Proteomics-Based Approach Identifying Autoantibody against Peroxiredoxin VI as a Novel Serum Marker in Esophageal Squamous Cell Carcinoma

Yoshihisa Fujita,1 Toyofumi Nakanishi,2 Masako Hiramatsu,1 Hideaki Mabuchi,1 Yoshiharu Miyamoto,1 Akiko Miyamoto,1 Akira Shimizu,2 and Nobuhiko Tanigawa1

Abstract

Purpose: Detection of novel tumor-related antigens and autoantibodies will aid in diagnosis of early-stage cancer and development of more effective immunotherapies. The purpose of this study was to identify novel tumor antigens in an esophageal squamous cell carcinoma (ESCC) cell line (TE-2) and related autoantibodies in sera from patients with ESCC using a proteomics-based approach.

Experimental Design: TE-2 proteins were separated by two-dimensional PAGE, followed by Western blot analysis in which sera of patients with ESCC, healthy controls, and patients with other cancers were tested for primary antibodies. Positive spots were excised from silver-stained gels and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS).

Results: Sera from patients with ESCC yielded multiple spots, one of which was identified as peroxiredoxin (Prx) VI by MALDI-TOF/TOF MS. Western blot analysis against recombinant Prx VI showed reactivity in sera from 15 of 30 (50%) patients with ESCC and 2 of 30 (6.6%) healthy individuals. Autoantibody against Prx VI was found in sera from 1 of 30 (3.3%) patients with other types of cancer (colon cancer).

Conclusion: We have identified for the first time an autoantibody against Prx VI in ESCC patients. The proteomic approach implemented here offers a powerful tool for identifying novel serum markers that may display clinical usefulness against cancer.

The identification of novel tumor antigens and related autoantibodies in patients with tumor is expected to facilitate diagnosis of early-stage malignant tumor and establish new and effective immunotherapies. Crawford et al. (1) first described autoantibodies against human p53 protein in 9% of breast cancer patient sera in 1982, and since then >150 articles have been published on anti-p53 autoantibodies in patients with various tumor types (2). Detection of anti-p53 autoantibodies may serve not only to diagnose early-stage malignancy but also to monitor tumor progression and recurrence (2). However, little work about autoantibodies against other proteins has been described (3, 4). This may be attributed to conventional methods for detecting antibodies: enzyme immunoassay for predefined specific proteins, such as oncogene products and glycoproteins.

In 2001, Brichory et al. (5) introduced a proteomics-based approach to identify panels of tumor antigens and related autoantibodies and discovered anti–Annexin I and II autoantibodies in sera from patients with lung cancer. The proteomics-based approach employed in that report allows for the identification of autoantibodies to proteins as occurring in the natural states, in lysates prepared from tumors or tumor cell lines. With the new developmental methods, several reports on autoantibodies against novel proteins have been published (6–8).

Why only a subset of patients with a particular tumor type develops humoral responses to a particular antigen remains unclear. Whether endogenous proteins acquire antigenicity may be attributed to self-modification of proteins (e.g., overexpression, posttranslational modifications, conformational changes, and other such protein processing; ref. 2). In this respect, a proteomics-based approach that can identify quantitative and qualitative protein changes associated with malignant phenotypes and can also be used to examine important posttranslational modifications such as glycosylation and phosphorylation (9) represents a useful method to identify autoantibodies against intracellular antigens.

Although a large number of reports are available on proteomics-based analysis of various tumors, little has been reported on esophageal squamous cell carcinoma (ESCC) using proteomics-based analysis (10, 11). ESCC is one of the most common and fatal malignancies in the world (12), with a 5-year survival rate of ~5% to 20% for stage III and stage IV
patients undergoing curative resection in Japan (13). The poor prognosis for ESCC involves not only the aggressive behavior of this tumor, which is associated with systemic involvement at diagnosis (14), but also the limited number of serum markers available for diagnostic purposes (15, 16). Identification of new serum markers may greatly advance the diagnosis and improve the prognosis of ESCC.

In the present study, we found novel tumor antigens in ESCC cell lines (TE-2) and related autoantibodies in sera from patients with ESCC using a proteomics-based approach. With this new method, we identified a novel autoantibody against peroxiredoxin (Prx) VI, which is a member of the Prx gene family (17). Prxs are ubiquitous enzymes, such as antioxidant enzymes, which control intracellular H2O2 levels by catalyzing reduction to water. These proteins are stress inducible and associated with cell signaling pathways and also participate in cellular antioxidant defense, with at least some inducing cell proliferation and protecting cells from undergoing apoptosis (18). The detection of autoantibody against Prx VI may serve not only to diagnose ESCC but also to develop new targeted therapies.

Materials and Methods

**Sera and cell lines.** Serum and tumor tissues were obtained at the time of diagnosis after obtaining written informed consent. The experimental protocol was approved by the Institutional Review Board of Osaka Medical College. Sera were obtained from 30 patients with ESCC. Sera from 30 healthy individuals and from 30 patients with other cancers (gastric cancer, n = 15; colon cancer, n = 15) were used as controls. Mean age of subjects who donated sera for this study was 53.2 years (range, 37-74 years). ESCC was stage I in 7 patients, stage II in 8 patients, stage III in 11 patients, and stage IV in 4 patients. The TE-2 human squamous cell carcinoma cell line was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Nikken Cell Media, Kyoto, Japan). Cells were harvested in 300 μL of solubilization buffer (9 mol/L urea, 2% NP40, 2% β-mercaptoethanol, 10 mmol/L phenylmethylsulfonl fluoride) using a cell scraper and stored at −80°C until use.

**Tumor tissue samples.** ESCC tissues and paired adjacent normal esophageal tissue were obtained during surgical resection from Osaka Medical College Hospital. After excision, sample tissues were frozen immediately at −80°C and stored until use.

**Two-dimensional PAGE and Western blotting.** To detect autoantibody against tumor antigens in sera from cancer patients, we followed the approach established by Brichory et al. (5). Proteins (50 μg) solubilized from cultured cells were applied to isoelectric focusing gel electrophoresis (one-dimensional electrophoresis) and subjected to SDS-PAGE (two-electrophoresis). Isoelectric focusing was conducted with the immobilized pH 3-10 nonlinear gradient strips (Amersham Biosciences, Pittsburgh, PA) at 500 V for 1 hour, followed by 3,500 V for an additional 3 hours using a Multiphor II Electrophoresis Unit (Amersham Biosciences). After isoelectric focusing gel electrophoresis, the isoelectric focusing gel strip was equilibrated with 50 mmol/L Tris (pH 6.8) containing 10% glycerol, 2% SDS, 1% DTT, and bromphenol blue, and then the treated gel strip was loaded on a two-dimensional gel. Separated proteins were transferred onto a Hybond P polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences) for 1 hour at a constant voltage of 20 V using a Mini Trans-Blot system (Bio-Rad, Hercules, CA) or visualized by silver staining. After transfer, PVDF membranes were incubated with blocking buffer consisting of PBS and 5% nonfat dry milk overnight at 4°C, then washed with washing buffer (PBS/0.05% Tween 20) and incubated with diluted sera from patients with ESCC or healthy individuals at 1:250 dilution for 1 hour at room temperature. After four washings, membranes were reacted with horseradish peroxidase–conjugated mouse anti-human immunoglobulin (Amersham Biosciences) at 1:10,000 dilution for 1 hour at room temperature and then washed. Immunodetection was accomplished using an enhanced chemiluminescence plus system (Amersham Biosciences) followed by autoradiography on Hyperfilm MP (Amersham Biosciences).

**In-gel enzyme digestion and mass spectrometry.** The two-dimensional gel was stained with silver nitrate and the pieces of gel corresponding to Western blot–positive spots were excised. Protein identification was done as previously reported (19). Gel pieces were alternately washed with 50 mmol/L ammonium bicarbonate (pH 8.5) and acetone and finally dehydrated with acetonitrile. These pieces were completely dehydrated in a Speedvac device at 30°C, then covered with 25 μL of 1-(tosylamido-2-phenyl) ethyl chloromethyl ketone–modifed trypsin (0.02 mg/mL; Promega, Madison, WI) in NH4HCO3 buffer (40 mmol/L, pH 8.5) and left at 37°C overnight. After enzymatic digestion, the resultant peptides were extracted in 100 μL of 0.5% (v/v) formic acid and then in 100 μL of acetonitrile/H2O + 1% (v/v) formic acid (50:50). Extraction was conducted in an ultrasonic bath for 15 minutes each time. Extracts were concentrated and desalted in ZipTip C18 microcolumns (Millipore, Bedford, MA). Extracted peptides were loaded onto the matrix-assisted laser desorption/ionization (MALDI) target plate by mixing 1 μL of each solution with the same volume of a matrix solution that was prepared fresh every day by dissolving 0.3 g/mL a-cyano-4-hydroxycinnamic acid (Wako Purified Reagent, Kyoto, Japan) in acetone/ethanol (1:1, v/v) solvent. Measurements were done with an Ultraflex MALDI-TOF/TOF mass spectrometer (Brucker Daltonics, Bremen, Germany) with an accelerating voltage of 20 kV. Laser wavelength was 337 nm and laser pulse frequency was 25 Hz. The peptide mass fingerprint was used for protein identification from the tryptic fragment size using the Mascot Search engine based on the entire NCBI and SwissProt protein databases.

**Prx VI detection by Western blot analysis.** Rabbit anti–non-selenium glutathione peroxidase (1-Cys Prx) polyclonal antibody (AB9248; Chemicon International, Temecula, CA) was used at 1:1,000 dilution for Western blotting and was processed as for incubations with patient sera, with donkey anti-sheep/goat immunoglobulin horseradish peroxidase–conjugated, affinity-purified, secondary antibody (AB324P; Chemicon International).

**Detection of autoantibody against Prx I, Prx II, Prx III, and Prx VI from patient sera by Western blot analysis.** Recombinant Prx I (LF-P0002; LabFrontier, Seoul, Korea), Prx II (LF-P0007), Prx III (LF-P0023), and Prx VI (LF-P0004) were dot-blotted on PVDF membranes and incubated with diluted individual sera from patients with ESCC, patients with other cancers, and healthy individuals at 1:5,000 dilution for 1 hour at room temperature. After three washings, membranes were reacted with horseradish peroxidase–conjugated mouse anti-human immunoglobulin (Amersham-Pharmacia Biosciences) as the secondary antibody at 1:25,000 dilution for 1 hour at room temperature.

**Immunohistochemistry.** Immunohistochemical staining was done with the standard avidin-biotin-peroxidase complex technique using an LV Dako LSAB kit (DAKO, Copenhagen, Denmark) as previously described (20). Sections were then incubated overnight at 4°C with rabbit anti–non-selenium glutathione peroxidase (1-Cys Prx) polyclonal antibody (AB9248; Chemicon International) diluted at 1:200 in a humidified chamber. After washing, biotinylated antimouse and antirabbit immunoglobulin (DAKO) were applied for 30 minutes at room temperature, followed by incubation with streptavidin-conjugated horseradish peroxidase. Finally, 3,3-diaminobenzidine was used for color development and hematoxylin was used for counterstaining.

**RNA isolation.** Total RNA was isolated from frozen tumor tissue by an acid guanidium-phenol-chloroform method using Isogen (Nippon Gene, Toyama, Japan). Isolated total RNA pellets were dissolved in RNase-free, diethylycarboxylate-treated water. For first strand cDNA synthesis, 5 μg of total RNA, 1 μL of oligo(dt)12-18 primer, and 6.5 μL of Advantage RT-for-PCR kit (Clontech Laboratories, Palo Alto, CA), including 1 μL of dNTP mix, 4 μL of 5× reaction buffer and 0.5 μL of
RNase inhibitor, and 1 μL of reverse transcriptase (SuperScript II RNase H− Reverse Transcriptase, Invitrogen, Carlsbad, CA), were mixed to make a total volume of 20 μL. Reverse transcription reactions were incubated at 50°C for 90 minutes. Finally, cDNA solution was diluted to a total volume of 100 μL.

Quantitative reverse transcription-PCR analysis. Quantitative reverse transcription-PCR amplifications were done on a LightCycler quick system 330 (Roche Molecular Biochemicals, Mannheim, Germany). PCR conditions were initial denaturation at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 62°C for Prx VI or at 63°C for glucose-6-phosphate dehydrogenase (G6PD) for 10 seconds, and extension at 72°C for 10 seconds. A standard curve was generated using fluorescent data from serial dilutions of the plasmid including a single PCR product. Data for Prx VI were normalized according to data for G6PD. All primers and probes used in this study were designed by Nihon Gene Research Laboratories (Sendai, Japan). Sequences of forward and reverse primers for Prx VI were 5′-AGCTCGTGTGGTGTGTT-3′ and 5′-TCTTCAGGGAGTGTTAGG-3′, respectively. Sequences of hybridization probes F and R for Prx VI were 5′-GAATCCTGCAAAATGTCGAGCCAGTGAGC-3′ and 5′-GGGTAGAGCATAGACGGTTCTGTT-3′, respectively. Sequences of forward and reverse primer for G6PD were 5′-TGGACCTGACCATGGAAAGATAGA-3′ and 5′-GGCCTTGATCTGGAACCC-3′, respectively. Sequences of hybridization probes F and R for G6PD were 5′-TTTCTACCCCAACTGAGCAACC-3′ and 5′-GATTGAGCTGAGAACCC-3′, respectively.

Results

Autoantibodies to ESCC proteins in sera from patients with ESCC. TE-2 cell line proteins were separated by two-dimensional PAGE and visualized by silver staining (Fig. 1A). To detect autoantibodies against proteins from TE-2, proteins were separated by two-dimensional PAGE transfer onto PVDF membranes, then incubated with sera from 10 patients with ESCC and 10 healthy control subjects. Each membrane was treated with one serum sample as the primary antibody and with horseradish peroxidase–conjugated mouse anti-human immunoglobulin as secondary antibody. Sera from patients with esophageal cancer reacted in multiple spots (Fig. 1B), whereas some reactive protein spots were observed in healthy controls and were considered representative of nonspecific reactivity. One of reactive spots, with an approximate isoelectric point 6.0 and molecular weight of about 27 kDa, was observed in 3 of 10 (30%) patients with ESCC (Fig. 1C), whereas no such reaction was observed with 10 healthy controls (Fig. 1D).

Identification of reactive proteins as Prx VI. The reactive spot shown in Fig. 1B was excised from silver-stained gel and the peptide was extracted from excised gel after in-gel enzyme digestion and then analyzed using Ultraflex MALDI-TOF/TOF mass spectrometry. The acquired spectra were processed and searched against a Mascot Search engine based on the entire NCBI and SwissProt protein databases (Fig. 2A and B). The protein was identified as Prx VI, which comprises 224 amino acids with a predicted molecular weight of 26.8 kDa and a theoretical isoelectric point of 6.0 (Fig. 1A). Identity with Prx VI was confirmed by Western blot analysis using rabbit anti–non-selenium glutathione peroxidase (1-Cys Prx) polyclonal antibody (Fig. 1E).

Confirmed autoantibody against Prx VI in sera from patients with ESCC, patients with other cancers, and healthy individuals by Western blot analysis. To screen autoantibody against Prx VI in sera from patients with ESCC or other cancers and from healthy individuals, recombinant Prx VI protein (100 ng) was analyzed by Western blot for 30 patients with ESCC, 30 patients with other cancers (gastric cancer, n = 15; colon cancer, n = 15), and 30 healthy individuals (Table 1). Individual analysis against recombinant Prx VI showed reactivity in sera from 15 of 30 (50%) patients with ESCC (Fig. 3A) and 2 of 30 (6.6%) healthy individuals (Fig. 3B). Autoantibodies against Prx VI were found in sera of 1 of 30 (3.3%) patients with other types of cancer (colon cancer). No reactivity against other Prxs, including Prx I, Prx II, and Prx III, was observed in sera from ESCC patients (data not shown). Reactivity was not limited to patients with advanced-stage disease, with sera reactivity identified in 8 of 15 (53.3%) patients with stage I or II disease. No correlation was noted between appearance of autoantibody against Prx VI and disease stage.

Expression of Prx VI mRNA. To examine whether the immunogenicity of Prx VI in ESCC could be due to elevated transcriptional mechanisms, expression levels of Prx VI mRNA in tumor tissue were compared between patients with and without autoantibody against Prx VI. To examine Prx VI expression in eight sample tissues (with autoantibody, n = 3; without autoantibody, n = 5), real-time reverse transcription-PCR was done with expression levels of G6PD as an internal control. After normalization, Prx VI/G6PD ratio was calculated in each group (Fig. 4). Although the number of sample tissues in which level of Prx VI mRNA was examined was small, level of Prx VI mRNA expression tended to be higher in patients with autoantibody than in patients without autoantibody. These findings suggest that overexpression of Prx VI contributes to immunogenicity.

Immunohistochemical analysis of Prx VI. Prx VI expressions in cancer and noncancer epithelia were assessed by immunohistochemistry with rabbit anti–non-selenium glutathione peroxidase (1-Cys Prx) polyclonal antibody. In normal...
epithelium and dysplastic lesion, little expression of Prx VI was observed (Fig. 5A and B). In early cancer, Prx VI was slightly expressed in cytoplasm (Fig. 5C). High expression of Prx VI in cytoplasm was observed mainly at invasive lesion in advanced cancer (Fig. 5D).

**Discussion**

In this study, we applied a proteomics-based approach to find both novel tumor antigens in the TE-2 ESCC cell line and related autoantibodies in sera from patients with ESCC, and we identified a novel autoantibody against Prx VI in the TE-2 cell line. A humoral response directed against Prx VI occurred in sera from 50% of 30 patients with ESCC, compared with 6.6% of 30 healthy individuals. In 30 patients with other types of cancer, one serum sample from a patient with colon cancer exhibited reactivity against Prx VI.

Prx VI is a member of the Prxs, which have received considerable attention in recent years as a new and expanding

<table>
<thead>
<tr>
<th>Subjects</th>
<th>No. subjects</th>
<th>Prx VI autoantibody positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophageal cancer</td>
<td>30</td>
<td>15 (50.0%)</td>
</tr>
<tr>
<td>Stage I</td>
<td>7</td>
<td>2 (28.5%)</td>
</tr>
<tr>
<td>Stage II</td>
<td>8</td>
<td>6 (75.0%)</td>
</tr>
<tr>
<td>Stage III</td>
<td>11</td>
<td>5 (45.4%)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>4</td>
<td>2 (50.0%)</td>
</tr>
<tr>
<td>Other types of cancer</td>
<td>30</td>
<td>1 (3.3%)</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>15</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>15</td>
<td>1 (6.6%)</td>
</tr>
<tr>
<td>Other control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>30</td>
<td>2 (6.6%)</td>
</tr>
</tbody>
</table>
family of thiol-specific antioxidant proteins (17). Prxs are ubiquitous enzymes, comprising 0.1% to 0.8% of the soluble proteins in many mammalian cells, and are classified as either 1-Cys or 2-Cys Prxs based on whether the protein contains one or two conserved cysteine residues (17). In mammalian cells, six members of the Prx family have been described. Prx VI is the sole mammalian 1-Cys Prx and reduced glutathione is indeed the physiologic electron donor and provides the mechanism for interactions with oxidized Prx VI (21). Prx VI was detected in cytosolic and lysosomal fractions from mammalian cells and displays antioxidant and phospholipase A2 activity.

Prx VI controls intracellular \( \text{H}_2\text{O}_2 \) levels by catalyzing reduction into water and reducing reactive oxygen species derived from free radicals, such as superoxide anion (\( \text{O}_2^- \)). At low or moderate intracellular levels, reactive oxygen species play important roles in signal transduction and regulation of redox-sensitive transcription factors (22). Conversely, elevated levels of reactive oxygen species are concomitant with an accumulation of genetic alterations associated with carcinogenesis (22). In addition, reactive oxygen species themselves are regulators of p53 and play roles downstream of mitochondria during destruction of the cellular components mediated by activated caspases (23). Interestingly, recent reports on the outcomes of Prx I, Prx II, and Prx VI inactivation in mice by homologous recombination have indicated that these enzymes are important for cell survival and tumor suppression in a cell context-dependent fashion (24–26). In agreement with these reports, tumor cells may have developed defense systems that involve antioxidant proteins to protect against oxidative stress.

The present study confirmed that expression of Prx VI is markedly higher in tumor tissue than in normal epithelium and dysplastic lesion at the protein level according to immunohistochemistry. Qi et al. (27) have reported up-regulation of Prx I in ESCC and similar observations have been reported in other types of cancers, such as malignant mesothelioma (28), lung cancer (29), oral cancer (30), and breast cancer (31). Previous studies have suggested that Prx I offers a potential tumor marker and may facilitate cancer diagnosis in the early stages (29, 30). However, Prxs are expressed not only in tumor cells but also in various nonmalignant cells, such as alveolar macrophages, endothelial cells, and sometimes also chondrocytes. Prxs may be highly expressed in malignant tumors but do not seem to be useful as tumor markers.

The present study showed for the first time that the presence of autoantibody against Prx VI was detected in patients’ sera with ESCC. To the best of our knowledge, only one report has mentioned autoantibody against Prxs in malignant tumor. Chang et al. (32) reported an autoantibody against Prx I in sera from 25 of 53 (47%) patients with non–small-cell lung cancer, whereas such activity was detected in 4 of 50 (8%) healthy individuals. Why some patients develop immunogenicity to a particular antigen remains unclear, but the suggestion has been made that autoantibody against Prx I represents a potential tumor marker for lung cancer screening. We confirmed that the presence of autoantibody against Prx VI detected not only advanced-stage ESCC but also early-stage ESCC. Little reactivity against Prx VI was observed in healthy individuals or patients with other types of cancer. Our results support the possibility...
that autoantibody against Prx VI can be used as a new tumor marker to detect ESCC patients.

Prx VI has not been reported before as eliciting a humoral response in malignant tumor. Generally, the immunogenicity of autoantibody in malignant tumor may depend on the level of expression, posttranslational modification, or other types of processing of a protein, the extent of which may vary among tumors of a similar type. Soussi (2) has shown that p53 antibodies are found predominantly in human cancer patients, and such antibodies are predominantly associated with p53 gene missense mutations and p53 accumulation in the tumor. Sensi et al. (33) reported a new unique antigen generated by a point mutation in the Prx V in an HLA-A*0201 Human metastatic melanoma. Although Prx VI autoantibodies were largely restricted to patients with ESCC among the subject groups investigated here, not all patients with overexpression of Prx VI displayed immunoreactivity against Prx VI. Further examination is needed to consider the mechanism of generation of Prx VI antibodies for ESCC, such as quantitative analysis of autoantibody against Prx VI by ELISA or whether Prx VI gene missense mutations may be found in ESCC patients.

In conclusion, we have first identified an autoantibody against Prx VI in sera from patients with ESCC. This autoantibody may be used as a tumor marker. In regard to the detection of novel autoantibodies such as Prx VI, the proteomic approach that we have implemented offers a powerful tool to identify novel proteins that may display clinical usefulness against cancer.

References
Proteomics-Based Approach Identifying Autoantibody against Peroxiredoxin VI as a Novel Serum Marker in Esophageal Squamous Cell Carcinoma

Yoshihisa Fujita, Toyofumi Nakanishi, Masako Hiramatsu, et al.


Updated version  Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/12/21/6415

Cited articles  This article cites 32 articles, 10 of which you can access for free at: http://clincancerres.aacrjournals.org/content/12/21/6415.full.html#ref-list-1

Citing articles  This article has been cited by 5 HighWire-hosted articles. Access the articles at: /content/12/21/6415.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.