S100A16 is a Prognostic Marker for Lung Adenocarcinomas

Keita Saito¹, Makoto Kobayashi¹,², Ryo Nagashio¹, Shinichiro Ryuge³, Ken Katono³, Hiroyasu Nakashima⁴, Benio Tsuchiya¹, Shi-Xu Jiang⁵, Makoto Saegusa⁵, Yukitoshi Satoh⁴, Noriyuki Masuda³, Yuichi Sato¹*

Abstract

**Background:** Many functional molecules controlling diverse cellular function are included in low-molecular weight proteins and peptides. **Materials and Methods:** To identify proteins controlling function in lung adenocarcinomas (AC), we performed two-dimensional gel electrophoresis employing tricine-SDS polyacrylamide in the second dimension (tricine-2-DE). This system was able to detect proteins under 1 kDa even with post-translational modifications. To confirm the utility of detected proteins as novel tumor markers for AC, we performed immunohistochemical analysis using 170 formalin-fixed and paraffin-embedded lung AC tissues. **Results:** Tricine-2-DE revealed that five proteins including S100A16 were overexpressed in lung AC-derived cells compared with lung squamous cell carcinoma, small cell carcinoma, and large cell neuroendocrine carcinoma-derived cells. Immunohistochemically, S100A16 showed various subcellular localization in lung cancer tissues and a membranous staining status was correlated with the T-factor (P=0.0008), pathological stage (P=0.0015), differentiation extent (P=0.0001), lymphatic invasion (P=0.0007), vascular invasion (P=0.0001), pleural invasion (P=0.0087), and gender (P=0.039), but not with the age or smoking history. More importantly, membranous staining of S100A16 was significantly correlated with a poorer overall survival of either stage I (P=0.0088) or stage II / III (P=0.0003) lung AC patients, and multivariate analysis confirmed that membranous expression of S100A16 was an independent adverse prognostic indicator (P=0.0001). **Conclusions:** The present results suggest that S100A16 protein is a novel prognostic marker for lung AC.

**Keywords:** Lung cancer - adenocarcinoma - S100A16 - tricine-2DE - tumor marker

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**Introduction**

Currently, lung cancer is the leading cause of cancer-related death worldwide for both males and females. According to cancer statistics for 2013 in the USA (Siegel et al., 2013), 228,190 patients were newly diagnosed and 159,480 patients died. In Japan, 71,518 patients were newly diagnosed and 71,486 patients died of lung cancer in 2013, according to the report by Center for Cancer Control and Information Services, National Cancer Center, Japan.

Analysis of low-molecular-weight protein is usually performed with so-called “peptidomics”. Peptides are intact small molecules, such as hormones, cytokines, and growth factors. Since the amount and repertoire of peptides in the circulation change dynamically according to the physiological or pathological state of an individual, it is possible that comprehensive peptide analysis may lead to the discovery of novel biomarkers or new diagnostic approaches (Diamandis, 2006). He et al. reported that measuring panels of peptide markers might be more sensitive and specific than conventional approaches (He et al., 2013). However, Tan et al. reported that two-dimensional gel electrophoresis (2-DE) is still the best method for the high-resolution separation of a complex mixture of proteins, and its efficacy in distinguishing post-translationally modified (PTM) proteins and protein isoforms is unparalleled (Tan et al., 2009). One of the tools for analyzing low-molecular-weight protein is shotgun proteomics, and it is widely used as an evolving method for determining the protein composition of a biological mixture. Dong et al. reported that shotgun proteomics most commonly involved the digestion of proteins by trypsin, followed by the identification of product peptides, and the use of this information to infer protein identities and possibly targeted PTMs (Dong et al., 2014). However, they also reported that since digestion is a highly complex chemical process, a large proportion of identifiable products are not specifically targeted for analysis.

In this study, we performed proteomic analysis using 5 lung cancer-derived cell lines and focused on low-molecular-weight proteins using tricine-2-DE, which employs tricine-SDS polyacrylamide gel electrophoresis (PAGE) in the second dimension. The results showed that S100A16 expression in lung adenocarcinoma (AC) cell
lines was higher than those of cell lines derived from lung cancer of other histologic types. To confirm the utility of S100A16 as a prognostic biomarker, we performed immunohistochemical analysis using a large series of formalin-fixed and paraffin-embedded lung cancer tissues.

**Materials and Methods**

**Cell lines**

The A549 and LC-2/ad cell lines, derived from lung AC, were purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and the RIKEN BioResource Center (Ibaraki, Japan), respectively. The RERF-LC-AI cell line, derived from a lung squamous cell carcinoma (SCC), was purchased from the RIKEN BioResource Center. The N231 cell line, derived from a small cell lung carcinoma (SCLC), was purchased from the American Type Culture Collection (Rockville, MD, USA). The LCN1 cell line, derived from a large cell neuroendocrine carcinoma (LCNEC), was established in our laboratory (Jiang et al., 2004). These cells were maintained in RPMI-1640 medium (SIGMA, Steinheim, Germany) supplemented with 10% fetal bovine serum (Biowest, Miami, FL, USA), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Life Technologies Corp., Carlsbad, CA, USA). After harvesting and washing twice with phosphate-buffered saline without divalent ions, cells were stored at -80°C for proteomic analysis or fixed in 10% formalin and embedded in paraffin for immunohistochemistry (IHC).

**Tissue specimens**

Tissues of a total of 170 consecutive surgically resected lung AC from January 2002 to December 2005 at Kitasato University Hospital were used in this retrospective cohort study. Patients who received preoperative chemotherapy and/or radiotherapy were excluded. Ten percent formalin-fixed and paraffin-embedded tissues were processed into 3-μm-thick sections, and used for IHC. The histological diagnosis was based on the criteria of World Health Organization/International Association for the Study of Lung Cancer (Travis WD, 1999), and each case was reassessed according to the 7th edition of the TNM classification (Goldstraw et al., 2007).

**Ethics statement**

All samples were collected in accordance with the ethical guidelines and written consent mandated, and this study was approved by the Ethics Committee of Kitasato University School of Medicine (B7-06) and followed the Declaration of Helsinki protocol. All patients were approached based on approved ethical guidelines, and those who agreed to participate in this study provided signed informed consent forms. Patients could refuse entry and discontinue participation at any time.

**Tricine two-dimensional gel electrophoresis and protein identification**

Sample preparation for this analysis was described previously (Kobayashi et al., 2014), and tricine 2-DE was performed with minor modification. The first-dimensional isoelectric focusing gel was produced with carrier ampholyte at pH 3-10 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). For isoelectric analysis, 150 μg of each of extracted proteins from 2 mixed AC cell lines or 3 other cell lines were separated by 300 V for 5 h at 4°C. After the first-dimensional gel electrophoresis, the agarose IEF gels were equilibrated with an equilibration buffer containing 0.05 M Tris-HCl at pH 6.5, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.02% bromophenol blue. For the second-dimensional gel electrophoresis, the proteins were separated by 16.5% polyacrylamide gels using Tris/Tricine/SDS-PAGE buffer (Bio-Rad, Hercules, CA, USA). After electrophoresis, the protein spots were visualized by coomassie brilliant blue R-350 (CBB, PhastGel Blue R, GE Healthcare, Uppsala, Sweden) staining. Stained gels were recorded as digitized images using a high-resolution scanner (GT-9800; Epson, Tokyo, Japan). Each spot was analyzed using Prodigy same spot software (Nonlinear dynamics Ltd., Newcastle, UK), and the abundance of the same proteins was compared. Protein spots that changed more than 1.5-fold between AC cells and other cells progressed to in-gel digestion. In-gel digestion and protein identification were conducted as previously described (Kobayashi et al., 2014).

**Immunohistochemical staining**

For immunohistochemical analysis, 3-μm-thick sections were reacted with 1,000-times diluted anti-S100A16 polyclonal antibody (Abcam, Cambridge, UK), and the details of the procedures were described previously (Nagashio et al., 2008).

**Evaluation of IHC**

S100A16 protein was observed at diverse sites of tumor cells with a variety of expression levels. Recently, Tanaka et al. reported that S100A16 membranous staining was associated with a poor outcome in patients with breast cancer (Tanaka et al., 2015). In this study, S100A16 membranous staining in tumor cells was considered to be positive. The staining intensity was categorized into four groups: 0 (negative), 1 (weak stain), 2 (moderate stain), or 3 (strong stain). The tumors with a staining score of 2 or 3 were judged as positive. Because in most S100A16-positive cases, >5% of tumor cells are usually recognized, the tissues consisting of >5% positive tumor cells were considered significant.

**Statistical analysis**

The relationships between S100A16 expression and clinicopathological parameters were assessed with the χ² test. The cumulative survival of patients was estimated using the Kaplan-Meier method, and the significance of the survival differences between S100A16-positive and -negative groups was tested using the log-rank test. The 5-year cumulative survival probability was estimated using the life table method with the interval length set at 1 month. Multivariable analysis was performed by employing the Cox proportional hazards regression model to examine the interaction between S100A16 expression and other clinicopathological variables, and the independent prognostic effect of S100A16 on survival 2
was estimated by adjusting for confounding factors. The conventional P-value of 0.05 or less was used to determine the level of significance. All reported P-values are two-sided. Analyses were performed using Stat Flex Ver. 6.0 software (Artech Co., Ltd., Osaka, Japan).

Results

Tricine two-dimensional gel electrophoresis

The proteins extracted from the two lung AC cell lines A549 and LC-2/ad, and the other three lung cancer cell lines RERF-LC-A1, LCN1, and N231 were separated by tricine 2-DE. When focusing on proteins under 25 kDa, 9 spots of the AC cell lines were changed more than 1.5-fold in quantity compared with the corresponding spots of the other three lung cancer cell lines (Figure 1A, B). Identification of these proteins was conducted using MALDI-TOF/TOF MS analysis, and identifications of five of the nine protein spots are listed in Table 1.

Expression of S100A16 protein in lung cancer tissues

S100A16 expression was observed at various levels as cytoplasmic, nuclear (Figure 2A), or membranous staining (Figure 2B) in cancer cells of lung ACs. While in non-cancerous peripheral lung tissues, S100A16 was not expressed in any components (Figure 2C). S100A16 membranous staining in tumor cells was observed in 73 of 170 (42.9%) ACs (Table 2). They were subdivided into 36 of 109 (33.0%) of stage I and 36 of 61 (59.0%) of stage II-III ACs.

Relationships between S100A16 membranous staining and clinicopathological characteristics in lung ACs

The relationships between S100A16 membranous staining and clinicopathological characteristics in ACs are summarized in Table 2. S100A16 membranous staining was significantly correlated with a more advanced T-factor (P=0.0008), higher p-TMN stages (P=0.0015), lower differentiation extent (P=0.0001), more frequent lymphatic invasion (P=0.0007) and vascular invasion (P=0.0001) and severer pleural invasion (P=0.0087), and the sex (P=0.039). There was also a tendency toward S100A16 membranous staining in tumors associated with a poor nodal status (P=0.0709). No significant correlation between S100A16 membranous staining and the smoking history was revealed.

The prognostic significances of S100A16 membranous staining

All the 170 lung AC patients were included in the survival analysis. The overall follow-up periods ranged from 3.6 to 127.1 months (median, 89.5 months). S100A16 membrane-positive staining was significantly correlated with a poorer survival of patients with either stage I or stages II and III ACs (P=0.0088 and P=0.0003).

Table 1. The Proteins Showing a 1.5-Fold Difference in Abundance between Lung Adenocarcinoma-Derived Cells and other Histologic Type-Derived Cells by Tricine 2-Dimensional Gel Electrophoresis

<table>
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<tr>
<th>Gene symbol</th>
<th>Protein name</th>
<th>M.W. (kDa)</th>
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<th>Function</th>
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<td>S100A6</td>
<td>Protein S100-A6</td>
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<td>Cytoplasm, Nucleus</td>
<td>Calcium ion binding</td>
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</tr>
<tr>
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<td>Protein S100-A10</td>
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<tr>
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<td>Protein S100-A16</td>
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<td>Plasma membrane, Nucleolus</td>
<td>Calcium ion binding</td>
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<td>SSBP1</td>
<td>Single stranded DNA binding protein 1</td>
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<td>Mitochondria, Nucleolus</td>
<td>DNA binding</td>
<td>1.9</td>
</tr>
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<td>SFRS3</td>
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<td>19,318</td>
<td>Nucleus, Cytoplasm</td>
<td>DNA binding</td>
<td>1.5</td>
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respectively, Figure 3). The 5-year survival probability was 84 or 28% for patients with membrane-positive stage I or stage II-III tumors, but 97 or 76% for patients with membrane-negative stage I or stage II-III tumors.

**Effects of S100A16 expression on patients’ survival by univariable and multivariable analyses**

Cox proportional hazard regression was applied to estimate the effect of S100A16 membranous expression and clinicopathologic variables on patients’ survival. As shown in Table 3, both univariable and multivariable analyses revealed that S100A16 membranous staining was an independent adverse prognostic factor (P<0.0001 and P=0.0001, respectively). Furthermore, multivariable analysis showed that higher p-TMN stages were also independent prognostic factors for lung AC patients.

**Discussion**

The S100 family of proteins, a group of EF-hand calcium-binding proteins, are expressed in a cell- and tissue-specific manner and exert a broad range of intracellular and extracellular functions (Hernandez et al., 2013). Twenty-five different types, including S100A proteins, have been shown to belong to the S100 protein family (Gross et al., 2014). Gross et al. reported that the
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References


<table>
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<th>Newly diagnosed with treatment</th>
<th>Persistence or recurrence</th>
<th>Remission</th>
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<td>Concurrent chemoradiation</td>
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