Clinicopathological correlation of Peroxiredoxin I in breast cancer

Pei-Jou Chua, Eng-Hong Lee, Chunhua Guo, George W-C Yip, Puay-Hoon Tan and Boon-Huat Bay

Abstract—Peroxiredoxins (Prxs) are antioxidant enzymes which have been linked with fundamental processes such as cellular proliferation and apoptosis. The Prx I protein, a member of the Prx family, is known to be expressed in several cancers. This study correlates the immunohistochemical expression of Prx I with clinicopathological parameters in 180 breast cancer tissues. Prx I was observed to be inversely correlated with histological grade 2 versus histological 3. Prx I expression was also demonstrated at the mRNA level by real-time PCR and protein level by Western blot, immunocytochemistry and immunofluorescence in MCF-7 and MDA-MB231 breast cancer cells. Higher Prx I expression appears to be associated with less aggressive breast tumors.

Keywords—breast carcinoma, histological grade, immunohistochemistry, Peroxiredoxin I, real-time PCR

I. INTRODUCTION

PEROXIREDOXINS (Prxs) were first discovered as radical scavenging ‘protector’ proteins in the yeast [1]. Prxs, are antioxidant enzymes which can reduce hydroperoxides and peroxynitrite generated by reactive oxygen species [2]. The Prx family of proteins are categorized into six subgroups based on the position of the cysteine residue involved in catalysis, viz. 2-cys subgroup (Prx-I-IV), 1-cys Prx subgroup (Prx VI) and atypical 2-cys Prx subgroup (Prx V) [2,3]. The Prx proteins have been linked with cellular apoptosis, differentiation and proliferation [4].

Prx I belonging to the 2-Cys Prx subgroup is also known as natural killer enhancing factor [5] and by other names such as 23-kDa macrophage stress induced protein [6], 23-kDa heme-binding protein [7] and osteoblast specific factor [8]. The Prx I gene is also called proliferation associated gene (PAG) [9]. Prx I expression is reported to be increased in esophageal carcinoma, oral cancer and astrocytic brain tumors [10-12].

In this study, we examined the immunohistochemical expression of Prx I in breast carcinoma tissues and correlated the expression with clinicopathological parameters. The expression of Prx I mRNA and Prx I protein level were also determined in breast cancer cells in vitro.

II. MATERIALS AND METHODS

Clinical samples
A total of 180 cases of archival invasive ductal breast cancers were obtained from female patients (age range 34-86 years) who had undergone surgery in the Singapore General Hospital. Tissue microarrays were constructed from 10% formalin fixed, and paraffin embedded breast cancer tissues. Ethics approval was obtained from the Institutional Ethics Committee for all the clinical samples used in this study.

Cell Culture
MCF-7 and MDA-MB-231 breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). MDA-MB-231 breast cancer cells were cultured in RPMI medium containing 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT). MCF-7 breast cancer cells were cultured in DMEM medium supplemented with 10% FBS. Cell cultures were propagated at 37°C in a 5% CO2 incubator.

Immunohistochemistry
Automated immunohistochemistry was carried out on breast cancer tissue microarrays with the Leica Bond™ system. Optimization of the rabbit polyclonal Prx I antibody (Abcam, Cambridge, UK) was carried out and a dilution of 1:800 was selected for immunohistochemical staining. The immunostaining was scored based on a semi-quantitative scoring system. The immunoreactive score (IRS) was computed as Σ (intensityn × percentage of cells stained with intensityn). The tissue sections were photographed using the Olympus BX51 microscope (Olympus Optical Co., Tokyo, Japan). For immunocytochemistry, cells were grown in four-well chambers and fixed with 4% paraformaldehyde. Rest of the procedure is as described above.

Real-time reverse transcription polymerase chain reaction (Real-time RT-PCR)
Total RNA was extracted from cells using the RNasy mini kit (Qiagen, Valencia, CA) and 1µg of RNA was reverse transcribed using...
transcribed using the SuperScript™ III First Strand Synthesis System (Invitrogen, Carlsbad, CA) with random hexamer according to manufacturer’s instruction. The synthesized cDNA was then used as a template for real-time PCR with the LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany). The primer sequences for Prx I gene were 5’ CAG CCT GTC TGA CTA CAA AGG A-3’ (forward) and 5’- CCA GTC CTC CTT GTT TCT TAG G-3’ (reverse). For GAPDH, the primer sequences were 5’-GAA GGT GAA GGT CGG AGT CAA-3’ (forward) and 5’-TGC CAT GGG TGG AAT CAT ATT GG-3’ (reverse). The PCR conditions used were initial denaturation at 95°C for 15 mins, followed by 45 cycles of denaturation at 94°C for 15s, annealing at 60°C for 25s and extension at 72°C for 15s.

Western Blot analysis
Cells were lysed and harvested with M-PER® Mammalian Protein Extraction Reagent (Pierce, Rockford, IL), containing EDTA and protease inhibitor. Cell debris was removed by centrifugation at maximum speed for 10 minutes. The protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). 20µg of proteins were loaded onto 10% SDS-PAGE and separated by gel electrophoresis. The separated proteins were then transferred to a PVDF membrane. The membrane was blocked with 5% skimmed milk overnight to prevent non-specific binding of proteins. Membrane was blotted with antibodies against Prx I rabbit polyclonal antibody (1:1000, Abcam, Cambridge, UK) and beta actin mouse monoclonal antibody (1:6000, Sigma, St Louis, MO). The primary antibodies were detected using anti-rabbit or anti-mouse HRP-conjugated secondary antibody (1:10,000) and visualized with the Super-Signal West Pico Chemiluscent Substrate (Pierce, Rockford, IL). Protein bands were then analyzed using the GS-7000 Imaging Densitometer (Bio-rad laboratories, Hercules, CA).

Statistical analysis
The statistical analysis was done using the Graph Pad Prism software (San Diego, CA). For immunohistochemistry results, Spearman’s correlation was used to compare between continuous variables. For discrete variables in each group, one way ANOVA with post hoc Tukey test was used. For the in vitro work, unpaired t-test was used to compare the difference between two groups. All the data are expressed as mean ± SEM of at least three independent experiments. P<0.05 was considered as statistically significant.

III. RESULTS
Prx I expression in breast cancer tissues
Immunohistochemical staining of Prx I in breast cancer tissue microarrays was performed. Prx I was found to be localized mainly in the cytoplasm of the breast tumor cells. The different staining intensity of Prx I in breast cancer tissues is classified as light (Figure 1B), moderate (Figure 1C) and intense staining (Figure 1D). As can be seen in Figure 1A, the negative control is devoid of any brownish staining.

Correlation of Prx I expression with clinicopathological parameters
The cohort consisted of predominantly Chinese patients with mean age 54.2 years. There were no significant differences between Prx I expression and tumor stage,
lymph node status and hormonal status. However, a significant difference was observed between the immunoreactive scores of Prx I in patients with histological grade 2 compared with histological grade 3 tumors (Table 1 and Figure 2, P<0.01).

Prx I immunostaining was lower in tumors with higher histological grade. Although histological grade 1 tumors had the highest intensity, the result was not statistically significant. It must also be noted that there was a much smaller number of patients with histological grade 1 tumors.

Table 1. Patient distribution for histological grade of breast tumors

<table>
<thead>
<tr>
<th>Histological grade*</th>
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<tbody>
<tr>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>89</td>
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*Histological grade of 5 cases were unknown

Prx I immunostaining was performed in the three histological subtypes of breast cancer viz., invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC) and mixed carcinoma (MC). Prx I staining in IDC and ILC are shown in Figure 3.

P<0.05. Error bar = SEM.

However, no significant differences were detected in the three histological subtypes with respect to Prx I staining (Figure 4).

P<0.05. Error bar = SEM.
Prx I expression in breast cancer cell lines.

Prx I mRNA expression was examined in non-invasive breast MCF-7 cancer cells and the highly metastatic MDA-MB-231 breast cancer cell line by using real time PCR. Prx I mRNA was found to be expressed in both cancer cell lines with MDA-MB-231 cells having lower expression as compared with MCF-7 cells although not statistically significant (Figure 5).

Figure 5: mRNA expression of Prx I and housekeeping gene GAPDH in breast cancer cell lines. Prx I expression was examined using real-time RT-PCR. Error bar = SEM.

The specificity of the Prx I primers are shown by agarose gel electrophoresis in Figure 6. Melting curve analyses also showed the presence of single peaks for the primers used (not shown).

Figure 6. RT-PCR products of Prx I by gel electrophoresis

Expression of the Prx I proteins detected by Western blotting showed a similar trend with MCF-7 cells having higher expression (Figure 7). The Prx I proteins appear as single bands of 22 kDa size. The housekeeping protein used was beta actin.

Figure 7. Prx I protein expression MCF7 and MDA-MB-231 breast cancer cells. Western blot analysis showing the protein bands of Prx I and beta actin for both cell lines. Bar chart shows the optical density ratio of Prx I with beta actin as the loading control. Error bar = SEM.

The Prx I proteins were seen to be present predominantly in the cytoplasm of the MCF-7 breast cancer cells by immunohistochemistry and immunofluorescence (Figure 8).

Figure 8. Prx I immunostaining of MCF-7 breast cancer cells. (A) Immunocytochemical staining. Hematoxylin counterstain. Bar = 30 µm. (B) Immunofluorescence staining observed by confocal microscopy. DAPI stains the nuclei blue. Red cytoplasmic stain denotes the presence of Prx I protein. Bar = 50 µm.
IV. DISCUSSION

The role of Prx I in breast cancer is still controversial. On the one hand, Prx I has been found to be overexpressed in breast cancer. Noh et al. [13] observed that Prx I was overexpressed in the breast cancer tissues from 21 out of 24 patients. Recently, Cha et al. [14] reported that Prx I mRNA and Prx I proteins (by western blotting) were expressed at higher levels in breast cancer tissues compared to normal tissues.

However, there are reports that Prx I functions as a tumor suppressor. Loss of Prx I expression has been observed to shorten the life span of mice which developed hemolytic anemia and cancer, implicating that Prx I possessed tumor suppressor properties [15]. This is supported by the finding that Prx1 interacts with the c-Myc oncogene and inhibits the suppressor properties [16]. Moreover, Cao et al. [17] has also reported that Prx I promotes PTEN tumor suppressive function and reduces susceptibility to H-Ras-induced mammary tumors.

Karihtala et al. [3] found no correlation between Prx I expression and clinicopathological features in breast cancer. A similar observation was also reported recently in a cohort of 224 patients with early stage (Stage I and II) invasive breast cancer by Woolston et al. [18]. However, the same authors noted that high expression of cytoplasmic Prx I was associated with higher risk of local recurrence after radiotherapy.

In this study, we found that histological grade 2 tumors had higher Prx I protein levels than histological grade 3 tumors. Histological grade is based on glandular differentiation, nuclear pleomorphism and mitotic counts [19]. A higher histological grade is known to have a poorer prognosis [20]. The in vitro data in this study seems to support the notion that higher expression of Prx I is associated with less aggressive tumors. Prx I mRNA levels were higher in non-invasive MCF-7 breast cancer cells compared to the highly malignant MDA-MB-2331 breast cancer cells although the difference was not statistically significant. This is also reflected in the higher protein expression in the more aggressive MDA-MB-231 cells.

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REFERENCES


