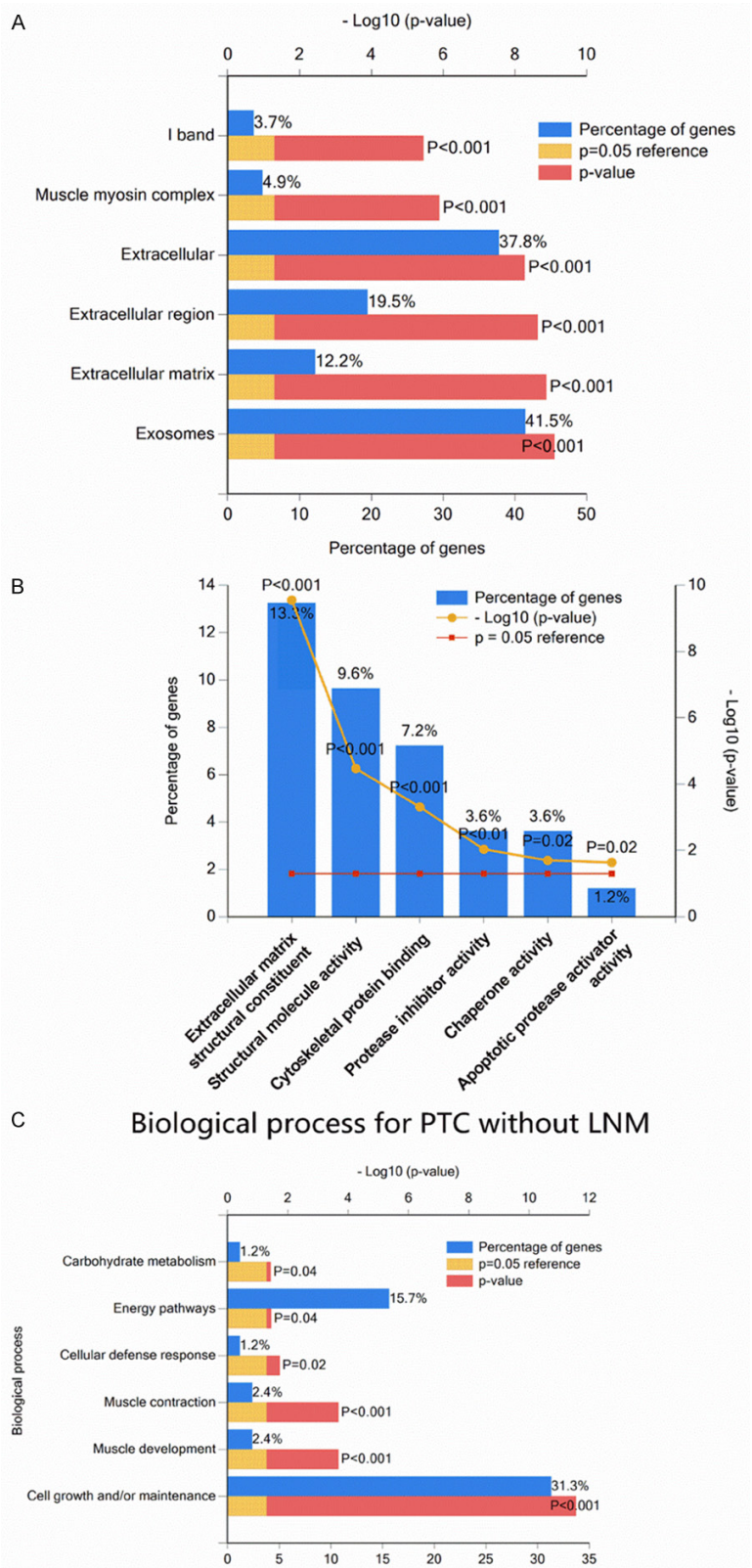

Figure 4. Enrichment analysis of proteins differentially expressed in PTC without LNM. A: Bar graph of cellular component enrichment analysis. B: Column graph of molecular function enrichment analysis. C: Bar graph of biological process enrichment analysis.

ligands by scavenger receptors were also upregulated (**Table 4**). These differences may provide biomarkers that can distinguish between PTC with and without LNM.

Discussion

PTC originates from thyroid follicular cells and is one of the most frequent endocrine malignancies, but fortunately prognosis is good providing metastasis does not occur. However, PTC with LNM requires aggressive treatment and mortality is increased dramatically [5]. Therefore, it is of great importance to distinguish PTC with LNM from PTC without LNM. Thus, with the aim of identifying potential biomarkers for this purpose, and to investigate the molecular mechanisms of lymphatic metastasis in PTC, we performed quantitative proteomics and bioinformatics analysis to determine characteristic proteome changes.

In the present study, we identified 153 proteins that were differentially expressed specifically in PTC with LNM, and 85 were differentially expressed specifically in PTC without LNM. Integrated analysis indicated that downregulation of proteins involved in ECM organization and a hyperactive TCA cycle might promote lymphatic metastasis in PTC, while upregulation of proteins involved in ECM organization and binding and uptake of ligands by scavenger receptors may distinguish PTC without LNM from PTC with LNM.

The ECM is a complex network of extracellular mole-

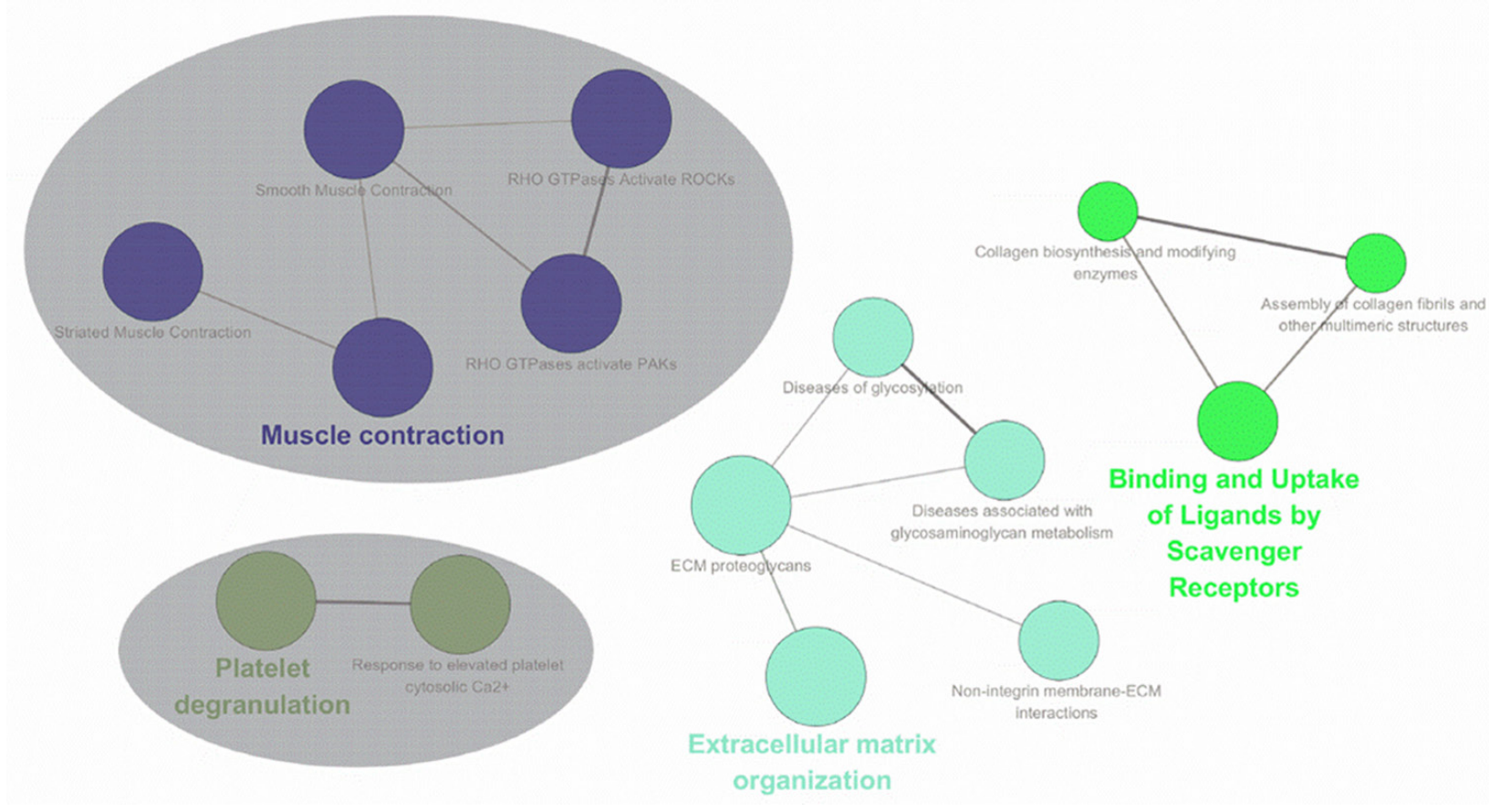

Figure 5. Protein-protein interaction network analysis of proteins differentially expressed in PTC without LNM. Proteins associated with muscle contraction and platelet degranulation were excluded for subsequent analysis and are highlighted with grey.

Table 4. Representative altered proteins in PTC without LNM

Accession	Description	Score	Coverage	# Unique Peptides	127/126 Adjust	131/130 Adjust
Q9BXN1	Asporin GN=ASPN SV=2 - [ASPN_HUMAN]	180.90684	39.47	17	1.449	2.576
P21810	Biglycan GN=BGN SV=2 - [PGS1_HUMAN]	735.62796	50.82	16	1.112	2.076
P02452	Collagen alpha-1(I) chain GN=COL1A1SV=5 - [CO1A1_HUMAN]	120.40386	12.43	12	0.858	1.538
P02461	Collagen alpha-1(III) chain GN=COL3A1 SV=4 - [CO3A1_HUMAN]	36.915877	2.66	2	0.876	1.537
P20908	Collagen alpha-1(V) chain GN=COL5A1SV=3 - [CO5A1_HUMAN]	10.247419	2.61	3	1.193	1.937
P11532	Dystrophin GN=DMD SV=3 - [DMD_HUMAN]	22.710677	3.09	8	0.802	0.447
O95967	EGF-containing fibulin-like extracellular matrix protein 2 GN=EFEMP2 SV=3 - [FBLN4_HUMAN]	17.444696	14	4	1.003	1.505
P24043	Laminin subunit alpha-2 GN=LAMA2SV=4 - [LAMA2_HUMAN]	41.806213	5.06	12	0.715	0.697
P51884	Lumican GN=LUM SV=2 - [LUM_HUMAN]	856.32837	47.34	14	0.937	1.561
P01033	Metalloproteinase inhibitor 1 GN=TIMP1 SV=1 - [TIMP1_HUMAN]	13.33347	28.99	4	1.130	1.997
P04004	Vitronectin GN=VTNSV=1 - [VTNC_HUMAN]	83.746291	21.13	7	0.730	1.508
P02768	Serum albumin GN=ALB SV=2 - [ALBU_HUMAN]	1719.516752	75.37	51	0.909	1.783

cules secreted by cells that provides biophysical and biochemical support to the surrounding cells, and includes laminin and collagen, both of which were differentially expressed in our study. The ECM may regulate tumor progression both structurally and functionally, and malfunctions in ECM physiology promote tumor-related angiogenesis, inflammation and metastasis [23]. The composition, turnover, processing and orientation of ECM alter dramatically during cancer progression [24]. During formation of brain tumor and breast tumor, deposition of components of the ECM such as collagen I, II, III, V, and IX is increased [25, 26]. Consistent with this, our results revealed upregulation of nine proteins differentially expressed specifically in PTC without LNM, including COL1A1 and COL3A1, and 10 ECM-related proteins that were downregulated in PTC with LNM. However, previous studies indicated that migration of tumor cells was promoted in tissues enriched in collagen [27], and increased deposition of collagens facilitates the invasion of tumor cells into adjacent tissues in breast cancer [28]. These apparent differences may be explained by different cancers and different types of metastasis in our study compared with previous studies. Collectively, our study suggests that ECM organization plays a role in lymphatic metastasis of PTC and proteins involved in this process could be used to distinguish PTC with LNM from PTC without LNM.

The TCA cycle is a crucial pathway of basal metabolism that takes place in the mitochondria, and anomalies lead to a variety of diseases [29, 30]. Isocitrate dehydrogenase (IDH), succinate dehydrogenase (SDH) and fumarate hydratase (FH) are three important TCA cycle enzymes [31-33], and mutations in IDH1 and IDH2 are reported to be associated with astrocytomas, oligodendromas and acute myeloid leukemia [31, 34]. In the present study, our data revealed that three proteins (IDH3A, MDH2 and NNT) associated with TCA cycle were upregulated in PTC with LNM. These results are consistent with those of Chen *et al.* who performed a proteomics analysis and found that the TCA cycle was stimulated in brain metastases of breast cancer [35]. Overactivation of TCA may therefore contribute to lymphatic metastasis of PTC.

Scavenger receptors (SRs) are a super family of membrane-bound receptors grouped into classes A-J, consisting of a diverse array of integral membrane proteins and soluble secreted extracellular domain isoforms, whose function is the clearance of modified lipoproteins and pathogens [36]. SRs are involved in a variety of disorders, including pathogen infections, immune surveillance and cancer [36-38]. Neyen *et al.* demonstrated that a deficiency in SR-A inhibited tumor progression and metastasis of ovarian and pancreatic cancer in a SR-A(-/-) mice model [39]. Consistent with this, our results revealed two proteins (COL1A1 and COL3A1) are scavenged by SR-A and that were upregulated in PTC without LNM, suggesting that inhibition of SR-A function could be involved. Our study implied that SR-A also participates in lymphatic metastasis of PTC, and

could help to distinguish PTC with LNM from PTC without LNM.

In addition, we identified five calcium-binding proteins that are potential biomarkers for lymphatic metastasis of PTC, namely ANXA5, ANXA6, ANXA7, S100A9 and S100A11. ANXA5 is a member of the annexin family and has been used to detect apoptosis as well as other forms of cell death [40]. Although the precise function of ANXA5 is still unclear, studies demonstrated a close relationship between ANXA5 and cancers [41, 42]. Sofiadis *et al.* indicated that ANXA5 could be used to discriminate between PTC and follicular thyroid carcinoma [6]. In the present study, downregulation of ANXA5 appeared to be characteristic of PTC with LNM, and this may therefore be a novel biomarker for PTC with LNM. ANXA6 promotes gastric cellular proliferation via Ras/MAPK signaling pathway [43, 44], and Lorenz *et al.* reported that ANXA6 was upregulated in rat thyroid cells (FRTL-5) stimulated with TSH [45]. In this study, expression of ANXA6 was decreased in PTC with LNM, indicating a role in lymphatic metastasis. ANXA7 is involved in lymphatic metastasis of hepatocellular carcinoma, and downregulation of ANXA7 was reported to promote lymph node metastasis of tumor cells in a mouse model [46, 47]. In line with this, ANXA7 was downregulated in PTC with LNM in our study, indicating involvement of ANXA7 in lymphatic metastasis of PTC. Finally, our results showed that S100A9 was downregulated specifically in PTC with LNM, while S100A11 was upregulated only in PTC without LNM. Previous studies revealed that PTC was negative for S100A9 and suggested this protein is characteristic of undifferentiated thyroid carcinoma, but not differentiated carcinoma such as PTC [48, 49]. Besides, Anaian *et al.* reported that overexpression of S100A11 contributes to PTC [50], but the role of these proteins in lymphatic metastasis of PTC has not been studied to date. The results of our study suggest S100A9 and S100A11 could be potential biomarkers for distinguishing PTC with LNM from PTC without LNM.

In conclusion, our study identified a number of proteins that are differentially expressed in PTC with or without LNM, and revealed ECM organization, the TCA cycle and binding and uptake of ligands by scavenger receptors as possible contributors to lymphatic metastasis of PTC. Moreover, our study identified five biomarker candidates that could be used to distinguish PTC with LNM from PTC without LNM. Our results also shed new light on the molecular mechanisms of lymphatic metastasis of PTC.

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Disclosure of conflict of interest

None.

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