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PDLIM5 identified by label-free quantitative proteomics as a potential novel biomarker of papillary thyroid carcinoma

Xiaodan Wei [a, b], Yuan Zhang [b], Shoujun Yu [c], Shasha Li [b], Wenguo Jiang [b], Yanping Zhu [b], Yuxue Xu [b], Chunhua Yang [b], Geng Tian [b], Jia Mi [b], Jonas Bergquist [a, e], Miaoqing Zhao [d, **], Fangzhou Song [a, *]

[a] Molecular Medicine and Cancer Research Center, Chongqing Medical University, Yuzhong District, No.1, Medical Road, Chongqing 400016, China
[b] Medicine and Pharmacy Research Center, Binzhou Medical University, Laishan District, No.346, Guanhai Road, Yantai, Shandong Province 264003, China
[c] Department of Radiology, Affiliated Hospital of Binzhou Medical University, 661 Second Huanghe Rd, Binzhou, Shandong Province 256603, China
[d] Department of Pathology, Provincial Hospital Affiliated to Shandong University, No. 324 Jingwu Weiqi Road, Jinan, Shandong Province 250021, China
[e] Department of Chemistry - BMC, Uppsala University, PO Box 599, Husargatan 3, Uppsala 75124, Sweden

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ABSTRACT

In order to better understand the mechanisms underlying the development of papillary thyroid carcinoma (PTC), and to identify new potential biomarkers, high-resolution label-free mass spectrometry was performed on PTC tissues and adjacent normal thyroid tissues from six patients. In this process, 2788 proteins were identified, out of which 49 proteins presented significant differences between PTC tissues and adjacent normal thyroid tissues. Gene ontology revealed that the majority of these proteins are involved in the catalytic activity and binding. We selected three proteins with differential expressions: PDZ and LIM domain 5 (PDLIM5), PDLIM1 and ALDH1A1; Protein expressions were further verified by RT-PCR and western blot. Among these, expression of PDLIM5 and PDLIM1 was up-regulated, while that of ALDH1A1 was down-regulated in PTC tissues. Next, we confirmed their expression through quantitative dot blot (QDB) technique. We found that knockdown of PDLIM5 expression in the B-CPAP cell line could inhibit the migration, invasion and proliferation of PTC cells. In addition, PDLIM5 knockdown reduced Ras and Phospho-ERK1/2 expression. Thus, we suggested that PDLIM5 promotes PTC via activation of the Ras-ERK pathway. Our research provides new molecular insight into the function of PDLIM5, which may assist in studying the mechanism of PTC. In addition, PDLIM5 could be further explored as a potential candidate for PTC treatment.

1. Introduction

Thyroid carcinoma is one of the most common malignancies of the endocrine system. Its incidence rate has rapidly increased over the last decades [1,2]. Among all types of thyroid carcinoma, 80% cases are that of PTC [3] which usually has an excellent prognosis [4]. However, subsequent disease relapse occurs in 10–15% of PTC patients. Patients with distant metastases usually do not response well to standard treatments, and show poor clinical outcomes [5]. Currently, there is a shortage of markers for the evaluation of PTC prognosis and monitoring of PTC recurrence. As a primary step to identify novel therapeutic targets, it is essential to understand the molecular mechanisms that underlie PTC.

Proteomics, especially quantitative proteomics, has been applied to improve our understanding of cancer pathogenesis, and to develop novel biomarkers associated with carcinogenesis and tumor progression. The use of nano-liquid chromatography coupled to tandem mass spectrometry (Nano-LC-MS/MS), which could determine trace amounts of proteins in biological samples, is an effective method to examine the potential biomarkers from various samples [6]. Various studies have shown that specific biomarkers in breast cancer, lung cancer, gastric cancer and other cancers [7–11] can be identified through proteomics.

In the present study, we identified totally 49 differentially expressed proteins through the proteomics technique by

* Corresponding author.
** Corresponding author.
E-mail addresses: zhaomqsd@163.com (M. Zhao), FangZhou_s@163.com (F. Song).

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comparing protein expression profiles between PTC tissues and adjacent normal thyroid tissues. Next, we focused on PDZ and LIM domain protein 5. PDZ-LIM proteins are a family of proteins that possess a 100-amino acid PDZ domain in the N terminus and one to three LIM domains in the C terminus [12]. PDZ-LIM proteins are involved in scaffolds for the formation of multiprotein complexes [13]. Scaffolding proteins are typically depicted as non-catalytic polypeptides that passively tether enzymes to their substrates. However, recent studies indicated that scaffolds can allosterically control specific catalytic events [14]. PDLIM5 is also known as enigma homolog (ENH), a member of the enigma subfamily, which features one N-terminal PDZ domain and three C-terminal LIM domains [15]. Multiple transcript variants that encode different isoforms have been found for this gene, though not all of them have been fully characterized [16]. PDLIM5 has been reported to be associated with mental illnesses [17–19] and heart diseases [20]. Some findings suggested that PDLIM5 expression may be associated with progression of gastric cancer [21], lung cancer [22], and breast cancer [23]. However, little is known regarding functional roles of PDLIM5 in PTC. Heiliger K.J et al. found that PDLIM5 mRNA expression is higher in PTC tissues than in normal thyroid tissues [24]. However, its role in PTC carcinogenesis is still poorly understood, and remains to be elucidated. Here, we investigated the expression and functions of PDLIM5, and showed that it can promote PTC through enhancement of the Ras/ERK signaling cascade. Ras/ERK signaling affects a wide set of cellular events, including growth, proliferation, differentiation, survival and migration [14]. Our research provides a basis for further screening of specific PTC markers.

2. Materials and methods

2.1. Clinical samples and ethics statement

Seventy-five pairs of papillary thyroid cancer tissues and adjacent normal thyroid tissues were obtained from surgical specimens at the Binzhou Medical University -affiliated hospital. All specimens were immediately frozen in liquid nitrogen, and stored at −80 °C until further processing. The present work was conducted in accordance with the protocols approved by the Binzhou Medical University Ethics Committee, and informed consent was obtained from all the patients.

2.2. Protein extraction, in-solution digestion, and peptide purification

Tissue samples were suspended and homogenized in lysis buffer containing 9 M Urea, 20 mM HEPES, and a protease inhibitor cocktail. Samples were briefly sonicated, followed by centrifugation at 12,000 g and 4 °C for 10 min. The total protein content of the supernatant was measured using the Bradford assay (Bio-Rad).

In order to study the differential expression of PTC and the adjacent normal thyroid tissues, in-solution digestion was carried out in six pairs of samples. For this, 35 μg protein was diluted in 100 μL digestion buffer (6 M urea, 100 mM TEAB). Next, 10 μL 45 mM DTT solution was added. The mixture was incubated at 50 °C for 15 min, followed by the addition of 10 μL 100 mM IAA solution. This was incubated at room temperature for 15 min in darkness. Trypsin/Lys-C (Wako Chemicals, Osaka, Japan) was dissolved in digestion buffer and added to each sample to obtain a final trypsin/protein concentration of 5% (w/w). An overnight incubation was carried out at 37 °C. Digestion was terminated by the addition of 1:1 with trifluoroacetic acid (TFA) in acetonitrile (ACN) and MilliQ water to the sample. A sample containing 20 μg of digested proteins was desalted using C18 Stage-tips with EmporeDisksC18 from Varian (Palo Alto, CA, USA), and subjected to complete dry in a vacuum centrifuge.

2.3. Lable-free quantitative mass spectrometer

Processed samples were analyzed for LC/MS. All analyses were performed using a QExactive plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoelectrospray ion source. Dried samples were dissolved in 0.1% formic acid in water. Peptides were separated by reversed phase liquid chromatography using an EASY-nLC 1000 system. A two-step column separation method was used. The pre-column was a 2 cm EASY-column (1D 100 μm, 5 μm, C18), whereas the analytical column was a 10 cm EASY-column (1D 75 μm, 3 μm, C18). Peptides were eluted with a 90-min long linear gradient from 4% to 100% ACN at 250 nL/min. The mass spectrometer was operated in positive ion mode to acquire a survey mass spectrum with a resolving power of 70000 and a consecutive high collision dissociation (HCD) fragmentation spectra of the 10 most abundant ions. Gene ontology and protein class analysis were performed with the PANTHER classification system.

2.4. Real time PCR

Total mRNA from tissues and cultured cells was extracted using TRizol (Invitrogen, CA, USA) according to the manufacturer's instructions. Total RNA(1.5 μg) was used by SuperScript II reverse transcriptase (Invitrogen, CA, USA) according to the standard instructions. Real-time PCR was performed with a final volume of 20 μL containing 1 μL cDNA template, 2 μL of each 10 nM primer, and 10 μL SYBR-Green Master mix (TransGen Biotech, Beijing, China). The primers used were as follows:

PDLIM5 F: 5′-TCAACATGCCTCTGACAATCTC-3′
R: 5′-GCCTTGCCGCCATCTTTTAG-3′
PDLIM1 F: 5′-CCCAGCAGATAGACCTCCAG-3′
R: 5′-TCTGAGCTTCCAAGTGTGTCATA-3′
ALDH1A1 F: 5′-CCGTGGCGTACTATGGATGC-3′
R: 5′-GCAGCAGACGATCTCTTTCGAT-3′
ERK F: 5′-GGC ATC CGA GAC ATC CTC AG-3′
R: 5′-TAT GTA CTT GAG GCC CCG GA-3′
RAS F: 5′-GGA CAA TCG CTA ACA ACC CCC T-3′
R: 5′-GGC ACT CTT TCC CAC GCC TCT A-3′
GAPDH F: 5′-TGTGGGCATCAATGGATTTGG-3′and
R: 5′-ACACCATGTATTCCGGGTCAAT-3′

GAPDH was used as internal control gene. Each sample was measured in triplicate. Changes of the expression were calculated using the $2^{-\Delta\Delta ct}$ method [25]. The relative expression ratio was presented as the fold change.

2.5. Western blot

Tissues and cell samples were lysed in radioimmunoprecipitation assay buffer. Equal amounts of protein (25 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred onto polyvinylidene fluoride membranes. Non-specific binding sites were blocked with PBS-Tween-20 and 5% fat-free milk for 1 h at room temperature. Next, the samples were incubated overnight at 4 °C with the primary antibody PDLIM5 (ab83060, Abcam), PDLIM1 (ab155788, Abcam), ALDH1A1 (#11388-H07E, Sino Biological Inc), Phospho-p44/42 MAPK (Erk1/2) (#4370, Cell Signaling Technology) and Ras (ab52939, Abcam). Membranes were washed three times for 10 min in TBST, and then incubated with appropriate secondary

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