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Identification of metastasis associated proteins in human lung squamous carcinoma using two-dimensional difference gel electrophoresis and laser capture microdissection

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ABSTRACT

A quantitative proteomic approach was used to discover potential protein markers associated with lymph node metastasis (LNM) in human lung squamous carcinoma (LSC). Laser capture microdissection was performed to purify LSC cells with LNM (LNM LSC) and LSC without LNM (non-LNM LSC). The differentially expressed proteins between pooled microdissected non-LNM LSC and LNM LSC cells were identified by two-dimensional difference gel electrophoresis (2D-DIGE) coupled with mass spectrometry (MS). 14 proteins were found to be differentially expressed between non-LNM LSC and LNM LSC. Among these proteins, ten proteins were overexpressed in LNM LSC compared with non-LNM LSC, and four proteins were downregulated in LNM LSC. Some of these identified proteins (Annexin A2, HSP27, CK19, and 14-3-3σ) were further confirmed by Western blotting and immunohistochemical analysis. These results show the value of LCM coupled with 2D-DIGE in identifying potential markers for lymph node metastasis of LSC, and also provide further insights into the prognosis of LSC.

1. Introduction

Lung cancer is the leading cause of cancer related death in men, and in women it has surpassed even breast cancer [1]. According to the WHO's estimation, China will become one of the countries that have a relatively high incidence of lung cancer in the 21st century. Squamous carcinoma is the most common type, accounting for 30–50% in all of lung cancer patients. In Chinese urban districts, the number of squamous carcinoma patients is rapidly increasing, affecting 50–70% of all of lung cancer patients [2,3]. It has been accepted that the most important predictor of survival in LSC is the use of tumor, node, metastasis (TNM) staging at diagnosis. However, approximately half of the patients present with metastastic disease at diagnosis. The long-term survival rate continues to be unsatisfactory, and no more than 50% of the cases who successfully undergo potentially curative resection survive for more than 5 years after surgery, the remaining cases eventually suffering widespread metastases or local recurrence [4,5]. Lymph node metastasis is the main route of metastases in LSC. Unfortunately, very little is known about how lung cancer cells give rise to LNM.

Metastasis is a complex pathologic course consisting of multiple stages and regulated by multiple genes. Differential proteome analysis allows the identification of aberrantly expressed proteins in cancer that might provide key information for finding biomarkers for treatment of cancer as well as an understanding of the mechanism of metastasis [6]. Clinical tissue sample is the most meaningful and direct material to carry out proteomic analysis. However, tumor tissue is composed of multiple kinds of cells. These other cells may influence the detection results. It is therefore very important to solve the cellular heterogeneity for a tumor molecular pathology

Abbreviations: LSC, lung squamous carcinoma; LNM, lymph node metastasis; 2D-DIGE, two-dimensional difference gel electrophoresis; 2-DE, two-dimensional electrophoresis; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; PMF, peptide mass fingerprint.

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study. Laser capture microdissection (LCM) overcomes this complication by collecting specific cell populations from primary tissue [7]. Although LCM can obtain homogeneous preparations of cells it is technically difficult and time consuming. The small volume of protein obtained also makes the analysis challenging. In our study, we have used 2D-DIGE as it allows more sensitive protein expression profiling with LCM samples. A relatively small quantity of protein extracts are first labeled using fluorescent dyes such as cyanine (Cy2, Cy3 or Cy5) dyes, then mixed equally and subjected to 2D separation. After scanning at different emission wavelengths, multiple images corresponding to different samples are generated from one 2D gel. 2D-DIGE reduces the gel-to-gel variability associated with traditional two-dimensional electrophoresis (2-DE) and improves accuracy in protein semiquantitation [8]. This novel technology is being applied to the analysis of protein expression in different neoplasia for the search of markers [9–11].

Recently, while employing the technologies of LCM and 2D-DIGE in combination, some medical and clinical applications of tissue proteomics promised to identify new diagnostic markers and drug targets in different types of tissues such as esophageal cancer, glomeruli, and gastric adenocarcinoma [11–14]. In this report, we first describe protein expression alterations by LCM and 2D-DIGE between LNM LSC and non-LNM LSC. After image analysis by software, it revealed changes in abundance of 14 proteins with statistical variance of LNM LSC versus non-LNM LSC. Some of the differentially expressed proteins that we identified were confirmed by Western blotting and immunohistochemistry (IHC). The results were consistent with the 2D-DIGE results, and proved the credibility of 2D-DIGE analysis. Our data indicate that these proteins have potential utility as biomarkers for LSC, and provide valuable insight into the metastasis mechanisms in LSC.

2. Materials and methods

2.1. Materials

Cy2, Cy3, and Cy5 were purchased from GE Healthcare (Uppsala, Sweden). Immobiline pH-gradient (IPG) DryStrips (pH 3–10 NL, 24 cm), IPG buffer (pH 3–10), DryStrip cover fluid, thiourea, urea, DTT, Pharmalyte (pH 3–10), bromophenol blue, Commassie Brilliant Blue G-250, Trisbase, SDS, molecular weight marker were purchased from Amersham Biosciences (Stockholm, Sweden). Modified trypsin (sequencing grade) was obtained from Promega (Madison, WI, USA). All other chemicals and biochemicals used were at least analytical grade and all buffers were prepared using Milli-Q deionized water (Millipore, Bedford, MA, USA).

2.2. Tissue samples

For proteomics analysis and Western blotting, 24 cases of fresh LSC were obtained from the First and Second Xiangya Hospitals of Central South University and the Cancer Hospital of Hunan Province, China. All lung cancer tissues were obtained from surgically treated patients and the study protocol was approved by the Medical Ethics and Human Clinical Trial Committee of Central South University. These samples were frozen in liquid nitrogen within 30 min after surgery until they were used for the experiments. All tissue samples were verified by histopathology before microdissection. The LSC samples were divided into two groups of 12 non-LNM LSC tissues and 12 LNM LSC tissues according to lymph node metastasis status. All of non-LNM LSC tissues had no distant metastasis. An additional group of formalin-fixed and paraffin-embedded archival tissue specimens consisting of 67 non-LNM LSC, 80 LNM LSC and 80 matched local positive lymph nodes (LN) specimens were used for IHC analysis. According to the 1997 version of lung cancer staging standard by International Union Against Cancer (UICC), TNM staging of these patients is from I to IV. The mean age of patients was 68 years (range 38–81 years). All the patients recruited in this study received neither chemotherapy nor radiotherapy before the surgery.

2.3. LCM and sample preparation

6–8 μm thick frozen sections of fresh non-LNM LSC and LNM LSC were prepared using a Leica CM 1900 cryostat (Leica, Milton Keynes, UK) at −25 °C. The sections were placed on membrane-coated glass slides (PEF Membrane 2.0 μm 50 pcs, Leica, Germany), fixed in 75% alcohol for 30 s, and stained with 0.5% violet-free methyl green (Sigma, St. Louis, MO). All solutions for staining were supplemented with protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Indianapolis, IN). Following methyl green staining, the sections were air-dried and microdissected by a Leica AS LMD system (Leica, Wetzlar, Germany). The power and pulse durations were adjusted as required during microdissection. Pooled microdissected cells from non-LNM LSC and LNM LSC were used for 2D-DIGE. Each cell population was determined to be 95% homogeneous by microscopic visualization of the captured cells (Fig. 1).

The microdissected cells of each sample were dissolved in lysis buffer (7 mol/L urea, 2 mol/L thiourea, 100 mmol/L DTT, 4% CHAPS, 0.5 mmol/L EDTA, 40 mmol/L Tris, 2% NP40, 1% Triton X-100, 5 mM PMSF, and 2% Phamarlyte) at 4 °C for 1 h, and then centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was transferred to a fresh tube, and stored at −80 °C until 2-DE. The concentration of the total proteins was measured by a 2D Quantification kit (Amersham Biosciences). Of 12 non-LNM LSC tissues group, every 4 cases were mixed equally to a set, thus non-LNM groups consisted of three sets as biological triplicate. The concentration of mixed total proteins of every set was measured again. The same procedure was carried out for the LNM groups.

2.4. 2D-DIGE

Cell lysates of each set were labeled with the cyanine dyes Cy2, Cy3 and Cy5 following the CyDye DIGE Fluors kit's instructions. Fifty micrograms of lysate was labeled with 400 pmol of either Cy3 (non-LNM group) or Cy5 (LNM group), while the same amount of the pool standard that contained all the samples was labeled with Cy2. Labeling reactions were carried out on ice and in the dark for 30 min before being quenched with 1 μl of 10 mM lysine for 10 min on ice. 50 μg Cy3- and Cy5-labeled samples from each set of non-LNM group and LNM group were combined before mixing with 50 μg Cy2-labeled internal standards. The biological triplicate non-LNM and LNM sets and the internal standard were run on 3 gels as analytic gels to generate 9 protein-spot maps. First, the IPG strips (24 cm, pH 4–7 NL) were rehydrated overnight with rehydration buffer (8 M urea, 4% CHAPS, 1% Bio-lyte pH 3–10, 13 mM DTT, and 0.5% (v/v) IPG buffer and trace amount of bromophenol blue). Equal volume of 2× sample buffers (8 M urea, 2 M thiourea, 4% CHAPS, 2% Bio-lyte pH 4–7, 130 mM DTT) were added to the labeled samples which were then left on ice for 10 min prior to IEF. Focusing was carried out on an IPGphor (Amersham Biosciences) according to the following conditions: 1 h at 500 V, 0.5 h at 1000 V, 0.5 h at 5000 V and 8.5 h at 8000 V to give a total of 68 kVh. Prior to the 2-D run, the IPG strips were subjected to a two-step reduction and alkylation step by equilibrating the strips for 15 min first in 50 mM Tris–HCl, pH 6.8, 6 M urea, 30% (v/v) glycerol, 1% (w/v) SDS, and 1% (w/v) DTT, followed by another 15 min in 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 1% (w/v) SDS, and 2.5% (w/v) iodoacetamide (IAA). The 2-D separation was conducted in 1 mm thick,