Detection of tumor-specific autoantibodies in sera of patients with lung cancer

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Summary The presence of autoantibodies (AAs) in sera from two pulmonary carcinoma patients, adenocarcinoma (AD) and small cell carcinoma (SCLC) was screened by immunoblotting using cell lysate of four cell lines (LCN1, large cell neuroendocrine carcinoma (LCNEC); N231, SCLC; A549, AD; RERF-LC-AI, squamous cell carcinoma (SCC)). To identify the antigens recognized by AAs, two-dimensional gel electrophoresis was immunoblotted and target spots were cut out from the membrane and gel. After trypsin digestion, the proteins were analyzed by mass-spectrometry using a liquid chromatography-tandem mass spectrometer. By this method, cytokeratin18 (CK18) and villin1 were identified with AAs in sera from patients with AD and SCLC, respectively. Thus, the expressions of CK18 and villin1 were further immunohistochemically studied on 124 formalin-fixed and paraffin-embedded pulmonary carcinomas of various histologic types (44 AD, 27 SCC, 29 SCLC, and 34 LCNEC) using commercially available CK18 and villin1 antibodies. Positive CK18 immunostaining was observed in almost all cases with staining

Abbreviations: 2-DE, two-dimensional gel electrophoresis; AA, autoantibody; Ab, antibody; AD, adenocarcinoma; ACN, acetonitrile; AEC, 3-amino-9-ethylcarbazole; BPB, bromophenol blue; CBB, Coomassie brilliant blue; CK18, cytokeratin 18; DTT, dithiothreitol; FBS, fetal bovine serum; HRP, horseradish peroxidase; IEF, isoelectric focusing; IHC, immunohistochemical staining; LC-MS/MS, liquid chromatography-tandem mass spectrometer; LCNEC, large cell neuroendocrine carcinoma; MW, molecular weight; NSS, normal swine serum; PVDF, polyvinylidene difluoride; RT, room temperature; SCC, squamous cell carcinoma; SCLC, small cell carcinoma; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCEP, tris(2-carboxyethyl)-phosphine hydrochloride; VP, vinylpyridine.

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1. Introduction

Autoantibodies (AAs) are antibodies (Abs) produced in the sera of patients with various autoimmune diseases. They are also frequently observed in the sera of patients with various carcinomas, and thus may be potential tumor markers [1—7]. Production of AAs is thought to depend on the level of expression, tumor-specific post-translational modification, and altered processing of a protein. Many factors, such as variability among individuals, major histocompatibility complex type of tumors, cytokines and growth factors, are known to affect these immune responses [8]. Many tumor-related AAs have been reported in pulmonary carcinomas [9—12], and the protein gene product 9.5 antigen as well as its AA in sera have been shown to be useful diagnostic markers [9—12]. The presence of SOX Group B and/or ZIC2 AAs in sera of patients with lung cancer [11] has the potential to yield early serodiagnostic markers in cancer patients.

Two-dimensional gel electrophoresis (2-DE) using an agarose for isoelectric focusing (IEF) [14—16] has advantages such as the separation of high molecular weight proteins and large amounts of protein, up to 1.5 mg; therefore, a low abundance of protein can be detected easily using this methodology. Antigens identified by AAs on immunoblotting membranes and corresponding protein spots on gels were cut out and detected by a liquid chromatography-tandem mass spectrometer (LC-MS/MS) [17]. In the present study, we detected AAs and their corresponding antigens by immunoblotting using one- and two-dimensional gel electrophoresis with pulmonary carcinoma cell lines and sera from adenocarcinoma (AD) and SCLC patients as primary Abs. Identified antigens in this study were further immunohistochemically confirmed using commercially available Abs on formalin-fixed and paraffin-embedded pulmonary carcinoma tissue microarray.

2. Materials and methods

2.1. Sera

Serum from two patients with pulmonary carcinoma, one each of T1N2M0 AD and T1N0M0 SCLC, and a healthy individual at Kitasato University Hospital was used in this study. Serum of the former patient was obtained at diagnosis and that of the latter after one course of cisplatin-based chemotherapy, and sera were kept at −80 °C until use.

This study was approved by the Ethics Committee of Kitasato University School of Medicine. All patients were informed of the aim of the study and gave consent to donate their samples.

2.2. Cell lines

Cell lines were one large-cell neuroendocrine carcinoma (LCNEC) of the lung (LCN1) [18], one SCLC (N231, American Type Culture Collection, Rockville, MD), one AD (A549) and one squamous cell carcinoma (SCC) (RERF-LC-AI), (RIKEN BioResource Center, Ibaraki, Japan). LCN1, N231, and A549 cells were grown in RPMI1640 (SIGMA, Steinheim, Germany) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco, Auckland, NZ). RERF-LC-AI was grown in Dulbecco’s modified Eagle’s medium (SIGMA) supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco). Harvested cells were washed with phosphate-buffered saline without bivalent ion (PBS-) and stored at −80 °C until use.

2.3. Tissues

Tissue microarray of 124 pulmonary carcinomas and their normal lung tissues fixed in 10% formalin and paraffin-embedded (42 ADs, 23 SCCs, 29 SCLCs, and 30 LCNECs) was performed according to the protocol of Tissue Microprocessor KIN-type 1 (Azumaya, Tokyo, Japan), and analyzed by immunohistochemical staining.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were extracted from each cell line with detergent lysis buffer [19] containing 2% (w/v) SDS, 62.5 mM Tris—HCl (pH 6.8), 0.001% (w/v) bromphenol blue (BPB), 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, and 1 M phenylmethyl-sulfonyl fluoride using an ultra-sonic homogenizer (UH-50; SMT Company, Tokyo, Japan). Ten micrograms of extracted proteins was boiled and separated by 12% SDS-PAGE.

2.5. Agarose 2-DE

Cell lines were solubilized in lysis buffer [14] containing 7 M urea, 2 M thiourea, 2% 3-[3-cholamidopropyl] dimethylammonio]propanesulfonic acid, 10 mM tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), 2.5% pH 3—10 pharmlate (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), and 0.1 tablet/mL of complete mini EDTA-free protease inhibitors (Roche Diagnostics, Mannheim, Germany) using

intensities significantly higher in AD and LCNEC than in SCC and SCLC. Villin1 was detected in 17/44 (38.6%) of AD and 21/34 (61.8%) of LCNEC, respectively, while in only one each of SCLC and SCC. Thus, villin1 and CK18 may be useful markers to distinguish LCNEC/AD from SCLC/SCC, and the present method might be useful to identify specific tumor-associated molecules in sera from pulmonary carcinoma patients with different histologic types.
an ultra sonic homogenizer, and centrifuged at 20,000 × g for 5 min at 4 °C. The supernatant was reduced by 10 mM TCEP, and then alkylated with 1/20 volumes of 400 mM 4-vinylpyridine (VP) for 1 h by mixing. The reaction was quenched by the addition of the same volume of 400 mM dithiothreitol (DTT), which destroys excess 4-VP. After centrifugation at 20,000 × g for 20 min at 4 °C, interfering components were removed by 2-D Clean-up Kit (GE Healthcare Bio-Sciences Corp.) according to the manufacturer’s instructions. Finally, protein samples were quantified by Bio-Rad Protein Assay solution (BIO-RAD Laboratories, Hercules, CA).

The Agarose-2-DE method used in this study was previously described by Oh-Ishi et al. with some modifications suitable for mini-gel [14,20]. The first-dimensional agarose IEF gel (75 mm in length and 2.5 mm in inner diameter) was made by single pharmalyte pH 3—10 (GE Healthcare Bio-Sciences Corp.). The second-dimensional SDS-PAGE used 12% polyacrylamide gel, as mentioned for SDS-PAGE. One hundred micrograms of extracted protein samples were applied at the cathodic end of the agarose IEF gel, and loaded in stepwise voltages as follows: 100 V, 20 min; 300 V, 15 min; 500 V, 15 min; 700 V, 60 min, 900 V, 60 min at 4 °C. After fixation in 10% (v/v) trichloroacetic acid and 5% (w/v) sodium metabisulfite acid for 3 min at room temperature (RT) with mild shaking, agarose gels were placed in distilled water for 15 min 3 times. The agarose gel was then placed on top of the second-dimensional SDS-PAGE gel, and loaded with a constant current at 20 mA. Two pieces of gel are prepared for one sample, one was transferred to polyvinylidene difluoride (PVDF) membrane for immunoblotting (MILLIPORE Corp., Bedford, MA) and another was visualized by Coomassie brilliant blue R-350 (CBB) staining (PhastGel Blue R; Amersham Pharmacia Biotech AB, Uppsala, Sweden).

2.6. Immunoblotting

Blotting membranes were blocked with ImmunoBlock (Dainippon Sumitomo Pharma, Osaka, Japan) for 1 h at RT. The membranes were then reacted with 100-times diluted sera with 2% normal swine serum (NHS; Dako, Glostrup, Denmark) for 1 h at RT, and then incubated with 1000-times diluted horseradish peroxidase (HRP) conjugated rabbit anti-human IgG polyclonal antibody (Dako) for 30 min at RT. Finally, signals were developed by Immobilon Western (Millipore Corp., Billerica, MA) for 1-D immunoblotting and 3-amino-9-ethylcarbazole (AEC) substrate (Dako) for 2-D immunoblotting. Immunoblot membrane visualized in AEC was further stained with amidoblock to confirm the corresponding protein spot with CBB-stained 2-DE gel. Both staining images were taken with a scanner, and the equivalent positive spot was cut out.

2.7. Identification of antigen proteins

2.7.1. In gel digestion

The methods used for gel digestion were previously described [16]. In brief, protein spots were excised from a 2-DE gel, destained with 50% (v/v) acetonitrile (ACN)/50 mM NH₄HCO₃, dehydrated with 100% (v/v) ACN, and dried under vacuum conditions. Tryptic digestion was performed for 24 h at 37 °C in a minimum volume of digestion solution which contained 0.5 ng/μL sequencing grade modified trypsin (Roche Diagnostics) and 50 mM Tris—HCl buffer (pH 9.0). After incubation, digested protein fragments eluted in solutions were collected, and gels were washed once in 5% formic acid/50% ACN and collected in the same tube.

2.7.2. On membrane digestion

AEC-stained spots were excised from the PVDF membrane, stripped and re-probed using Restore™ Western Blot Stripping Buffer (PIERCE, Rockford, IL) for 15 min at RT, and destained with 50% (v/v) ACN/50 mM NH₄HCO₃ for 30 min. The membrane pieces were dried under vacuum conditions, and then treated and collected using the above methods.

2.7.3. Protein identification

 Solutions containing digested protein fragments were loaded on to a LC-MS/MS system, which consisted of a Nanospace SI-2 (Shiseido Fine Chemicals, Tokyo, Japan), an HPLC system (LCQ Deca: ThermoQuest, San Jose, CA) and an ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The SEQUEST program was used to identify a protein from measured masses of the tryptic peptides and their MS/MS fragments. The SEQUEST program is well documented at the following website http://fields.scripps.edu/sequest/index.html.

2.8. Immunohistochemical staining (IHC)

Three micrometer-thick sections were deparaffinized in xylene and rehydrated in a descending ethanol series, and then treated with 3% hydrogen peroxide for 10 min. Antigen was retrieved by autoclaving in 0.01 M citrate buffer (pH 6.0) with a final concentration of 0.1% Tween 20 at 121 °C for 10 min. After blocking with 2% NSS for 10 min, the sections were reacted with 500-times diluted mouse anti-human cytokeratin 18 (CK18; Novocastra, Newcastle, UK) or mouse anti-human Villin1 monoclonal antibodies (Novocastra) for 2 h at RT. After rinsing in TBS (0.01 M Tris—HCl pH 7.5, 150 mM NaCl) 3 times for 5 min each, the sections were reacted with ChemMate ENVISION (Dako) for 30 min at RT. Finally, the sections were visualized by Stable DAB solution (Invitrogen, Carlsbad, CA) and counterstained with Mayer’s hematoxylin.

2.9. Evaluation of immunohistochemical staining

Immunohistochemical staining was scored by multiplication of the percentage of positive tumor cells and staining intensity. The percentage of positive tumor cells was scored as negative (0%), 1+ (1—25%), 2+ (26—50%), 3+ (51—75%), or 4+ (76—100%). Staining intensity was also scored as 1+ (weakly positive), 2+ (moderately positive), or 3+ (strongly positive). The Mann—Whitney U-test was used for statistical evaluation of IHC data. Statistical significance was recognized when p < 0.05.
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Fig. 1 Immunoblot analysis of whole cell lysates from LCN1, N231, A549, and RERF-LC-AI cells using sera from patients with adenocarcinoma and small cell carcinoma of the lung as the first antibody. (A) The autoantibody existing in the serum from the adenocarcinoma patient recognized an approximately 45 kDa protein in A549 cells. (B) The autoantibody in the serum from the small cell lung carcinoma patient recognized an approximately 90 kDa protein in LCN1 cells.

2.10. Immunoblot analysis of CK18 and villin1

The expressions of CK18 and villin1 were confirmed by 1-D immunoblotting with the above antibodies in pulmonary carcinoma cell lines. The details of the method are mentioned in the sections on SDS-PAGE and immunoblotting. Concentrations of primary antibodies were the same as for immunostaining. A 5000-times diluted anti-human β-actin antibody (SIGMA; A5441) was used as a control.

3. Results

3.1. 1-D immunoblot analysis with sera from pulmonary carcinoma patients

The presence of several AAs was found in sera from patients. On the other hand, no obvious AA was detected in serum from a healthy donor. The molecular weights of antigens recognized by sera from AD and SCLC patients were different. Two AAs that showed strong reactivity were further analyzed to identify their corresponding antigen (Fig. 1); roughly, 45 and 90 kDa proteins were recognized in sera from patients with AD and SCLC, respectively. We designated the two antigens as LAA-1 and LAA-2 (lung cancer-associated antigen-1 and -2), respectively.

3.2. Identification of LAA-1 and LAA-2 antigens by 2-D immunoblotting

In order to purify and identify LAA-1 and LAA-2 proteins, we performed 2-DE with cell lysates of A549 and LCN1 cells that showed a high expression of the proteins, respectively, followed by immunoblot analysis using the same serum for 1-D immunoblotting as the primary antibody. The results of each 2-D immunoblot are shown in Fig. 2. LAA-1 protein had an estimated molecular weight (M.W.) of 45 kDa and an isoelectric point of 5.6–5.8 by using serum from an AD patient. On the other hand, LAA-2 protein had an estimated M.W. of 90 kDa and an isoelectric point of 6.2–6.4 using serum from an SCLC patient. After SEQUEST search, LAA-1 was identified as cytokeratin 18 (accession: 05783), which is composed of 429 amino acids with a predicted M.W. of 47,927 Da and theoretical isoelectric point of 5.44. On the other hand, LAA-2 was identified as villin1 (accession: P09327), which is composed of 826 amino acids with a predicted M.W. of 92,564 Da and theoretical isoelectric point of 6.35.

3.3. IHC of CK18 and villin1

To evaluate the utility of CK18 and villin1 as diagnostic markers, immunohistochemical staining was performed using a tissue microarray of pulmonary carcinomas and their corresponding normal lung tissues. The expression of CK18 was observed in the cytoplasm of tumor cells at various strengths by pulmonary carcinomas. Strong expression of CK18 was also recognized in the cytoplasm of almost all normal bronchial epithelial cells and type II pneumocytes; therefore, the stainability of normal lung tissues in each case was used as an internal control. CK18 was detected in all 42 cases in AD, 17 of 23 (73.9%) in SCC, 28 of 29 (96.6%) in SCLC, 29 of 30 (96.7%) in LCNEC, and 2 of 2 (100%) in LCC, and mean staining scores of CK18 were 10.9, 2.7, 6.1, 8.2, and 7.0, respectively. The mean staining scores were lowest in SCC and highest in AD (Fig. 3). The staining score of CK18 in AD was significantly higher than in SCC and SCLC (both p < 0.001). The staining score of CK18 in LCNEC was also significantly higher than in SCC and SCLC (p < 0.001, p < 0.01, respectively).

The expression of villin1 was barely observed at the cilia of normal bronchial epithelium. Within cancer tissues, the expression of villin1 was generally limited to AD and LCNEC cases. Only one SCLC showed faint cytoplasmic staining. Positive rates of villin1 were 17 of 44 (38.6%) in AD, 21 of 34 (61.8%) in LCNEC and 1 of 28 (3.6%) in SCLC. No
Fig. 2 2-D immunoblot analysis of autoantibodies on pulmonary carcinoma cell lines. A549 (A) and LCN1 (B) cell lysates were separated by 2-DE in duplicate. (A) 45 and 90 kDa proteins were detected by sera from the adenocarcinoma (A-a) and the small cell lung carcinoma (B-a) patients, respectively. (A-b) Amidoblack staining was performed after immunoblotting. (A-c) 2-DE protein pattern after CBB staining. (B) A 90 kDa protein was recognized by autoantibody in serum from the small cell lung carcinoma patient. (B-a) Immunoblot analysis performed with serum from the small cell lung carcinoma patient as the primary antibody. (B-b) Amidoblack stain after immunoblotting. (B-c) 2-DE protein pattern after CBB staining. Arrows indicate the antigens recognized by autoantibodies.

obvious membranous staining was observed in AD and SCLC cases, whereas 61.9% of LCNEC showed membranous staining (Fig. 4).

3.4. Immunoblot analysis using commercially available antibodies

We confirmed the expression levels of CK18 and villin1 in cell lines by commercially available antibodies (Fig. 5). The results were similar to those obtained by sera from patients in this study. For CK18, strong expression was detected in AD and LCNEC cell lines and the expression of villin1 was limited in LCNEC and AD cell lines.

4. Discussion

In this study, we demonstrated the existence of AAs in sera of patients with AD and SCLC by 1-D immunoblot analysis, and specified the corresponding antigen proteins of two AAs by 2-D immunoblot analysis followed by LC-MS/MS. One of the two AAs identified reacted with CK18 and another with villin1. It has been reported that CK18 AA was found in the sera of patients with toluene diisocyanate-induced asthma and idiopathic pulmonary fibrosis [21,22], but there are no reports regarding pulmonary carcinomas. Our study demonstrated, for the first time the existence of autoantibodies against CK18 in serum from AD patients. Fragments of CK18 in blood have been reported to be a useful serodiagnostic markers for unfavorable prognosis and malignancy in patients with AD of the mammary gland, stomach, and lung [23,24]. In the present immunohistochemical study, both positive rates and staining scores of CK18 were higher in AD and LCNEC. Although both LCNEC and SCLC were categorized as high-grade neuroendocrine carcinoma of the lung, the staining scores of CK18 in LCNEC were significantly higher than those of SCLC. LCNEC was newly categorized as a distinct entity of pulmonary carcinoma in 1991 by WHO Histological Typing [25], and was graded as a high-grade neuroendocrine carcinoma along with SCLC. SCLC and LCNEC have many common properties and their histologic distinction is occasionally difficult [26–28]. The present study showed that CK18 is a potential marker distinguishing LCNEC from SCLC. We also found that the staining score of CK18 in LCNEC was similar to that in AD. Recently, Nitadori et al. have suggested that significantly higher expression of CK7, CK8, E-cadherin, and beta-catenin is more characteristic of LCNEC than SCLC, and both tumor types are separate entities morphologically and immnophenotypically [29].

Microvilli are composed of actin and an actin-binding protein termed villin1, which has been shown to be a calcium-regulated protein present in the cytoskeleton of intestinal microvilli [30]. Villin1 is mainly located at the brush border of epithelial cells. Under normal physiological conditions, villin1 is expressed in epithelial cells of the intestinal mucosa, gall bladder, renal proximal tubules, and ductuli efferentes of the testis, but not in normal lung cells such as the bronchiolar epithelium, alveolar cells, or bronchial gland cells [31–34]. Rimm et al. reported that the AA against villin1 was detected in the blood of colon cancer patients, and they stated that villin1 AA may provide a unique noninvasive approach for the detection of gastrointestinal malignancy [35]; however, as far as we know, there is no report that villin1 AA was detected in pulmonary carcinomas. In the present study, we demonstrated the existence of villin1 AA in SCLC patients for the first time. Immunohistochemically, villin1 expression patterns in pulmonary carcinomas were divided into three types: (1) brush-border pattern, (2) cytoplasmic pattern with minor brush-border staining, and (3) a diffuse cytoplasmic pattern without brush-border staining [31]. Nambu et al. reported that the positive rate and expression pattern of villin1 in primary pulmonary AD differed from those in metastatic pulmonary carcinomas originating from the digestive tract, and some primary pulmonary AD possessed novel smaller villin1 mRNA. Thus, they suggested that villin1 might be a useful marker for distinguishing primary from secondary pulmonary ADs [31]. Furthermore, Zhang et al. investigated neuroendocrine tumors arising from various organs and found that villin1 was predominantly restricted to gastrointestinal neuroendocrine tumors (except islet cell tumor), although a small number of bronchial carcinoids may also be posi-
Fig. 3  Expression of cytokeratin 18 in pulmonary carcinomas. Cytokeratin 18 was observed at various degrees in the cytoplasm of pulmonary carcinomas. In general, strong stainability was detected in both adenocarcinomas (A) and large cell neuroendocrine carcinomas (D); however, weaker stainability was observed in both squamous cell carcinomas (B) and small cell carcinomas (C). Moderate to strong expression of cytokeratin 18 was also observed in bronchial epithelia (E) and type II pneumocytes (F).

tive. These results suggest that villin1 has different roles in different neuroendocrine tumors and may be a useful marker for the differential diagnosis of neuroendocrine tumors [36]; however, no report has been published concerning the expression of villin1 in LCNEC. In the present study, we demonstrated that the expression of villin1 was generally limited in AD and LCNEC cases, and positive rates in AD were lower than in AD arising from other organs. With regard to the staining pattern of villin1, a brush-border pattern was found in 5 cases of primary pulmonary AD, different from a previous report [31]. Zhang et al. found faint villin1 expression in the cytoplasm of a few SCLC cases [36]; however, in the present study, the expression of villin1 was observed in LCNEC, but was hardly found in SCLC cases. Furthermore, the expression of villin1 was uniformly observed along the cytoplasmic membrane, which is different from a previous finding reported in AD or SCLC. In the present study, we showed that villin1 as well as CK18 may be a useful marker to
Fig. 4  Expression of villin1 in pulmonary carcinomas. Villin1 was observed in adenocarcinomas and large cell neuroendocrine carcinomas, but not in squamous cell carcinomas and small cell carcinomas. Three different staining patterns were observed in adenocarcinomas: brush (A), apical (B), and cytoplasmic pattern (C). On the other hand, only a membranous staining pattern was detected in large cell neuroendocrine carcinomas (D). No obvious staining was observed in small cell carcinomas (E), squamous cell carcinomas (F) or normal lung tissues (G).

differentiate LCNEC from SCLC or AD. In this study, although villin1 was generally absent in SCLC tissues, the autoantibody of villin1 was found within the serum of an SCLC patient who received chemotherapy before serum collection. Thus, there is a possibility that the nature of the tumor varied with anticancer agents. Because biopsy specimens were not available, we could not evaluate villin1 staining in the tumor prior to therapy.

Circulating AAs in pulmonary carcinoma have been reported to have potential for either diagnosis or prognosis [9—11,13,37,38]. Pereira-Faca et al. identified tumor-associated AAs by the proteomics technique and demonstrated that a panel of 14—3—3ζ, annexin 1, and protein gene product 9.5 proteins exhibited 55% sensitivity with 95% specificity in discriminating lung cancer at the preclinical stage from matched controls [38]. Furthermore, several
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Fig. 5 Validation of CK18 and villin1 expressions by commercially available antibodies on pulmonary carcinoma cell lines. (A) A strong band of CK18 at 47.9 kDa was observed in A549 and LCN1 cells, and minor bands at 29.0 and 25.2 kDa were also detected in A549 cell. (B) A band of villin1 at 92.6 kDa was observed strongly in LCN1 cells and weakly in A549 cells, and minor bands were detected in LCN1 cell. (C) The expression of β-actin was almost equal in each cell line.

proteomics-based analyses are emerging as useful means to discover autoantibody biomarkers, including our present study [11,39,40].

In this study, we identified AAs using 1-D and 2-D immunoblot analysis, and antigens by the combination of in gel and/or on membrane digestion and LC-MS/MS. Our data suggest that AAs against tumor-related proteins could be identified by using the simple methodologies presented.

5. Conclusion

In this study, the presence of AAs in two types of sera from AD and SCLC patients was detected by immunoblotting using cell lysates of various types of pulmonary carcinomas. To identify the antigens recognized by AAs, two-dimensional gel electrophoresis, immunoblotting and a liquid chromatography-tandem mass spectrometer were utilized. By this method, CK18 and villin1 were identified as antigens by AAs in sera from patients with AD and SCLC, respectively. The expression of CK18 and villin1 was immunohistochemically studied on 124 formalin-fixed and paraffin-embedded pulmonary carcinomas and their corresponding normal lung tissues. The staining score of CK18 was significantly higher in AD and LCNEC, and villin1 expression was generally limited to AD and LCNEC. These results indicate that both CK18 and villin1 may be useful markers to distinguish LCNEC and/or AD from SCLC and SCC. We think that the present method might be simple and reliable to identify tumor-associated molecules in sera from patients with specific histologic types of cancer.

Conflict of interest

None declared.

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