Peroxiredoxin-I is an autoimmunogenic tumor antigen in non-small cell lung cancer

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Abstract In eukaryotic cells, peroxiredoxins are both antioxidants and regulators of H_2O_2-mediated signaling. We previously found that peroxiredoxin-I (Prx-I) was overexpressed in non-small cell lung cancer (NSCLC) tissue. Since overexpressed protein can induce a humoral immune response, we examined whether serum from NSCLC patients exhibited immunoreactivity against Prx-I using Western blotting. We found that 25 (47%) of 53 NSCLC patients tested had autoantibodies against Prx-I in their sera, whereas such activity was detected in 4 (8%) sera from 50 healthy subjects. Prx-I itself was detected in the sera from 18 (34%) of 53 NSCLC patients but in only 1 (2%) serum from 50 controls. Moreover, 17% of NSCLC sera were positive to both Prx-I antibody and antigen but none in control sera. The data indicate both Prx-I autoantibody and circulating antigen are potential biomarkers for use in serological diagnosis of NSCLC. Interestingly enough, we found that Prx-I was secreted by lung adenocarcinoma cells (A549) but not by non-cancer lung cells (BEAS 2B) or breast cancer cells (MCF7). This cell culture study suggests the possibility of Prx-I secretion from NSCLC tumor tissues.
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1. Introduction

Lung cancer is the leading cause of cancer death [1] and its prevention is a major worldwide challenge. At present, diagnosis of lung cancer mainly relies on physical examination. By the time a physician discovers malignant tissue in the lung, it is likely to contain at least one billion cancer cells with high probability of metastasis, which underlies the high mortality rate for this disease [2]. Therefore, there is much interest in identifying biomarkers for early detection of lung cancer [3,4]. A number of tumor antigens have been evaluated as biomarkers, including α-fetoprotein, neuron-specific enolase (NSE), carcinoembryonic antigen (CEA), cytokeratin 19 fragment (CYFRA 21-1), squamous cell carcinoma (SCC) antigen, cancer antigen 125 (CA125) and tissue polypeptide antigen [5–11]. Autoantibodies against several tumor antigens such as p53, embryonic neural proteins and antineural/antinuclear antigens have also been tested [12–15]. Recently, autoantibodies against PGP9.5, annexin-I and annexin-II were found in the sera of lung cancer patients using a proteomics approach [16,17]. However, expression of these molecules does not appear to be sufficiently sensitive and specific enough to be reliable for early diagnosis. At present, great efforts are being made in order to discover better biomarkers for the earliest possible diagnosis of lung cancer.

We previously found that peroxiredoxin-I (Prx-I) is overexpressed in non-small cell lung cancer (NSCLC) tissue [18], and other groups have presented similar data [19–21]. Prx-I is one of six members of the Prx family, which are recently discovered enzymes with antioxidant function [22,23]. Cancer cells may become resistant to reactive oxygen species (ROS) stress through Prx-I overexpression.

Although the mechanism underlying autoantibody generation is not fully understood, protein overexpression may trigger a humoral autoimmune response [24–26]. In the present study, Western blot analysis was used to screen sera from cancer patients for the presence of autoantibodies that react against Prx-I. We found that 25 (47%) of 53 NSCLC patients had autoantibodies against Prx-I in their sera. In addition, Prx-I antigen itself was detected in 18 (34%) out of 53 sera from NSCLC patients. Meanwhile, 4 and 1 sera from 50 controls were detected as Prx-I antibody and antigen positives, respectively. Based on these findings, we propose that Prx-I is an autoimmunogenic human tumor antigen of NSCLC.

2. Materials and methods

2.1. Subjects
Tumor tissues and sera were collected at the Chonnam National University Medical School Hospital (Gwangju, Korea). Sera from 53 lung cancer patients were obtained at the time of diagnosis after informed consent. Patients comprised 34 males and 19 females with an age range of 44–81 years (median, 63.8 years). The diagnoses were adenocarcinoma (28 patients) and squamous carcinoma (25 patients). Healthy controls comprised 31 males and 19 females with an age range of 44–85 years (median, 64.8 years).

2.2. Cell culture and preparation of conditioned media

The human cell lines BEAS 2B (non-cancer lung cells), A549 (lung adenocarcinoma cells) and MCF7 (breast cancer cells) were obtained from the American Type Culture Collection. Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 (v/v). Cultured cells were harvested when 90% confluent after washing twice with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4). To obtain conditioned media, once cells were 70% confluent they were cultured in serum-free medium (3 ml per 60 mm dish culture), which was then collected at the indicated times.

2.3. Immunohistochemical staining

Tissues were fixed with 4% formaldehyde in PBS at 4 °C overnight, dehydrated with ethanol and penetrated with 100% xylene. Tissues were coated with pre-warmed paraffin for 1 h and sliced using a microtome. After removal of excessive paraffin with xylene and ethanol, tissues were incubated in 1% BSA for 1 h and then with anti-Prx-I antibody (LabFrontier, Seoul, Korea) overnight. After incubation with secondary anti-rabbit antibody, immunoreactivity was determined by the AEC method (streptavidin–biotin) using a DAKO kit (DAKO, Carpinteria, CA). Tissues were counterstained with Mayer's hematoxylin for nuclei staining.

2.4. Polyacrylamide gel electrophoresis (PAGE)

Protein samples were incubated with SDS sample buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.1% bromophenol blue) at 95 °C for 5 min and then subjected to SDS–PAGE using the method of Laemmli [27]. For two-dimensional electrophoresis (2-DE), harvested cells were directly disrupted in urea lysis buffer (9 M urea, 2% Chaps, 1% DTT, and 0.8% (v/v) carrier ampholytes), centrifuged at $100\,000 \times g$ at 4 °C for 1 h and total protein was recovered in the supernatant. Immobilized pH gradient (IPG) strips were prepared using the procedures described by Görg et al. [28] and 2-DE was performed as previously described [18]. Briefly, samples were applied to the IPG strips during rehydration in IPGphor (Pharmacia) and electrophoresed at 20 V for 12 h. Proteins then underwent isoelectric focusing (IEF) using the following voltage sequence: 500 V for 1 h, 1000 V for 1 h, a gradual increase over 1 h to 8000 V, and finally 8000 V for 1 h. After IEF, IPG strips were incubated for 15 min in 50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 1% DTT, and then for an additional 15 min in the same solution containing 2.5% iodoacetamide instead of DTT. The strips were then placed on 10–16% gradient polyacrylamide gels and electrophoresis was performed at 120 V in 25 mM Tris, pH 8.8, 192 mM glycine, and 0.1% SDS. The protein concentration was determined using the Bradford method [29].

2.5. Western blotting

Proteins separated by 1-DE or 2-DE were transferred to nitrocellulose membranes, which were incubated for 2 h with 5% (w/v) non-fat dry milk in TBS-T (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% (w/v) Tween-20). After washing with TBS-T, membranes were incubated for 1 h with 2000-fold diluted anti-Prx-I or anti-Prx-IV antibody (LabFrontier, Seoul, Korea) or diluted sera (1:500). Membranes were then washed with TBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5000) (Jackson Immuno-Research Laboratories Inc., West Grove, PA) or anti-human secondary antibody (1:5000) (Amersham Bioscience, Uppsala, Sweden). Immunoreactive proteins were detected using ECL reagents.

2.6. Preparation of hexahistidine-tagged peroxiredoxin-I (H_6Prx-I)

The Prx-I gene was amplified from a human lung cDNA library (Clontech, Palo Alto, CA) by polymerase chain reaction using the following primers: 5′-ggatccgcgatgtcttcaggaaatgc-3′ and 5′-cccaagcttggtcacttctgcttggag-3′. Amplified DNA fragments were ligated between the BamHI and HindIII sites of the pET28a vector (Novagen, Darmstadt, Germany) to create a pET28/H_6Prx-I, and this plasmid was transformed into BL21(DE3) E. coli. Recombinant H_6Prx-I protein was expressed following induction with 1 mM isoprophy-β-D-thiogalactoside, and purified by affinity chromatography using a Ni–nitrilotriacetic acid column (Novagen).

2.7. Mass spectrometry

Protein spots in Coomassie blue-stained gels were identified by peptide mass fingerprinting (PMF) using matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry as described previously [18]. Proteins in conditioned media were digested with modified trypsin (Promega, Madison, WI) and analyzed using tandem mass spectrometry directly connected with biphasic microcapillary chromatography (LC/LC_MS/MS). Briefly, the digested product was acidified and injected on to a biphasic microcapillary HPLC column. The first phase contained 4 cm of Partisphere strong-cation exchange media (Whatman, Clifton, NJ) and the second phase contained 7 cm of reversed-phase Polaris C18-A 5 μm resin (Phenomenex, Torrance, CA). Peptides eluted from the capillary column were electrosprayed into a LCQ Deca XP ion trap mass spectrometer (ThermoFinnigan, Woburn, MA). Bioworks Ver 3.1 was used to filter the search results.

3. Results

3.1. Prx-I is overexpressed in NSCLC tissue

Our previous proteome analysis comparing A549 lung cancer and BEAS 2B lung non-cancer cells showed that Prx-I was overexpressed in A549 cells, and subsequent Western blotting showed that Prx-I was also overexpressed in malignant lung tissue from NSCLC patients [18]. In the present study, we used immunohistochemical methods to compare Prx-I expression between malignant and non-malignant lung tissue sampled from the same NSCLC patient. We found that Prx-I was more highly expressed in cancer tissues (Fig. 1). Interestingly enough, relatively higher expression of Prx-I was frequently observed in cells exposed to airways or in peripheral cells of the cancer mass (arrows in Fig. 1B and C).

3.2. Autoantibodies against Prx-I are present in NSCLC patient sera

Given the increased expression of Prx-I in NSCLC patient cancer tissue, we investigated whether NSCLC patient sera
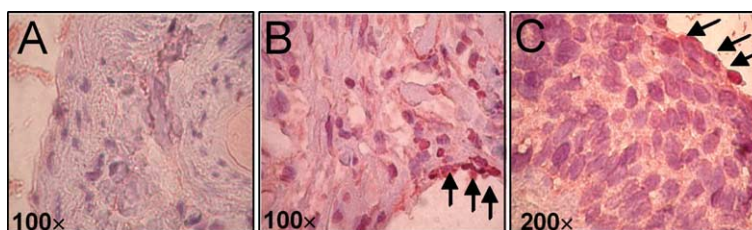

Fig. 1. Overexpression of Prx-I in malignant lung tissue. Normal (A) and cancer (B and C) tissue from the same NSCLC patient was immunostained using an anti-Prx-I antibody. Arrows indicate strongly Prx-I-positive cells located near airways or on the periphery of the cancer mass. A and B = 100× magnification. C = 200× magnification.
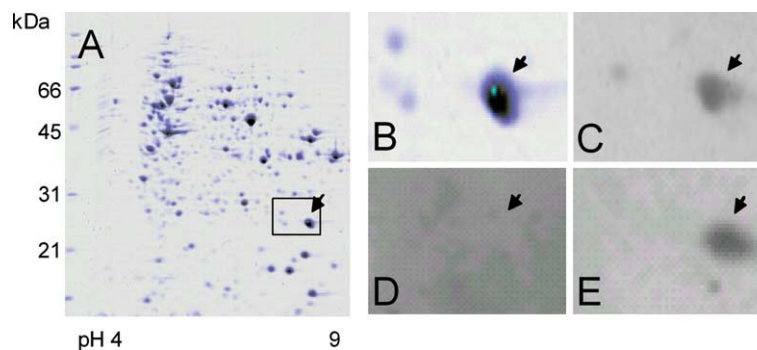
Fig. 2. Prx-I autoantibodies in serum from NSCLC patients. (A) Total protein extracts from A549 cells were separated by 2-DE and stained with Coomassie blue. The protein spot indicated by the arrow was identified as Prx-I using peptide mass fingerprinting. (B) An enlargement of the region containing the Prx-I protein (box in panel A). A549 cell lysate proteins were separated by 2-DE, transferred to nitrocellulose membranes and then immunoblotted with either an anti-Prx-I antibody (1:2000) (C), or with diluted (1:500) sera combined from 10 healthy control subjects (D) or 10 NSCLC patients (E). Membranes were then incubated with appropriate secondary antibodies and data visualized using chemiluminescence.

contained Prx-I autoantibodies. A549 cell lysates were subjected to 2-DE (Fig. 2A), and the position to which Prx-I migrated (arrows in Fig. 2B) was confirmed by MALDI-TOF mass spectrometry (data not shown) and Western blotting with an anti-Prx-I antibody (Fig. 2C). To determine whether NSCLC patient sera contained Prx-I autoantibodies, A549 cell lysates were subjected to 2-DE, transferred to membranes, incubated with pooled sera from 10 healthy control subjects or 10 NSCLC patients, and immunoreactive spots identified. We found that an antibody present in lung cancer patient sera reacted with Prx-I (Fig. 2E), while no such reaction was observed with healthy sera (Fig. 2D). These data indicate that lung cancer patient sera contain autoantibodies against Prx-I. We then screened individual patient serum for Prx-I autoantibodies. Purified, recombinant Prx-I with hexahistidine at the N-terminus (H6Prx-I) was Western blotted and probed with individual patient's serum. We found that the combined patient sera used above was able to detect as low as 5 ng H6Prx-I whereas the control sera could not detect 40 ng H6Prx-I (Fig 3A). Individual analysis against 10 ng H6Prx-I showed that sera from 25 (47%) of the 53 NSCLC patients contained immunoreactivity, whereas 4 (8%) positive signals were detected from age- and sex-matched 50 control sera (Fig. 3B and Table 1). We also examined whether Prx-I antigen itself was circulating in sera from NSCLC patients. By Western blot analysis using Prx-I antibody, Prx-I was detected in sera from 18 (34%) of the 53 patients, whereas it was detected in only 1 out of the 50 healthy control sera (Fig. 3C and Table 1). Although the number of subjects is not enough to determine any differences in Prx-I antibody and antigen positives among cancer types and stages (Table 1), the data strongly suggest that both Prx-I antibody and antigen are potential biomarkers for use in serological diagnosis of NSCLC.

3.3. Secretion of Prx-I from A549 cells

The detection of Prx-I in patient sera was somewhat unexpected since this protein is known to be cytosolic and has no signal peptide [23]. We examined whether Prx-I was secreted from cultured lung cancer cells. Fresh media was placed on BEAS 2B (human lung non-cancer) and A549 (human lung cancer) cells, and was then collected after 1, 2, 4 and 8 h and analyzed by Western blotting using an anti-Prx-I antibody. Prx-I was detected in A549-conditioned media but not in BEAS 2B-conditioned media (Fig. 4A). In addition, Prx-I

was not detected in media conditioned by MCF7 breast cancer cells (Fig. 4B). Among Prx family members, only Prx-IV has a signal sequence and a potential to be secreted [30,31]. We did not detect Prx-IV in A549-conditioned media by Western blotting using an anti-Prx-IV antibody (data not shown). The presence of Prx-I in A549-conditioned media was confirmed by LC/LC_MS/MS analysis (Fig. 4C), while any other Prxs including Prx-IV were not detected by this analysis.

4. Discussion

We previously found that Prx-I is overexpressed in NSCLC tissue [18], and Prx-I overexpression in lung cancer has also
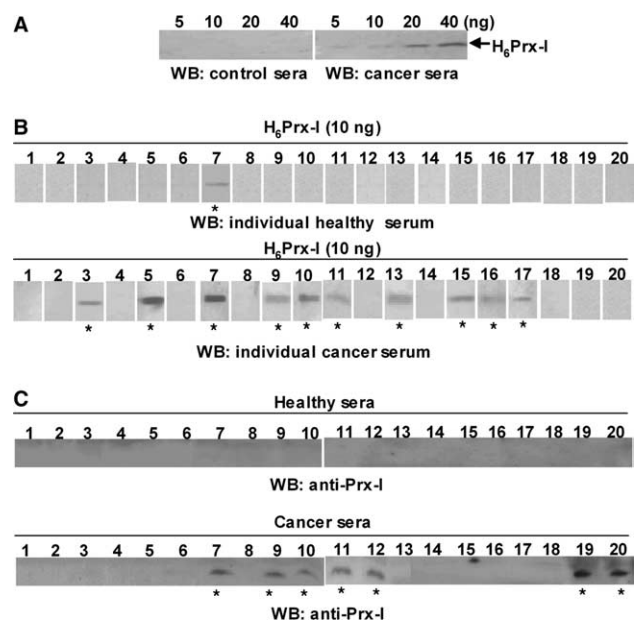

Fig. 3. Prx-I autoantibodies and antigen in serum from NSCLC patients. (A) Purified recombinant H6Prx-I (5, 10, 20 and 40 ng) was Western blotted and probed with combined sera from 10 healthy controls or 10 NSCLC patients. (B) H6Prx-I (10 ng) was Western blotted and probed with individual serum from healthy control subjects and NSCLC patients. (C) Individual serum (10 μl) from healthy control subjects and NSCLC patients was Western blotted and probed with an anti-Prx-I antibody. Results from 20 sera from patients or controls were shown as the representative data in B and C.

Table 1
Prx-I Ab and Ag positive sera from NSCLC patients and control subjects

	Number of subjects	Prx-I Ab positive	Prx-I Ag positive	Prx-I Ab/Ag positive
Non-small cell lung cancer	53	25 (47.1)	18 (33.9)	9 (17.0)
Types				
Adenocarcinoma	28	14 (50.0)	10 (35.7)	5 (17.9)
Squamous carcinoma	25	11 (44.0)	8 (32.0)	4 (16.0)
Stages				
I/II	12	4 (33.3)	3 (25.0)	0 (0)
III	20	12 (60.0)	6 (30.0)	4 (20.0)
IV	21	9 (42.9)	9 (42.9)	5 (23.8)
Controls	50	4 (8.0)	1 (2.0)	0 (0)

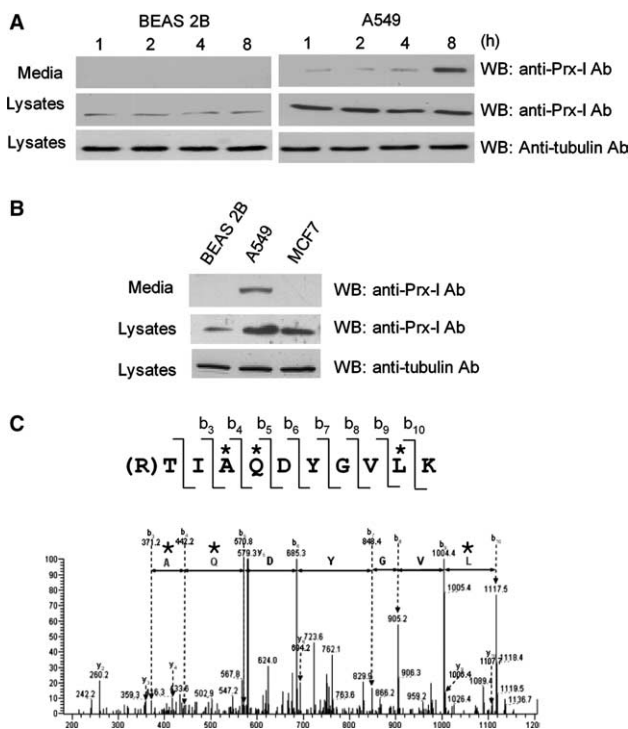Numbers in parentheses are percentage. Ab, antibody; Ag, antigen.

Fig. 4. Secretion of Prx-I from A549 cells. (A) Conditioned media from cultured BEAS 2B and A549 cells were collected at the indicated times and analyzed by Western blotting using an anti-Prx-I antibody. (B) Conditioned media from cultured BEAS 2B, A549, and MCF7 cells were collected at 8 h and analyzed by Western blotting with the same antibody. Prx-I and tubulin in cell lysates were monitored by Western blot analyses (A and B). (C) Prx-I in A549-conditioned medium was confirmed by tandem mass spectrometry. Asterisks indicate Prx-I-specific amino acids comparing to Prx-IV.

been reported by others [19–21]. We believe that increased Prx-I expression may have an important role in oncogenic transformation of human lung cells. Prx-I overexpression may protect cancer cells from oxidative stress, which may increase cell survival [32]. Therefore, down-regulation of Prx-I may improve outcomes of lung cancer therapy.

Many biomarkers have been suggested and evaluated for early diagnosis of lung cancer [5–17]. However, they are not routinely used clinically because their sensitivity and specificity are not high enough. While some groups have used several biomarkers in combination in attempts to improve specificity and sensitivity, there is still a great need for better early diagnosis biomarkers of lung cancer.

Although it is not clear why some patients develop immunogenicity to a particular antigen, the majority of tumor-associated antigens are not products of mutated genes but are overexpressed proteins or antigens related to cell differentiation [24–26]. The present study demonstrated the presence of Prx-I autoantibodies and antigen in NSCLC patient serum. Although we screened only 53 sera from cancer patients, the frequency with which these proteins were identified (47% for antibody and 35% for antigen) is high enough to consider Prx-I as a potential clinical biomarker of NSCLC.

Over 80% of lung cancer patients suffer from NSCLC, which represents a mixed group of tumors with differing histology, clinical course and response to treatment. The heterogeneous nature of NSCLC limits the use of biomarkers. Cancer typing can improve diagnosis because biomarkers specific to certain cancer types will improve sensitivity and specificity. Recently, proteomics approaches provided a novel method for lung cancer typing [33,34]. Levels of Prx-I overexpression can be used as a criterion for NSCLC typing.

We found that despite a lack of signal peptide, Prx-I was secreted by A549 lung cancer cells. Prx-I secretion was also reported from cultured astrocyte cells [35]. Furthermore, PGP9.5, which does not have signal peptide, was also shown to be secreted from A549 cells [16]. However, Prx-I overexpression per se does not appear to cause secretion since there was no Prx-I secretion from MCF7 cells, which also overexpress Prx-I. We are currently investigating the mechanisms involved in Prx-I secretion from A549 cells and its functional relevance in lung cancers.

Our findings indicate that overexpressed Prx-I in NSCLC induces a humoral immune response and suggest that Prx-I autoanitbody and antigen are potential biomarker for clinical serological screening of lung cancer.

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