Comparative Proteomics Uncovers Correlated Signaling Network and Potential Biomarkers for Progression of Prostate Cancer

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Abstract

Background/Aims: Prostate cancer is one of the most common cancers for males worldwide, and it is prone to show the metastatic foci in lymph node and bone with high mortality. To date, the potential mechanism and the corresponding biomarkers for metastatic prostate cancer are still lacking. Hence, our study aims to clarify the mechanism of prostate cancer progression and identify the useful biomarkers for metastatic prostate cancer. Methods: The proteins and network tightly associated with tumor metastasis were identified using quantitative proteomics. Furthermore, the mRNA level of differential expressed proteins were confirmed using qRT-PCR, and the functional cluster analysis was performed using String and Cytoscape. Results: Totally, our study identified 203 differential proteins closely associated with tumor cell migration, and the mRNA expression of those proteins were verified by qPCR. Moreover, the migration associated molecular network was established using bioinformatics analysis. Conclusion: These data raveled the critical proteins for the cell migration of prostate cancer, and identified the potential markers for diagnosing the metastasis of prostate cancer.

Introduction

Prostate cancer is the second most common diagnosed cancer and the fifth cause of cancer-related mortality for males worldwide [1]. In United States, approximately 238,590 new cases and 29,720 deaths were reported during 2013 [2]. Part of those prostate cancer would develop to the castration-resistant prostate cancer (CRPC), and CRPC is readily to show the bone and lymph node metastases. Clinical studies reported that the median survival of CRPC varied from 9 to 30 months [3], and this indicated that prostate cancer, particularly the metastatic prostate cancer, would significantly shorten the lifespan and lower the life quality.
At the beginning of the prostate cancer progression, the tumor cells grew locally, and those cells were sensitive to hormone therapy. However, the tumor will be resistant to the hormone after 1 to 3 years of treatment, and this is an obvious characteristic of CRPC [4]. The advanced prostate cancer mainly metastasize to the bones and lymph nodes, and the 5-year survival rate of patients with distant metastases (28%) was significantly lower than those with local prostate cancer (nearly 100%) [5]. Hence, the metastasis of prostate cancer is a crucial factor for mortality. Firstly, it is extremely important and valuable to accurately identify the genes or proteins tightly associated with prostate cancer progression, and the clear molecular mechanism for prostate cancer metastasis will be helpful for the treatment, especially for the precision medicine. Secondly, the biomarkers for the advanced progression were also critical for the diagnosis and treatment.

Proteomics approach is a powerful tool to simultaneously identify a large amount of differential expressed proteins among different samples, including cell lines and tissue samples from patients. Importantly, the most attractive points for this technique are the identification of cancer-related proteins for effective treatment, and the establishment of biomarkers for diagnosis of tumor metastasis [6].

For improved insight into the molecular mechanisms and novel biomarkers of prostate cancer metastasis, we utilized two human prostate cancer cell lines, PC3 and LNCaP, with high and low metastatic potentials, respectively [7]. These two cell lines were derived from Caucasian male patients with lymph node and bone metastases, respectively, and PC3 cells showed higher metastatic capability than LNCaP cells [7]. Therefore, these two cell lines are excellent cell models for studying the cell migration in vitro and metastasis in vivo.

Our study identified large quantity of differential expressed proteins associated with cell migration (metastasis) from PC3 and LNCaP cells using quantitative proteomics, and the differential expression was further confirmed by qPCR at mRNA level. Moreover, bioinformatics analysis indicated that proteins associated with cell junction, adherens and cytoskeleton regulation may play crucial roles in cell migration. These findings partially explain the mechanism of tumor cell migration (metastasis) and also provide the potential biomarkers for the diagnosis of tumor metastasis. These novel data of differential proteins will promote the development for treatment and diagnosis of metastatic prostate cancer.

Materials and Methods

Cell lines

Human prostate cancer cell lines, PC3 (CRL-1435) and LNCaP (CRL-1740), were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). PC3 and LNCaP cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, 30-2002, ATCC, Manassas, VA, USA) and RMPI-1640 (30-2001, ATCC, Manassas, VA, USA) supplemented with 10% of fetal bovine serum (FBS, 10437-028, Life Technologies, Grand Island, NY, USA) in a humidified atmosphere of 5% CO₂.

Wound healing assay

Wound healing assay was performed as described previously [8]. Briefly, 3 x 10⁵ cells were seeded into a 6-well plate, and the complete culture medium was replaced with serum-free culture medium 6 h prior to the wound scratch. The monolayer cells were scratched using a 10-µL tip, and the cells were cultured with serum-free medium continuously. The images of healed wound at 0 h and 24 h were recorded using a common microscope, and the width of the wound was obtained using Image J software.

One-Dimensional SDS-PAGE analysis

Firstly, total proteins were extracted from cells using radio-immunoprecipitation assay (RIPA) buffer obtained from ThermoFisher Scientific (89900, Waltham, MA, USA), and the protein concentration was measured using a bicinchoninic acid (BCA) kit from ThermoFisher Scientific (23225, Waltham, MA, USA). Then the same amount of total proteins from the control and experiment groups were analyzed using a 4–20% Mini-PROTEAN TGX Precast Gel (4561093SEDU, Bio-Rad, Hercules, CA, USA) at 80 voltage for 1.5
h. Proteins in gel were further stained with coomassie brilliant blue R-250 (1610400, Hercules, CA, USA). The densities of differential protein bands were obtained using Quantity One software (1709600, Hercules, CA, USA).

In Gel Digestion

Gel slices were placed into 1.5 mL eppendorf tubes with 0.5 mL of water for 15 min. Then 50 µL of 250 mM ammonium bicarbonate was added, 20 µL of a 45 mM 1, 4 dithiothreitol (DTT) was added and the samples were incubated at 50°C for 20 min. For alkylation, samples were incubated with 30 µL of a 100 mM iodoacetamide solution for 15 min. Furthermore, 1 mL of 50:50 ratio of 50 mM Ammonium Bicarbonate: Acetonitrile was placed into each tube and samples were incubated at RT for 1 h. The solution was then removed and 300 µL of acetonitrile was added to each tube. Gel slices were rehydrated with 75 µL of 2 ng/µL trypsin. Additional bicarbonate buffer was added to completely submerse the gel slices. Gel slices were dehydrated with 100 µL of 80:20 ratio of Acetonitrile: 1% formic acid. The extract was combined with the supernatants of each sample. Finally, samples were dissolved into 25 µL of 5% Acetonitrile in 0.1% trifluoroacetic acid.

LC/MS/MS on Q Exactive

A 4.0 µL aliquot was injected onto a custom packed C₁₈ Magic 5µ particle trap column. Peptides were eluted and sprayed from a custom packed emitter in 30 min at a flow rate of 300 nanoliters/min using a Waters Nano Acquity UPLC system. Data dependent acquisitions were performed with a Q Exactive mass spectrometer (Thermo Scientific, Waltham, MA, USA). Raw data files were processed with Proteome Discoverer (version 1.4) prior to searching with Mascot Server (version 2.5) against the Uniprot database. The parameters of fixed modification of carbamidomethyl cysteine and variable modifications of acetyl (protein N-terminal), pyro glutamic for N-terminal glutamine, oxidation of methionine and serine/threonine phosphorylation were considered. Finally, Scaffold Viewer was used to assess the protein identification probabilities and label free quantitation.

Real-time qPCR

Total RNA was extracted using Trizol reagent from ThermoFisher Scientific (15596-026, Waltham, MA, USA). The first strand cDNA was obtained from 1 µg of total RNA by reverse transcription using a high-capacity cDNA reverse transcription Kit (4374967, Waltham, MA, USA). Next, the quantitative PCR (qPCR) was performed with Viia™ 7 Real-Time PCR System from ThermoFisher Scientific (4453535, Waltham, MA, USA) using cDNA, iTaq™ Universal SYBR Green Supermix from Bio-Rad (1725125, Hercules, CA, USA) and the primers listed in Table 1. Beta-actin was used as an internal control.

Bioinformatics analysis

Firstly, the heat map for the differential expression (> 1.5 fold upregulation or downregulation) of identified proteins were obtained using both Cluster and TreeView softwares. Secondly, the significant differential proteins were further analyzed by bioinformatics using String software (http://string-db.org/). Multiple protein names were input into String sequentially, and the organism was set as Homo sapiens. Several active prediction methods were used by String, at the same time, including neighborhood, gene fusion, cooccurrence, coexpression, experiments, databases, textmining and homology. During the analysis, we used the medium confidence (0.4). The parameter of the shown interactors was set as "custom limit=0", and the parameter of the additional (white) nodes was set as 0. By using above parameters, a signaling network was automatically generated by String database, which is composed of predicted or known protein interactions [9]. Moreover, the identified proteins were also clustered and analyzed using Cytoscape (ClueGo + CluePedia) as per their corresponding biological processes, molecular functions, cellular components and KEGG pathways [10].

Statistical analysis

Data were represented as means ± the standard deviation (SD). Statistical significances for comparisons between groups and among multiple groups (> 3) were determined using a Student’s paired t-test and analysis of variance (ANOVA) in Prism 5.0, and p < 0.05 was used as the level of significance.
Results

**PC3 cells show higher capability of migration than LNCaP cells**

To demonstrate the different abilities of cell migration between PC3 and LNCaP cells, the wound healing assay was employed to observe the cell migration *in vitro*. As shown in Fig. 1A and B, the wound of mono-layer PC3 cells was almost healed after 24 h of culture with serum-free medium, and the width of the wound for LNCaP cells did not show obvious change. These findings suggested that PC3 cells indeed present higher migration ability than LNCaP cells.

**SDS-PAGE analysis show differential protein bands**

To identify the differential proteins from PC3 and LNCaP cells, the proteins were firstly extracted, and the protein concentration was further measured. As shown in Fig. 2A, the R² value of the standard curve is 0.99, indicating that the protein concentration in this study was measured accurately. After the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel analysis and coomassie brilliant blue staining, the differential protein bands can be obviously observed (Fig. 2B), and the relative intensity of those bands were further analyzed by Quantity One. Totally, six significant differential bands were selected in this study, including two upregulated bands and four downregulated bands (LNCaP group vs. PC3 cells).

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**Table 1. Primer information of qPCR of selected genes**

<table>
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<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
<th>Amplicon size (bp)</th>
</tr>
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<tr>
<td>ACTN1</td>
<td>CCGAGCTGGATGACTACGGG</td>
<td>GCACTTCAAAAGATCTCTCG</td>
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<td>NLE3</td>
<td>TTCGATCGTGATACCACTCTG</td>
<td>CAGATGATGTTTTCATGGGT</td>
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<td>POLR2A</td>
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<td>GTGTTCCATCCGCAATGAC</td>
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<tr>
<td>PPP2R4</td>
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<td>GGGACTCTTGACTGAAAGT</td>
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</tr>
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<td>GGCAGGACAGTGTCTCAA</td>
<td>101</td>
</tr>
<tr>
<td>FN1</td>
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<td>AAACCTCGCTCTGTAATAA</td>
<td>130</td>
</tr>
<tr>
<td>GULP1</td>
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<tr>
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<td>AGTCCATACAGTAGCAGCA</td>
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</tr>
<tr>
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<td>105</td>
</tr>
<tr>
<td>VCL</td>
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<td>CATTGGTCGAGAACACTC</td>
<td>105</td>
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**Fig. 1.** PC3 cells show higher capability of migration than LNCaP cells. (A) Monolayer tumor cells were scratched with a 10 µL tip, and the images of healed wound were recorded at 0 h and 24 h using a microscope. (B) The width of the healed wound were obtained using Image J software. Experiment was repeated three times ***, p < 0.001.
Fig. 2. SDS-PAGE analysis of total proteins of PC3 and LNCaP cells. (A) Standard curve for measuring the protein concentration. X axis: absorbance value; Y axis: protein concentration. (B) Same amount of total proteins from PC3 and LNCaP cells and the protein markers were separated by SDS-PAGE, and the differential proteins bands were labelled after coomassie brilliant blue staining. (C) The gray intensity of differential protein bands were obtained using Quantity One software. All experiments were repeated in triplicate. *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

Fig. 3. Differential expressed proteins identified by LC-MS/MS. (A) The heat map was generated using Cluster and TreeView softwares sequentially. The red cubes represent upregulated proteins and green cubes indicate downregulated proteins, respectively. (B) The expression of upregulated differential proteins at the mRNA level was confirmed by real time-qPCR. (C) The expression of downregulated differential proteins at the mRNA level was confirmed by real time-qPCR, *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

PC3 group), and the molecular weight of proteins ranging above 25 kilo Dalton (kDa) (Fig. 2B and C). Our results indicated that PC3 and LNCaP cells actually expressed a large amount of differential proteins that may be associated with their distinct migration potentials.
Quantitative proteomics identify differential expressed proteins

To know the specific proteins in those differential bands in gel, liquid chromatography (LC)/MS/MS was used to quantitatively identify the differential proteins based on the intensity based absolute quantification (iBAQ). As shown in Fig. 3A, the heat map clearly showed 203 differential proteins in total. The downregulated proteins were from differential bands 2, 4, 5 and 6, and the upregulated proteins were from differential bands 1 and 3. Expectedly, the expression of these differential proteins was confirmed at the mRNA level (Fig. 3B and C). These data suggested that a large amount of proteins were differentially expressed in LNCaP and PC3 cells, and their expression of protein levels were consistent with that of mRNA levels. In summary, those tumor cell migration or metastasis associated proteins were accurately identified using quantitative proteomics analysis.

Bioinformatics analysis reveal the associations of differential proteins with ability of cell migration

To figure out the associations between differential proteins and the cell behavior, we firstly analyzed the proteins by String software. As shown in Fig. 4, the network of the proteins were established according to known or predicted protein-protein interactions. Furthermore, the genes of these differential proteins were clustered by ClueGo and CluePedia of Cytoscape as per the related biological process (Fig. 5A), cellular component (Fig. 5B), KEGG pathway (Fig. 5C) and molecular function (Fig. 5D). In terms of biological process, many genes were closely related to cell junction assembly and organization (Fig. 5A), and almost half of them belong to stress fiber and fascia adherens in terms of cellular component (Fig. 5B). KEGG pathway analysis indicated that one in third of genes were associated with actin cytoskeleton regulation (Fig. 5C). Consistent with the network analysis by String, these findings further confirmed that proteins associated with cell junction, adherens and...
Cytoskeleton regulation may play critical roles in cell migration. Interestingly, the cluster of molecular function showed that approximately half of the genes were tightly correlated with ATPase activity, oxidoreductase activity, actin filament binding and NAD binding (Fig. 5D), and these functions markedly influence the tumor progression. Taken together, the bioinformatics analysis in our study uncovered a molecular network and clarified the specific roles of those differential expressed proteins in prostate cancer cell progression.

Discussion

Prostate cancer is one of the most diagnosed and death-related cancer for males worldwide [1]. On one hand, many cases showed slow-growing and harmless of prostate cancer, and no treatment is required. On the other hand, the 5-year survival rate for those patients with distant metastasis sharply decreased [5]. Therefore, the efficient treatment for prostate cancer was markedly dependent on their stages. During the prostate cancer progression, the biomarker and the molecular mechanism were two key points for the efficient diagnosis, surveillance and treatment.

In general, early prostate cancer locally grew in prostate tissue, and the carcinogenesis is accompanied with increasing level of prostate specific antigen (PSA) and cell proliferation, and the hormone therapy is efficient at this stage. However, the prostate cancer will be insensitive to hormone therapy for some unclear reasons, i.e. CRPC [4]. Finally, the distant metastases of prostate cancer will be found in bone, lymph node, rectum, bladder and lower ureters [3, 11]. The metastases will seriously threaten the lifespan of patients. Hence, it is
urgent to find more efficient biomarkers and uncover the mechanism of prostate cancer metastasis.

PC3 and LNCaP cell lines were derived from bone and lymph node metastases of patients, respectively, and these two tissues were the most favorable metastatic sites of prostate cancer cells. Thus, PC3 and LNCaP cell lines are two excellent cell model for discovering novel biomarkers and molecular mechanism for metastatic prostate cancer.

Comparative proteomics is a powerful tool to analyze thousands of proteins simultaneously, and it was popularly used for identifying the useful biomarkers and signaling network in many diseases, particularly in the field of cancer research. Here, the iBAQ technique will be benefit for the quantification of specific proteins between two groups. Moreover, the qPCR data demonstrated that the iBAQ based quantification was reliable for further analysis (Fig. 3).

Bioinformatics analysis using String showed that proteins tightly associated with cell junction, adherens, and cytoskeleton, including PTK2, ACTN1, TJP1, FLNA, MYH9, SHC1, FLNB and FLNC [8, 12-16], constituted the core part of the interaction network (Fig. 4). Interestingly, FLNA and FLNB were also upregulated in hepatocellular carcinoma cells with high metastatic potential [8]. These findings suggested that proteins associated with cell junction, adherens, and cytoskeleton played important roles in cell migration or metastasis. Meanwhile, some proteins closely related to malignancy, including ACACA [17], PKN2 [18], MTOR [19], and TOP2A [20], were also presented in this network. Overall, the String network analysis indicated that the cell migration was promoted by these different function of proteins synergistically.

Cluster analysis by Cytoscape on the basis of biological process, molecular function, KEGG pathway and cellular component revealed a lot of critical information for the proteomics data [10]. The cluster results of Cytoscape were highly consistent with that of String, pointing the critical roles of cell junction assembly, organization, fascia adherens and cytoskeleton regulation in prostate cancer cell migration (Fig. 5). Also, other tumor associated function was characterized, such as mRNA metabolic process, ATPase activity, NAD binding and so on.

Taken together, our study firstly identified the differential proteins between PC3 cells and LNCaP cells using quantitative comparative proteomics. Bioinformatics analysis indicated the important roles of those differential proteins in prostate cancer cell migration. The data provided a better insight into the mechanism and also identified many candidates of biomarkers for prostate cancer metastasis. Our findings pave an avenue for the development of novel drug and biomarker, and then patients with prostate cancer could be benefit from more efficiently diagnosis and treatment.

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Disclosure Statement

None conflict of interest.

References


