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Proteomics-based identification of secreted protein dihydrodiol dehydrogenase as a novel serum markers of non-small cell lung cancer

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Summary Identification of secreted proteins of lung cancer could provide new candidates of serum biomarkers for cancer diagnosis and prognosis evaluation. In this study, non-small cell lung cancer (NSCLC) cell line A549 was cultured. Proteins in the conditioned medium of A549 were recovered and the proteome analysis was subsequently performed. Secreted proteins of A549 were identified using mass spectrometry and database search. Fourteen human proteins were identified, including peptidyl—prolyl *cis*—*trans* isomerase A, manganese superoxide dismutase, peroxiredoxin 1, phosphatidylethanolamine-binding protein, glutathione *S*-transferase P, PGP9.5, alpha enolase, phosphoglycerate mutase 1, galectin-1 and dihydrodiol dehydrogenase (DDH). DDH was selected for further analysis using RT-PCR, immunoblotting, immunohistochemical staining and ELISA in NSCLC patients. Compared with normal lung tissues, higher DDH mRNA and protein expression level were found in 15 NSCLC cancer tissues ($p < 0.05$). DDH overexpression was identified to be located in cytoplasm and cell membrane by immunohistochemical staining in NSCLC tissue. The serum level of DDH was significantly higher in NSCLC patients ($n = 64$) than nonmalignant lung tumor ($n = 20$) and healthy controls ($n = 20$) ($p < 0.05$). The results show that DDH was one of the secreted proteins in NSCLC. It can serve as a tissue marker and a novel serological marker of NSCLC. Identification of secreted proteins could be a feasible and effective strategy to search potential serum biomarkers of cancer.
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1. Introduction

Secreted proteins of cancer cells can be shed into blood. Their serum levels may increase in the early stage of cancer, and correlate with cancer cell proliferation and/or

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secreted protein overexpression. As a result, secreted proteins may be used as potential serum biomarkers of cancer [1,2]. Well-known secreted protein serving as serum biomarkers include CEA for colon cancer, prostasin and human kallikrein 10 for ovarian cancer [3,4], cysteine-rich secretory protein-3 and prostate-specific antigen (PSA) for prostate cancer [5,6]. Secreted protein analysis, together with genomic approaches have facilitated identification of a number of serum biomarkers [7] for malignant tumors, such as macrophage inhibitory cytokine 1 in metastatic prostate, breast, and colorectal carcinomas [2], osteopontin and prostasin in ovarian carcinomas [4,8], tissue inhibitor of metalloproteinase-1 (TIMP-1) in pancreatic adenocarcinoma [9]. Another good example is showed in the investigation of nasopharyngeal carcinoma (NPC) cell-secreted proteomes using SDS-PAGE and matrix-assisted laser desorption/ionization-time of flight mass (MALDI-TOF MS), three serum biomarkers of NPC were identified which included fibronectin, Mac-2-binding protein (Mac-2 BP), and plasminogen activator inhibitor 1 (PAI-1) [1]. We postulated that analysis of the secreted proteins of lung cancer cells may be an effective approach for delineating potential serum biomarkers.

Lung cancer is the most common cancer and the leading cause of cancer-related death worldwide. The early diagnosis of lung cancer is critical for successful therapy. Serological biomarkers can play important roles in cancer screening, monitoring of cancer progression, treatment response and surveillance for recurrence [10]. Serum protein biomarkers, such as carcinoembryonic antigen (CEA), carbohydrate antigen 125 (CA-125), cytokeratin 19 fragment marker (CYFRA 21-1) are not ideal tools in detection of lung cancer due to their low specificity and/or sensitivity [11,12]. For more effective lung cancer diagnosis, we need to find new biomarkers, which have higher specificity and sensitivity.

Lung cancer is divided clinically into small-cell lung cancer (SCLC) and non-SCLC (NSCLC). NSCLC comprises more than 80% of lung cancers. In this study, human NSCLC cell line A549 was cultured and the secreted proteins in conditioned medium were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and MALDI-TOF MS. Secreted protein dihydrodiol dehydrogenase (DDH) was identified and confirmed. High DDH expression was detected in NSCLC cancer tissues. The serum level of DDH was evaluated in NSCLC patients, benign lung tumor patients and healthy individuals using enzyme-linked immunosorbent assay (ELISA) which indicated high serum DDH level in NSCLC.

2. Material and methods

2.1. Cell culturing and sample preparation

A549 cells (ATCC no. CCL-185) were grown in RPMI 1640 supplemented with 15% fetal bovine serum (FBS) at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 (v/v). To obtain culture supernatants, cells were grown to confluence in 15 cm tissue culture dishes, then washed with serum-free medium and incubated in 5% FBS medium for 48 h. Five milliliters conditioned medium were collected and centrifuged to eliminate the intact cells. The supernatant was treated with DOC—TCA (deoxycholate—tricarboxylic acid) sediment method to recover the total protein. Added 0.1 mL 0.15% DOC (w/v) into per milliliter supernatant and mixed well, incubated on ice for 10 min, then added 50 μL 100% TCA (v/v) per milliliter to the mixture, centrifuged at 20,000 × g for 30 min, carefully discarded the supernatant, and air dry at last. Protein in medium of RPMI-1640 with 5% FBS was collected in the same way as the conditioned medium and used as negative control. Precipitated protein samples were resuspended in cell lysis buffer (8 mol/L urea, 4% CHAPS, 40 mmol/L Tris, 65 mmol/L DTT) and the supernatant was used for proteomic analysis. Cells left on the dishes were washed twice with PBS, and disrupted in cell lysis buffer for 30 min. The protein extracts were centrifuged at 20,000 × g for 30 min and the supernatants were used for immunoblotting. Protein concentrations of samples were determined by the BCA protein assay reagent from Pierce (Rockford, IL, USA).

2.2. Patient population and clinical specimens

Fifteen newly pathological identified NSCLC tumors samples and distant (distance to the edge of tumor >5 cm) nonmalignant lung samples of the same patient were obtained at the time of surgery at the Department of cardiothoracic surgery, Xiangya Hospital, China, and stored at −80 °C. These were used for reverse transcription polymerase chain reaction (RT-PCR) analysis and immunoblotting. Samples used for immunohistochemical staining were fixed in 10% buffered formalin and embedded in paraffin. There were 5 adenocarcinomas and 10 squamous carcinomas. The median age was 55 years (range 45—72 years); 5 were female and 10 were male. Eighty-four serum samples were collected which included 34 adenocarcinomas, 30 squamous carcinomas and 20 benign lung tumor patients. The clinical data was listed on Table 1. Sera were obtained from the Department of cardiothoracic surgery, Xiangya Hospital. The control group for ELISA consisted of 20 apparently healthy blood donors (ages 45—66 years; mean 51 years; 10 females and 10 males). Blood samples (5 mL) were collected, clotted at 4 °C, centrifuged at 3000 rpm for 10 min then stored at −80 °C. The study was approved by the Medical Ethics and Human Clinical Trial Committee at Xiangya Hospital.

2.3. Protein separation by two-dimensional electrophoresis (2-DE)

Total protein (1200 μg) of conditioned medium and control were separated by 2-DE which separated proteins according to their isoelectric points and molecular weights. Isoelectric focusing was performed using IPGstrip (pH 3-10L, 24 cm) on IPGphor isoelectric focusing cell (Amersham Biosciences) and second-dimension SDS-PAGE using Ettan Dalt II system (Amersham Biosciences) was conducted as described by manufacturer and Gorg [13]. After electrophoresis, the protein spots were visualized by ''blue silver'' staining technique described by Candiano et al. [14]. Stained with 0.12% Coomassie blue G-250 (Sigma, USA), 10% ammonium sulfate, 10% phosphoric acid, and 20% methanol for 24 h, and the background was destained with 10% methanol and 10% acetic acid. The stained 2-DE gels were scanned using the

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