ORIGINAL RESEARCH

Profiling Analysis Reveals the Crucial Role of the Endogenous Peptides in Bladder Cancer Progression

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Background: Peptide drugs provide promising regimes in bladder cancer. In order to identify potential bioactive peptides involved in bladder cancer, we performed the present study.

Methods: Liquid chromatography/mass spectrometry assay was used to compare the endogenous peptides between bladder cancer and normal control. The potential biological functions of these dysregulated peptides are assessed by GO analysis and KEGG pathway analysis of their precursors. The SMART and UniProt databases are used to identify the sequences of the dysregulated peptides located in the functional domains. The Open Targets Platform database was used to investigate the precursors related to metabolic diseases.

Results: A total of 9 up-regulated peptides and 110 down-regulated peptides in bladder cancer compared with normal control were identified (fold change > 1.2, $P < 0.05$). The MW of these dysregulated peptides ranged from 500 Da to 2500 Da and the MW of all identified peptides was below 3500 Da. The GO and KEGG pathway analysis indicated that these dysregulated peptides could play an important role in bladder cancer. Our further analysis revealed that [45]HFNPRFNAHGDAN[57] derived from LGALS1 and those peptides derived from P4HB and SERPINA1 might be the promising diagnostic biomarkers and therapeutic targets of bladder cancer.

Conclusion: In the present study, we have identified the profile of the peptides significantly dysregulated in bladder cancer. Moreover, using bioinformatic analysis, we found the peptides derived from LGALS1, P4HB and SERPINA1 could be the promising diagnostic biomarkers and therapeutic targets of bladder cancer.

Keywords: peptide, therapeutic target, progression, bladder cancer

Introduction

Bladder cancer (BC) is the ninth most common cancer worldwide, including approximately 430,000 incident cases and 165,000 attributable deaths annually worldwide.[1,2] It is a complex disease associated with high morbidity and mortality without effective therapy.[3] Approximately 80% of BC are classified as non-muscle invasive BC (NMIBC) depending on the degree of tumor infiltration in the vesical wall, while the remainder are muscle invasive BC (MIBC).[4] Patients with NMIBC have to undergo invasive cystoscopy regularly after TURBT to detect tumor recurrence.[5] Nevertheless, up to 70% of NMIBC patients still experience disease recurrence and progression.[6] Furthermore, patients have to undergo radical cystectomy or chemotherapy in case of tumor recurrence and progression, which imposes a great deal of pain and injury on them.[7,8] Therefore, in order to prevent BC progression more effectively, it is urgent to reveal the potential mechanisms in the progression of BC.

Peptide is a type of organic compound produced by endocrine glandular tissues or organs, containing 3–50 amino acids.[9] As an important role, peptide participates in almost all physiological activities, including cell differentiation, immune regulation and, especially, tumor formation.[10] In recent years, the application of peptides in the diagnosis and treatment of BC has aroused increasing interest. Based on the different metabolomic characteristics, two urinary peptides (AspAspGlyTrp and GlyCysAlaLys) were developed as early BC diagnostic biomarkers.[11]

Maria Frantzi et al exploits two urine-based peptide biomarker panels for detecting primary and recurrent urothelial BC.[4] Some other endogenous peptides such as Six-transmembrane epithelial antigen of the prostate (STEAP),[12] antimicrobial peptide magainin II,[13] DEP domain containing 1 (DEPDC1) and M-phase phosphoprotein 1 (MPHOSPH1)[14] are promising peptides in the treatment of BC.

However, due to technical limitations, the mechanisms of peptides in the pathogenesis of BC have not been fully studied in the past decades. With the technical development of proteomics, we have the opportunity to further investigate the role of endogenous peptides in disease.[15] Using peptidomics technology, a group of peptides have been identified to function in cells.[16,17] Therefore, as a new field focusing on the function of endogenous peptides, peptidomics has emerged, which attracted increasing interests of researchers recently.[18] However, there is still very limited information on differentially expressed peptides between normal urothelium and BC tissue. Therefore, it is of great importance to perform an in-depth peptidomic analysis to reveal the mechanism of BC progression.

In the present study, we identified the endogenous peptides that differentially expressed in BC. Furthermore, we analyzed the basic characteristic, cleavage sites patterns of these dysregulated peptides. GO analysis, KEGG pathway analysis, PPI analysis and functional domain analysis of the identified endogenous peptides were also conducted. We hope that our work could help to reveal the mechanism of bladder cancer progression from the aspect of peptidomics.

Materials and Methods
Patients and Tissue Specimens
The present study was conducted according to the recommendation of the Declaration of Helsinki and was approved by the Ethics Committee of Nanjing Drum Tower Hospital. Patients with a history of other cancers and any preoperative chemotherapy or radiotherapy were excluded. BC tissues and adjacent normal control tissues were collected from patients undergoing radical cystectomy. Finally, a total of 3 high-grade BC tissues and the matched normal control tissues were collected (Table 1). All collected samples were placed in frozen storage tubes and immediately snap-frozen in liquid nitrogen and stored at −80°C until use. Each patient has signed the informed consent before we collected the samples.

Peptides Extraction and Purification
All collected samples were added to the protease inhibitor mixture (Complete Mini EDTA-free, Roche, Basel, Switzerland) after isotonic PBS washing. The tissue specimen was cut into pieces and then ultrasonically homogenized for 20 min. Then, we added 20% acetonitrile to the lysate to block the connection between the proteins and peptides. Then, the samples were centrifuged at 12,000 g in the condition of 4°C for 15 min to obtain the supernatant. According to the manufacturer's instructions, the samples were centrifuged by AMICON 10 kDa molecular weight cut off (MWCO) centrifuge filter (Millipore). Then, the supernatant was filtered through a molecular filter, with the flow-through containing the peptides. Afterward, the BCA method was used to measure the concentration of the supernatant peptides. Lastly, supernatants were vacuum-dried by a Speed-vac system (RVC 2–18) and immediately frozen at −80°C for subsequent experiments.

TMT Labeling and LC-MS/MS Analysis
The freeze-dried peptide was dissolved in 0.1% formic acid and the solution was filtered through a 0.45-μm membrane. The filtrate was reduced with 10 mM DTT at 60°C for 1 h and afterwards alkylated via 50 mM iodoacetamide at room temperature in the dark for 45 min. Next, the peptides were desalted and dried, followed by the manufacturer's

Table 1 Clinical Characteristic of the BC Patients

Characteristics	Age (y)	Grade	Gender	Tumor Size(cm)	T stage	Muscle Invasion
Patient 1	58	High	Male	3	T2	Yes
Patient 2	53	High	Female	2	T3	Yes
Patient 3	67	High	Male	3	T2	Yes

recommendation, 5 μL of the TMT reagent (TMT 6-plex Label Reagent; Thermo Fisher Scientific, Waltham, MA, USA) were used to label with each 20 μg of the samples. Afterwards, the peptides labeled by TMT reagent were frozen at −80°C for further liquid chromatography (LC)-tandem mass spectrometry (MS/MS) analysis.

The separation was carried out by NanoAcuity ultra-high pressure nanoscale upgrading liquid chromatography system. Liquid phase A is 0.1% formic acid-aqueous solution, and B solution is 0.1% formic acid-acetonitrile solution. The sample was dissolved in 200 μL A phase. After 2 μL sample was absorbed by automatic injector, the sample was transmitted to the trap column at 10 μL/min flow rate for 2 minutes. The samples on the capture column were separated by chromatography on the analytical column with a flow rate of 300 mL/min. The spray voltage of Q-Exative mass spectrometer ion source is 2.0 kV, and the heating capillary is set to 300. The data-dependent mode is used to automatically switch the collection between MS and MS/MS. The full-scan MS uses Orbitrap for a first-level scan with a scan range of 350m 1600 and a resolution set to 70,000 (mz 200). The maximum introduction time of the ion is 50 Ms, the automatic gain control (Automatic gain control, AGC) is set to 5×10^5, and then the top 10 parent ions which meet the conditions of cascade (MS/MS) fragmentation were broken by high energy C-trap dissociation (HCD) and scanned with orbitrap. The scanning resolution was set to 17,500. The scan range is controlled automatically according to the mother-child mass–charge ratio, and the lowest scan range is fixed at m/z=100, up to 2000. The minimum ionic strength for MS/MS is set to 500, 000. The maximum ion introduction time is 100 Ms in MS/MS, the AGC control is set to 1.0×10^5, and the parent ion selection window is set to 2 Dalton. The 2,3,4 charge number ions are collected by MS/MS, and the dynamic exclusion is set to remove 30 seconds, 30% collision energy after one MS/MS, per parent ion in 10 seconds.

Peptide Quantification

We performed MS analysis according to the previous study.[19–21] We injected the samples into the LTQ-Orbitrap Velos mass spectrometer. Full-scan analysis was performed over the m/z range 400–5000 at 3 spectra/s. We, respectively, maintained the capillary voltage and cone voltage at 3.9 kV and 40 V. In mass spectrometry analysis, 2000 single spectra were accumulated from 10 random

positions on each sample, and each position was illuminated with 200 laser pulses.

The tolerance to the precursor ion mass was 10 ppm, the fragment tolerance was 0.05 Da and for miss cleavage, a single amino acid. In particular, we conducted an automatic decoy database search in Mascot via selecting the decoy checkbox where a random database sequence was generated and the raw spectrum as well as the real database was tested. In order to reduce the probability of false peptide identification, we applied the manufacturer's recommended isotope correction factors, and only the peptides with significance scores (≥20) at the 99% confidence interval by a Mascot probability analysis greater than "identity" were counted as identified. In the search, all peptides contained are at least 6 amino acids in length. We also used PEAKS software to search the databases via MS/MS spectral data.

Bioinformatics Analysis

For further research, the molecular weight (MW) and iso-electric point (pI) of each peptide were calculated using the online tool pI/MW (http://web.expasy.org/compute.pi/). We map the genes to the nodes of the Gene Ontology database and use GO (http://www.geneontology.org/) for functional enrichment analysis. The potential functions of these dysregulated peptide's precursors are mainly containing the following three categories: cellular component, biological process, and molecular function. KEGG pathway analysis was performed to predict biologic, putative biochemical and molecular functions of the selected peptides. The UniProt Database and SMART were applied to detect whether the dysregulated peptide sequences are located in the conservative domain structure of their precursor proteins. We used the Open Targets Platform database to reveal the relationship between the differentially expressed peptides and human disease. The protein interaction information of PPI network was constructed by using STRING database (v10, string-db.org).[22,23] The precursor proteins of these differentially expressed peptides were searched in *Homo sapiens* of the STRING database, and the network interaction map was constructed based on the interaction information searched.

Statistical Analysis

The differences between groups were assessed by a Student's *t*-test and one-way ANOVA using the SPSS 20.0 software package (SPSS, Chicago, IL, USA). *P* <

0.05 was considered to indicate a significant difference. The threshold value | fold change | > 1.2 was used to screen differentially expressed peptides. All experiments were performed in triplicate.

Results

Identification of Differentially Expressed Peptides Between Bladder Cancer and Normal Control

Endogenous peptides from the BC and normal control were directly analyzed by LC-MS/MS. Totally, 4408 non-redundant peptides have been identified. Among these peptides, we found that 119 peptides derived from 62 precursor proteins were significantly dysregulated, including 9 up-regulated peptides and 110 down-regulated peptides in BC compared with normal control (| fold change | > 1.2, $P < 0.05$). The dysregulated peptides are visualized in heat map (Figure 1A) and volcano plot (Figure 1B). In addition, Table 2 shows part of the differentially expressed peptides in BC tissues compared with normal control tissues.

Characteristics and Cleavage Site Patterns of the Dysregulated Peptides

Most peptides have molecular weights (MW) ranging from 500 Da to 2500 Da and the MW of all identified peptides was below 3500 Da (Figure 2A). Furthermore, the pI distribution of the dysregulated peptides ranged from 3.4 to 12.3 (Figure 2B). Because the pI distribution is related to the MW distribution and the amino acid composition, we also investigated the distribution of the MW relative to the pI (Figure 2C).

Previous researches[24,25] have proved that the regulation of the expression of peptides is associated with the enzymatic cleavage of the precursor proteins. Therefore, we analyzed the cleavage sites at the N- and C-terminals of the dysregulated peptides. As shown in Figure 2D, the top four most common cleavage sites included the amino acids ala (A), serine (S), lysine (K) and leucine (L). Meanwhile, we found that some dysregulated peptides derived from the same precursor protein. As shown in Figure 2E, the precursor proteins which more than one peptide shared were listed, and we found that SERPINA1 has the largest number of related peptides.
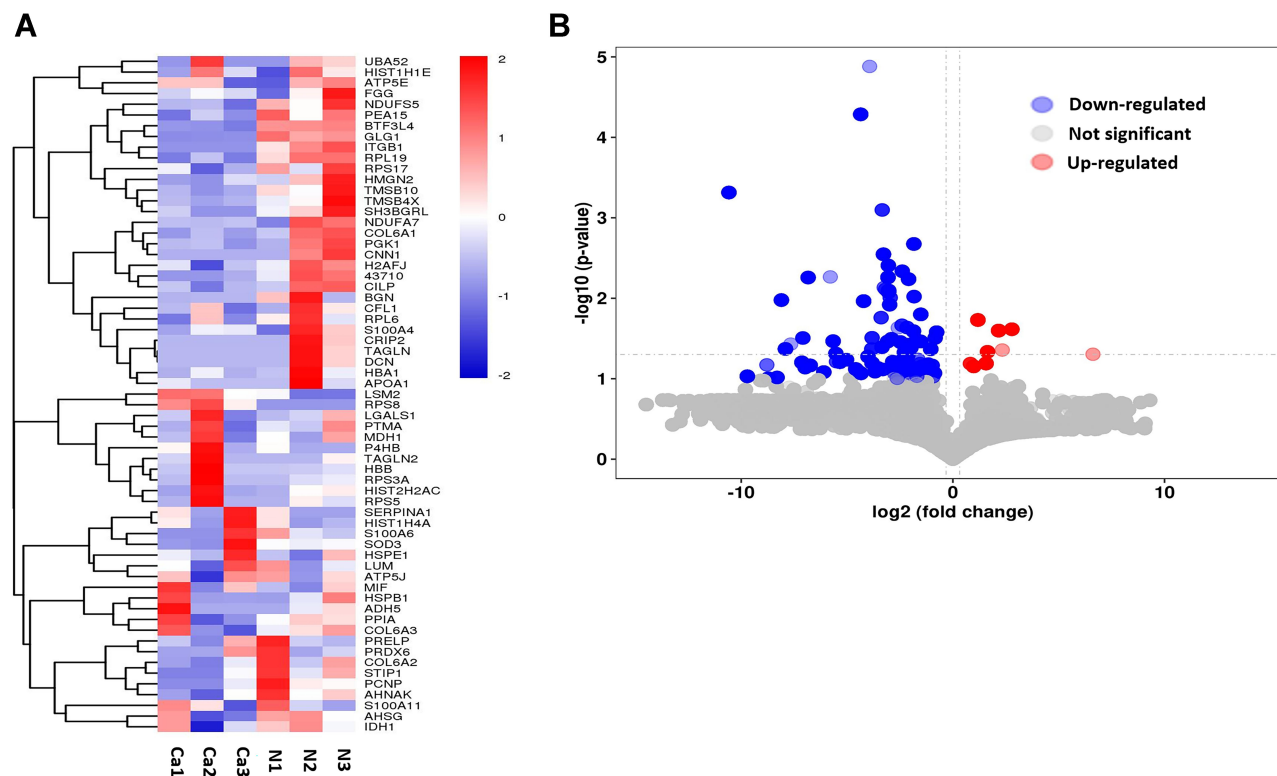

Figure 1 Differentially expressed peptides in bl3adder cancer compared with normal urothelium tissues. (**A**) Heatmap of expression profiles for the 119 dysregulated peptides containing 9 up-regulated peptides and 110 down-regulated peptides. (**B**) Volcano plots of the dysregulated peptides in BC.

Table 2 Part of the Differently Expressed Peptides in Bladder Cancer Tissues Compared with Normal Urothelium Tissues

Gene	Protein	Sequence	MW (kDa)	Fold Change	*P*-value
Up-Regulated Peptides					
RPS8	40S ribosomal protein S8	YLRKIKARKGK	1360.71	98.19565075	0.04963442
S100A6	Protein S100-A6	LDRNKDQEVNF	1377.48	6.90802353	0.0242696
LSM2	U6 snRNA-associated Sm-like protein LSm2	LQDAARKEALQQKQ	1626.83	5.040512325	0.04398991
HBB	Hemoglobin subunit beta;LVV-hemorphin-7; Spinorphin	KGTFATLSELHCDKLHVDPEN	2354.62	4.478971853	0.02523424
HSPE1	10 kDa heat shock protein, mitochondrial	FRDGDILGKYVD	1397.55	3.107810767	0.04627051
HBA1	Hemoglobin subunit alpha	SVSTVLTSKYR	1240.42	2.962531037	0.03464964
HBA1	Hemoglobin subunit alpha	ASVSTVLTSKYR	1311.50	2.267838831	0.01861224
S100A11	Protein S100-A11;Protein S100-A11, N-terminally processed	FLKAVPSQKRT	1274.53	1.977297757	0.02023438
HIST1H1E	Histone H1.4	SETAPAAPAAPAPAEKTPVK	1904.15	1.775721232	0.03494765
Down-regulated peptides					
TMSB4X	Thymosin beta-4;Hematopoietic system regulatory peptide	SDKPDMAEIEKFDKSKLK	2109.42	0.000652869	0.00048494
TAGLN	Transgelin	IIVQCGPDVGRPDRGRLG	1908.21	0.00119247	0.02324176
FGG	Fibrinogen gamma chain	TYNPDESSKPNMIDA	1681.79	0.002260979	0.02727634
PRELP	Prolargin	DLQHNRLSDGVFKPDTFHGLKN	2538.81	0.0023698	0.02988005
PTMA	Prothymosin alpha;Prothymosin alpha, N-terminally processed;Thymosin alpha-1	SDAAVDTSSEITTKDLKEKKEVVEEAEN	3066.28	0.003162826	0.01644275
MIF	Macrophage migration inhibitory factor	TQQLAQATGKPPQY	1530.70	0.003639171	0.02053066
SERPINA1	Alpha-1-antitrypsin;Short peptide from AAT	QNTKSPLFMGKVVNPTQ	1889.20	0.0041546	0.04265418
ITGB1	Integrin beta-1	SAVTTVVNPKYEGK	1492.69	0.004947969	0.03713312
SERPINA1	Alpha-1-antitrypsin;Short peptide from AAT	LRTLNQPDSQ	1171.28	0.007127744	0.0267898

Gene Ontology (GO) and KEGG Pathway Analysis of Peptide Precursors

GO and KEGG pathway analysis were performed to explore the potential functions of these dysregulated peptides and their precursor proteins. Cadherin binding, oxidoreductase activity. RNA binding, structural molecule activity and so on were the most highly enriched GO terms in molecular functions, whereas focal adhesion, extracellular matrix, extracellular exosome, collagen containing extracellular matrix and so on were the most highly enriched cellular component. Finally, in biological processes, the most highly enriched GO terms are translational initiation, mRNA catabolic process, protein targeting to ER and so on (Figure 3A).

As shown in Figure 3B, the KEGG pathway enrichment analysis indicates that Ribosome, Carbon metabolism, Glycolysis/Gluconeogenesis, Biosynthesis of amino acids, Systemic lupus erythematosus, Necroptosis, African trypanosomiasis, Pentose phosphate pathway, Fructose and mannose metabolism were the 10 most common pathways

in which the differentially expressed peptides and their corresponding precursor proteins were involved in.

Furthermore, KEGG pathway analysis revealed that these dysregulated peptides are involved in oxidative phosphorylation. Oxidative phosphorylation was found to be a crucial pathway in cancer progression[26,27] (Figure S1). Meanwhile, we found that these dysregulated peptides are involved in retrograde endocannabinoid signaling which have been proved to play an important role in multiple cancer progression[28–30] (Figure S2). These results showed that the dysregulated peptides might play an important role in BC.

The Protein–Protein Interaction Network of Peptide Precursors

Previous studies proved that the function of bioactive peptides is related to the function of their precursor proteins.[31] Therefore, to further investigate the role of these dysregulated peptides in BC, a protein–protein interaction (PPI) network was constructed based on the STRING database. As shown in Figure 4, UBA52, HSPE1, P4HB and RPS3A are
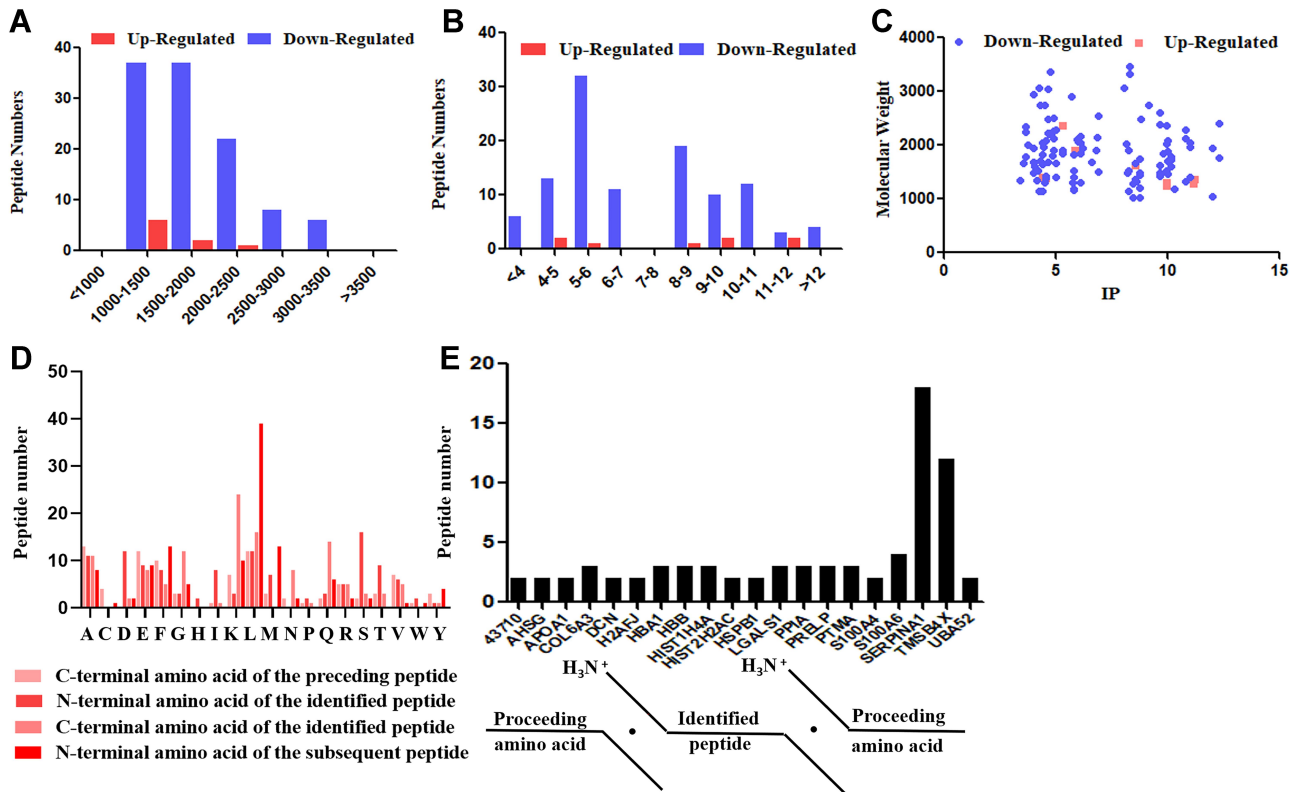
Figure 2 Basic features of differentially expressed peptides identified by LC-MS/MS. (**A**) Molecular weight (MW). (**B**) Isoelectric point (pI). (**C**) Scatter plot of MW versus pI. (**D**) Distribution of the four cleavage sites in the identified dysregulated peptides. (**E**) Peptides shared the same precursor proteins.
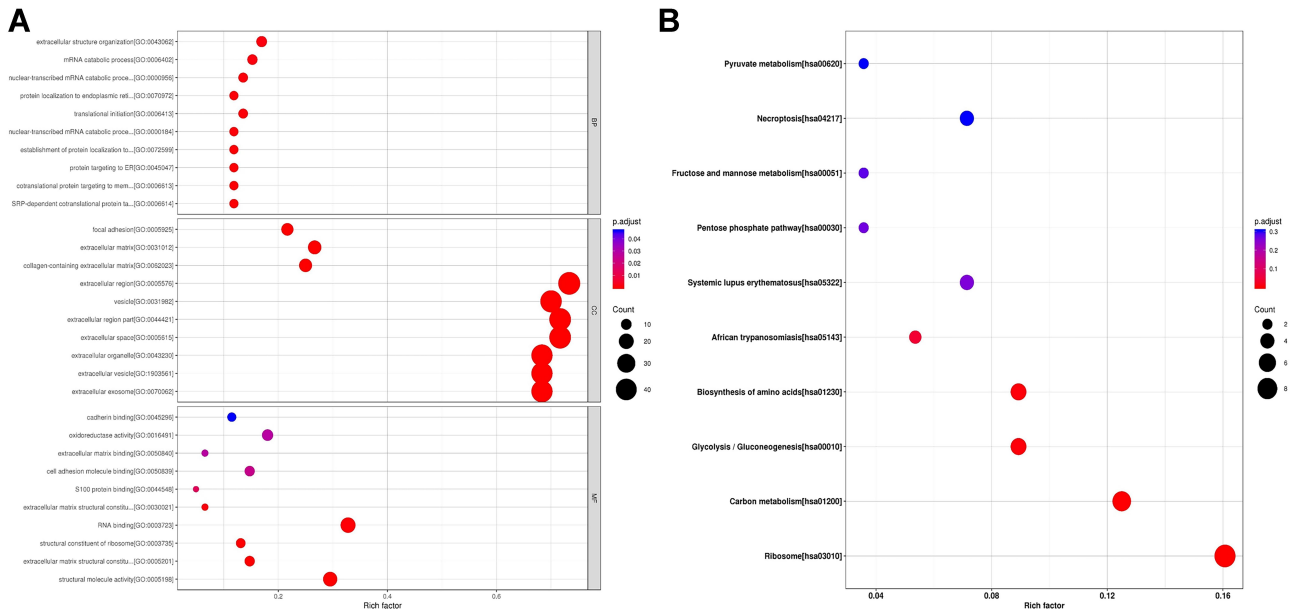

Figure 3 Gene ontology (GO) and KEGG pathways analysis of precursor proteins from which differentially expressed peptides were derived. (**A**) GO analysis of precursor proteins containing biological process categories, molecular function categories and cellular component categories. The horizontal coordinates in the diagram are the Rich factor values of the enrichment degree, and the vertical coordinates are GO Term. (**B**) KEGG pathways analysis of precursor proteins. The horizontal coordinates in the diagram are the Rich factor values of the enrichment degree, and the vertical coordinates are the KEGG Pathway information.
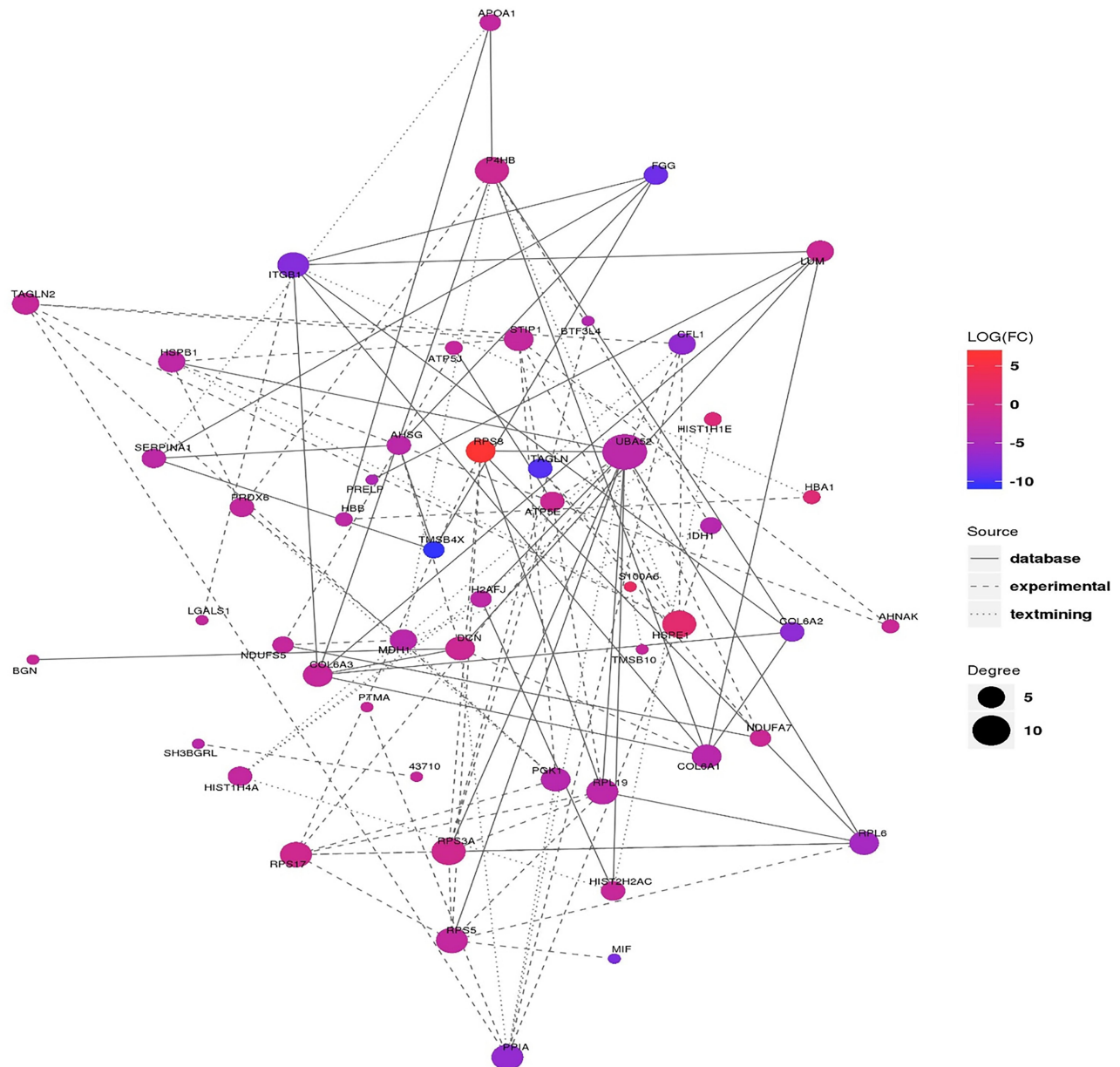
Figure 4 PPI network of altered peptide precursors. The color indicates the expression level of the differentially expressed protein, the red indicates the difference is up-regulated, the blue indicates the difference is down-regulated, and the size of the circle indicates the connective degree. The types of connections represent the source of interaction relationships, the real lines represent interactions from databases, dashed lines represent interactions from experiments, and dots represent interactions from text mining.

the top four precursor proteins which got the largest number of interacting proteins.

Putative Bioactive Peptides Associated with Bladder Cancer

Meanwhile, peptides derived from the functional domains of their precursor protein were analyzed using the online tool SMART and UniProt database. Totally, 37 peptide sequences were located in the functional domains of their corresponding

precursor proteins (Table 3). Furthermore, to reveal the potential peptides that play a role in BC, the Open Targets Platform database was used to investigate protein precursors. As shown in Table 4, a total of 50 precursor proteins were considered to be related to urogenital neoplasm.

Discussion

In recent years, with the technical development of proteomics and related methodologies, endogenous peptides are

Table 3 Differentially Expressed Peptides Located in Functional Domain

Protein Accession	Protein Name	Peptide Sequences	Locations		Domain	Description	P-value	Fold Change
			Start	End				
P01009	SERPINA1	DAAQKTDTSHHDQDHPTFNKITPNLA	30	55	25–418	Alpha-1-antitrypsin	0.037766	0.235678487
P01009	SERPINA1	DLKSVLGQLGITK	322	334	25–418	Alpha-1-antitrypsin	0.020822	0.219475983
P51888	PRELP	DLQHNRLSDGVFKPDTFHGLKN	201	222	21–382	Prolargin	0.031288	0.0023698
P01009	SERPINA1	EAIPMSIPPEVK	378	389	25–418	Alpha-1-antitrypsin	0.005499	0.11953535
P01009	SERPINA1	EAIPMSIPPEVKFNKPF	377	394	25–418	Alpha-1-antitrypsin	0.028919	0.242561409
P01009	SERPINA1	EDPQGDAAQKTDTSHHDQDHPTFNKITPNL	25	54	25–418	Alpha-1-antitrypsin	0.042966	0.484470234
P07237	P4HB	EEAEEPDMEEDDDQKAVKDE	488	508	18–508	Protein disulfide-isomerase	0.032335	0.371330153
P01009	SERPINA1	ELDRDTVFALVNYIFFK	199	215	25–418	Alpha-1-antitrypsin	0.039258	0.245571206
P11766	ADH5	ELMHSGKSIRTVVKI	360	374	2–374	Alcohol dehydrogenase class-3	0.007736	0.110520237
P08294	SOD3	FRQLAPRAKLD	84	94	19–240	Extracellular superoxide dismutase [Cu-Zn]	0.024958	0.34129121
P09382	LGALS1	HFNPRFNAHGDAN	45	57	2–135	Galectin-1	0.047342	0.203717747
P01009	SERPINA1	IFFKGKWERPFEVKDTEE	212	229	25–418	Alpha-1-antitrypsin	0.043157	0.234425789
P62987	UBA52	IIEPSLRQLAQKYN	78	90	77–128	60S ribosomal protein L40	0.009829	0.128413123
P62987	UBA52	IIEPSLRQLAQKYNCDK	78	93	77–128	60S ribosomal protein L40	0.000799	0.098916084
P18859	ATP5J	KFEDPKFEVIEKPQA	99	108	33–108	ATP synthase-coupling factor 6, mitochondrial	0.023106	0.223383459
P01009	SERPINA1	KQINDYVEKGTQGKIVDL	179	196	25–418	Alpha-1-antitrypsin	0.062222	0.024736355
P01009	SERPINA1	LEAIPMSIPPEVKFNKPFV	377	395	25–418	Alpha-1-antitrypsin	0.010855	0.053820508
P02765	AHSG	LGSPSGEVSHPRKT	326	339	301–340	Connecting peptide	0.025629	0.275022454
P01009	SERPINA1	LRTLNQPDSQ	124	133	25–418	Alpha-1-antitrypsin	0.032679	0.007127744
P01009	SERPINA1	LRTLNQPDSQLQ	124	135	25–418	Alpha-1-antitrypsin	0.015484	0.247967175
P01009	SERPINA1	LRTLNQPDSQLQL	124	136	25–418	Alpha-1-antitrypsin	0.008369	0.121866796
P01009	SERPINA1	LSLGTKADTHDEILEG	88	103	25–418	Alpha-1-antitrypsin	0.017135	0.252738434
P01009	SERPINA1	MIEQNTKSPLF	398	408	25–418	Alpha-1-antitrypsin	0.028238	0.218920467
P01009	SERPINA1	MIEQNTKSPLFMGKVVNPTQK	398	418	25–418	Alpha-1-antitrypsin	0.027029	0.36449943
P01009	SERPINA1	PLKLSKAVHKAVLTIDEKGTEAAG	349	373	25–418	Alpha-1-antitrypsin	0.023814	0.070881347
P01009	SERPINA1	QNTKSPLFMGKVVNPTQ	401	417	375–418	Short peptide from AAT	0.042654	0.0041546
P08708	RPS17	QVTQPTVGMNFKTPRGPV	118	135	2–135	40S ribosomal protein S17	0.034457	0.521711245
P51888	PRELP	RLSQNHISRIPPGVFSK	177	193	21–382	Prolargin	0.021329	0.04887646
P51888	PRELP	RLSQNHISRIPPGVFSKLEN	177	196	21–382	Prolargin	0.033068	0.304093
P09382	LGALS1	RVRGEVAPDAK	19	29	2–135	Galectin-1	0.004617	0.190800247
P05204	HMGN2	SAKPAPPKPEPKPK	29	42	2–90	Non-histone chromosomal protein HMG-17	0.042794	0.070127871
P10412	HIST1H1E	SETAPAAPAAPAPAEKTPVK	2	21	2–219	Histone H1.4	0.026948	1.775721232
P02765	AHSG	SPSGEVSHPRKT	328	339	301–340	Connecting peptide	0.00284	0.102972747
P01009	SERPINA1	SVLGQLGITK	325	334	25–418	Alpha-1-antitrypsin	0.031978	0.133968595
P09382	LGALS1	VLNLGKDSNNL	32	42	2–135	Galectin-1	0.037586	0.261031856
O75874	IDH1	WTRGLAHRAKLDNNKEL	336	352	2–414	Isocitrate dehydrogenase [NADP] cytoplasmic	0.03095	0.070958369

Table 4 Protein Precursors and Identified Peptides Related to Urogenital Neoplasm

Protein Name	Association Score	Peptide Numbers	Target Gene Name
IDH1	1	1	Isocitrate dehydrogenase (NADP(+)) 1, cytosolic
NDUFA7	1	1	NADH:ubiquinone oxidoreductase subunit A7
NDUFS5	1	1	NADH:ubiquinone oxidoreductase subunit S5
UBA52	0.81199992	1	Ubiquitin A-52 residue ribosomal protein fusion product 1
HIST2H2AC	0.8006457	2	Histone cluster 2 H2A family member c
RPL19	0.79641324	1	Ribosomal protein L19
ITGB1	0.76437047	1	Integrin subunit beta 1
HIST1H1E	0.76371103	1	Histone cluster 1 H1 family member e
FGG	0.75537863	1	Fibrinogen gamma chain
H2AFJ	0.75382293	2	H2A histone family member J
RPS3A	0.73017717	1	Ribosomal protein S3A
APOA1	0.61937796	2	Apolipoprotein A1
HBB	0.46185019	3	Hemoglobin subunit beta
HBA1	0.45942741	3	hemoglobin subunit alpha 1
P4HB	0.42665308	1	Prolyl 4-hydroxylase subunit beta
COL6A1	0.425827	2	Collagen type VI alpha 1 chain
COL6A3	0.4070895	3	Collagen type VI alpha 3 chain
COL6A2	0.40516103	1	Collagen type VI alpha 2 chain
PGK1	0.37178884	1	Phosphoglycerate kinase 1
STIP1	0.31365796	1	Stress induced phosphoprotein 1
PPIA	0.31318047	3	Peptidylprolyl isomerase A
PRELP	0.29236622	3	Proline and arginine rich end leucine rich repeat protein
MDH1	0.25592816	1	Malate dehydrogenase 1
LGALS1	0.24935391	3	Galectin 1
S100A6	0.24904395	1	S100 calcium binding protein A6
S100A4	0.24179143	1	S100 calcium binding protein A4
S100A11	0.22317851	1	S100 calcium binding protein A11
BGN	0.14883029	1	Biglycan
PRDX6	0.13666088	1	Peroxiredoxin 6
LUM	0.11588513	1	Lumican
DCN	0.11182324	2	Decorin
TMSB4X	0.09082789	1	Thymosin beta 4 X-linked
TAGLN	0.08225353	1	Transgelin
HSPB1	0.07331017	2	Heat shock protein family B (small) member 1
CFL1	0.07314591	1	Cofilin 1
SOD3	0.06748032	1	Superoxide dismutase 3
SERPINA1	0.06422752	1	Serpin family A member 1
TMSB10	0.06252222	1	Thymosin beta 10
PEA15	0.06080867	1	Proliferation and apoptosis adaptor protein 15
PTMA	0.05742044	3	Prothymosin alpha
MIF	0.05565778	1	Macrophage migration inhibitory factor
TAGLN2	0.05050489	1	Transgelin 2
CNN1	0.04811111	1	Calponin 1
HSPE1	0.04301032	1	Heat shock protein family E (Hsp10) member 1
HMGN2	0.04088889	1	High mobility group nucleosomal binding domain 2
AHSG	0.03716489	2	Alpha 2-HS glycoprotein
GLG1	0.03544518	1	Golgi glycoprotein 1
PCNP	0.0228	1	PEST proteolytic signal containing nuclear protein
LSM2	0.0208	1	LSM2 homolog, U6 small nuclear RNA and mRNA degradation associated
RPL6	0.0068	1	Ribosomal protein L6

going to become a promising research focus, attracting the attention of a large number of researchers.[21] A number of researches have been performed to identify the endogenous peptides which participated in adiposity,[32] preeclampsia,[33] bronchopulmonary dysplasia,[31] polycystic ovarian syndrome[9] and, especially in cancer.[34] These studies further revealed the pathogenesis of corresponding diseases and provided new ideas and choices for the diagnosis and treatment of these diseases. However, identification and further researches on peptides related to BC are still not been performed. Therefore, in order to develop new diagnostic markers and therapeutic targets, we conducted the present study to reveal the peptide expression profile of BC.

When a protein is cleaved by a protease, a peptide fragment is produced. According to previous researches, the cleavage sites at the N- and C-termini of endogenous peptides are highly conserved, despite differences in the sample preparation conditions used to analyze specific samples across individuals. In addition, the degradation of proteins and peptides varies greatly from disease to disease depending on the activity and species of their respective proteolytic enzymes.[18,35] The cleavage sites in the peptides that were differentially expressed in our study are summarized in Figure 2D. The probability that any of these 20 amino acids are located at these four cleavage sites is very different. Because each protease has its own specific rules for cleavage of proteins, our results reveal that a distinct set of proteases is active in the progression of BC. This is worth our further study.

Previous studies have revealed that the functions of endogenous peptides are closely related to their precursor proteins.[36,37] Therefore, in order to further analyze the potential roles of these dysregulated peptides in BC, we performed GO and KEGG pathway analysis based on their precursor proteins. As the most important enzyme in the cell, oxidoreductase mediates a large number of important life activities in the cell. In molecular function, we found that the dysregulated peptides were tightly associated with "oxidoreductase activity". Prasai et al have proved that oxidoreductase plays a crucial role in facilitated visualization and detection of human cancer cells.[38] Meanwhile, as revealed by Hernandez-Fernaud et al, secreted CLIC3 can drive cancer progression through its glutathione-dependent oxidoreductase activity.[39] These researches indicate that the differentially expressed peptides may participate in BC through their regulation of oxidoreductase activity. Meanwhile, in cellular component, many different secreted

peptides and their precursor proteins are associated with extracellular matrix. Previous studies have revealed that extracellular matrix could play an important part in diagnosis and targeted therapy of cancer.[40,41] Focal adhesion (FA) is a subcellular structure which provides strong adhesion to the extracellular matrix and acts as a scaffold for many signaling pathways involving integrin or the mechanical force exerted on cells.[42] Recent studies have revealed the dynamic cycle of "FA assembly–cytoskeleton remodelling–FA disassembly", which allows cells to move, and the regulation of FA is considered an important step in tumor invasion.[43,44] Furthermore, Kong et al have proved that the inhibition of focal adhesion kinase could induce apoptosis in BC cells via Src and the phosphatidylinositol 3-kinase/Akt pathway.[45] In our analysis, a number of dysregulated peptides were found to be tightly related to focal adhesion, which indicates that these peptides may participate in BC invasion through the regulated of focal adhesion.

Endogenous peptides have been proved to play biological function closely associated with their precursor proteins. Proteins maintain a temporal and spatial coherence by forming a protein-protein-interaction (PPI) network while performing biological functions. By constructing an interaction network of differentially expressed proteins, we can find the trend of differentially expressed peptides from the proteome level, which further helps us to find key nodes in differentially expressed peptides. In the PPI analysis, we finally find UBA52, HSPE1, P4HB and RPS3A are the top four hub protein in the network. Previous studies have revealed that P4HB played a crucial role in the progression of multiple malignant tumors,[46,47] indicating that peptides derived from P4HB protein may be the promising targets of the treatment of BC.

A protein domain is a relatively conserved portion of a given protein sequence and structure and it exists, evolves, and functions independently of the rest of the protein chain. In order to find more potential bioactive peptides, we identified 36 peptide sequences located in the functional domains using the online tools SMART and UniProt. In addition, we further used the Open Target Platform database to explore the precursor proteins related to urogenital neoplasm. Previous researches have proved that these progenitor proteins are involved in BC. For example, SERPINA1 was revealed to be a urinary protein biomarker which could help us detect the BC in early stage.[48,49] Rosser et al also found that SERPINA1 protein could be applied in the early diagnosis of BC

recurrence.[50] These efforts have proved that peptides derived from SERPINA1 would be promising tumor diagnostic biomarkers. Meanwhile, Wu et al found that LGALS1 is associated with tumor invasiveness and is a possible independent prognostic marker of urinary bladder urothelial carcinoma.[51] In addition, their further research revealed that LGALS1 could enhance urinary bladder urothelial carcinoma cell invasion through the JNK pathway.[52] The functions of peptides are closely related to their precursor protein.[36,37] Therefore, our results revealed that [45]HFNPRFNAHGDAN [57] derived from LGALS1 might play a crucial role in BC and could be the promising diagnostic biomarker and therapeutic target of BC.

Several strengths were observed in the present study. First, we identified a series of peptides that are significantly differentially expressed in BC, and these peptides may play a potential biological function in the progression of BC. Based on our research, researchers can further reveal the pathogenesis of BC from the perspective of peptides. Secondly, peptidomics is currently not widely used in the field of BC. Our innovative study provides a methodological reference for future researchers. Finally, peptide drugs have been developed and used in the treatment of multiple diseases. Our research has laid the foundation for the development of peptide research in BC and the development of peptide drugs. However, follow-up studies are essential to validate the roles of the dysregulated peptides.

Conclusion

In the present study, we have identified and compared the peptidomic profiles of BC tissue and normal control and finally observed significantly different expression patterns. Our further analysis revealed that [45]HFNPRFNAHGDAN [57] derived from LGALS1 and those peptides derived from P4HB and SERPINA1 might be the promising diagnostic biomarkers and therapeutic targets of BC. Our research provides us new insights into the pathogenesis mechanism of BC and could help us to discover novel biomarkers to better predict and prevent BC.

Abbreviation

BC, bladder cancer; NMIBC, non-muscle invasive BC; MIBC, muscle invasive BC; TURBT, transurethral resection of bladder tumor; STEAP, six-transmembrane epithelial antigen of the prostate; DEPDC1, DEP domain containing 1; MPHOSPH1, and M-phase phosphoprotein 1; LC-MS/MS, liquid chromatography tandem mass spectrometry; TMT, tandem mass tag; GO, gene ontology; MWCO, molecular weight cut off; PPI, protein–protein interaction; pI, isoelectric point; STRING, the Search Tool for the Retrieval of Interacting Genes.

Data Sharing Statement

The datasets used or analyzed in the present study are available from the corresponding author on reasonable request.

Disclosure

The authors report no conflicts of interest for this work and declare that there is no duality of interest associated with this manuscript.

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