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SWATH™- and iTRAQ-based quantitative proteomic analyses reveal an overexpression and biological relevance of CD109 in advanced NSCLC☆

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ABSTRACT

To identify cancer-related proteins, we used isobaric tags in a relative and absolute quantitation (iTRAQ) proteomic approach and SWATH™ quantification approach to analyze the secretome of an isogenic pair of highly metastatic and low metastatic non-small-cell lung cancer (NSCLC) cell lines. In addition, we compared two groups of pooled serum samples (12 early-stage and 12 late-stage patients) to mine data for candidates screened by iTRAQ-labeled proteomic analysis. A total of 110 proteins and 71 proteins were observed to be significantly differentially expressed in the cell line secretome and NSCLC sera, respectively. Among these proteins, CD109 was found to be highly expressed in both the highly metastatic cell line secretome and the group of late-stage patients. A sandwich ELISA assay also demonstrated an elevation of serum CD109 levels in individual NSCLC patients (n = 30) compared with healthy subjects (n = 19). Furthermore, CD109 displayed higher expression in lung cancer tissues compared with their matched noncancerous lung tissues (n = 72). In addition, the knockdown of CD109 influenced several NSCLC cell bio-functions, for instance, depressing cell growth, affecting cell cycle phases. These phenomena suggest that CD109 plays a critical role in NSCLC progression.

Biological significance
We simultaneously applied two quantitative proteomic approaches—iTRAQ-labeling and SWATH™—to analyze the secretome of metastatic cell lines, in order to explore the cancer-associated proteins in conditioned media. In this study, our results indicate that CD109 plays a critical role in non-small-cell lung cancer (NSCLC) progression, and is overexpressed in advanced NSCLC.

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1. Introduction

Lung cancer remains the leading cause of cancer death, and approximately 90% of all lung cancer deaths are due to metastases [1]. Once the tumor cells have metastasized and spread throughout the lungs, the cancer becomes very difficult to treat [2]. Imaging techniques and biopsy with histopathological examination have been widely used; however, further studies are necessary to identify cancer-associated proteins that could be used to monitor cancer progression, predict the risk of cancer metastasis and contribute to understanding the biology of lung cancer metastasis [3–6].

Mass spectrometry-based proteomics is a powerful tool for biological and clinical research and enables relatively comprehensive global analyses [7,8]. Relative quantitation with stable isotope labels or label-free methods has been widely used to study differential protein expression profiles [9–11]. For example, isobaric tags for relative and absolute quantification (iTRAQ), which can simultaneously label and quantify four to eight samples, are employed widely in discovery-based proteomics [12,13]. Alternatively, label-free methods based on a data-independent acquisition (DIA) strategy have emerged as powerful quantification tools because of recent developments in high-resolution quantitative proteomics [14]. SWATH™ was recently reported as a label-free quantification approach based on a DIA strategy and can successfully quantify compounds with high complexity. The technique is performed using a fast, high-resolution Q-TOF instrument that can repeatedly cycle through 32 consecutive 25 Da precursor isolation windows (swaths) and can generate a time-resolved fragment ion spectrum for all of the analytes detectable within the 400 to 1200 m/z precursor range [15–17]. As every method has its unique advantages, we simultaneously applied two quantitative proteomic approaches—iTRAQ-labeling and SWATH™—to analyze the cell secretome to explore cancer-related proteins and provide guidelines for approach selection based on special experimental designs.

The cancer cell secretome, particularly the secretome of an isogenic pair of highly metastatic and low metastatic model cell lines, contains rich underlying information about cancer progression and metastasis, making it a good source for discovering proteins involved in cancer [18]. In our study, we compared the secretome of two lung cancer cell lines, SPC-A-1 sci and SPC-A-1, which possess different lung metastatic capacities [19]. The cell line SPC-A-1 sci was isolated from lung metastases in NOD/SCID mice after several cycles of subcutaneous injection of the cell line SPC-A-1. Using this pair of model cell lines, our previous studies revealed several critical aspects of lung cancer metastasis based on different characteristics of the proteome and the genome [20,21]. Therefore, studying the secretome of these two cell lines is an attractive approach for identifying new candidate proteins associated with cancer progression and metastasis. In addition, it has been reported that the proteins secreted by tumors and other cells could enter their microenvironment and subsequently enter the bloodstream or other body fluids, eventually affecting the cancer cell malignancy phenotypes [22–24]. Therefore, we compared the secretome of two cell lines using iTRAQ-labeling coupled with two-dimensional liquid chromatography/tandem mass spectrometry (2D-LC–MS/MS) and SWATH™ coupled with one-dimensional liquid chromatography/tandem mass spectrometry (1D-LC–MS/MS) to explore cancer-related proteins. We then analyzed serum samples of two pooled groups (early-stage and late-stage NSCLC, n = 12 samples/group) with iTRAQ-labeling coupled with 2D-LC–MS/MS after immuno-depleting the 14 most highly abundant proteins. After combining the aforementioned analysis datasets, we identified CD109 as a candidate protein for further study.

2. Experimental methods

2.1. Cell lines and cell-secreted protein preparation

Cells were cultured in DMEM supplemented with 10% fetal bovine serum (Biowest, South America Origin) at 37 °C and 5% CO_2. After reaching 70% confluence, these two cell lines were washed three times with PBS to remove serum residue in the conditioned media (CM) and incubated for 48 h in serum-free DMEM. With cell viability higher than 97% (determined with 0.4% Trypan Blue solution; Invitrogen), the CM was collected with sterile filtration (pore size: 0.22 μm, Millipore) and centrifugated (1000 *g* for 10 min, 4 °C) to remove the floating cells and cellular debris. Then, Amicon Ultra-15 Centrifugal Filter Devices and Amicon® Ultra-0.5 Centrifugal Filter Devices (Millipore) were used to concentrate the CM by ultrafiltration according to instructions. Protein concentrations were determined by a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford).

2.2. Patients and serum collection

The Human Ethics Committee of Shanghai Jiao Tong University School of Medicine approved this study. When collecting serum, the patient blood was allowed to coagulate for 30 min, centrifuged at 1200 *g* for 10 min at 4 °C and subsequently stored at −80 °C. Twenty-four patients with lung cancer were divided into 2 groups (n = 12), early or late stage, respectively (Supplementary Table 4). Serum samples from 12 patients were pooled in equal volumes for each group, which was followed by Agilent Technologies column-based depletion. Another 19 healthy and 6 NSCLC samples (pathological stages III and IV) from the same hospital were used for further verification of CD109 expression. The 19 healthy serum samples were collected from the healthy individuals on physical examination in the health care department of the same hospital. All serum samples were collected prior to the administration of any treatment or surgery.

2.3. Affinity depletion of serum samples

Highly abundant proteins in serum samples were depleted using a multiple affinity removal system column (Agilent hu14 4.6 * 100 mm) that could selectively fractionate 14 high-abundance proteins from human serum [25,26]. According to the manufacturer's instructions, approximately 35 μL of serum was processed per sample and analyzed using an Agilent 1260 HPLC system. Low-abundance proteins were concentrated and desalted using 10-kDa molecular cutoff