

Peroxiredoxins in Breast Carcinoma¹

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ABSTRACT

Purpose: Peroxiredoxins (Prxs) are a novel group of peroxidases containing high antioxidant efficiency and some of them having also effects on cell differentiation and apoptosis. The mammalian Prx family has six distinct members located in various subcellular locations, including peroxisomes and mitochondria, places where oxidative stress is most evident.

Experimental Design: We examined immunohistochemically a large set of samples from patients with breast carcinoma and investigated associations with parameters such as tumor-node-metastasis classification, hormone receptor status, and patient survival. Three biopsies of healthy breast tissue were used as controls.

Results: Expression of peroxiredoxins I, III, IV, and V was found in $\geq 80\%$ of cases, whereas the expression of Prx II and VI was less frequent. Increased expression of Prx III was found to associate with the presence of progesterone ($P = 0.02$) and estrogen ($P = 0.03$) receptors, and Prxs IV ($P = 0.009$) and VI ($P = 0.04$) were overexpressed in progesterone receptor positive cases. Prx V was the only isoform that associated with items of tumor-node-metastasis classification, it was connected to a larger tumor size ($P = 0.05$) and positive lymph node status ($P = 0.04$). Prx V positivity was also connected with shorter survival ($P = 0.04$), whereas Prxs III ($P = 0.002$) and IV ($P = 0.02$) were related to better prognosis, probably resulting from their connection with a positive hormone receptor status.

Conclusions: In conclusion, we found that expression of peroxiredoxins, especially III, IV and V, is increased in

breast malignancy, suggesting the induction of Prxs as response to increased production of reactive oxygen species in carcinomatous tissue.

INTRODUCTION

Organisms living under aerobic conditions are exposed to ROS³ such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and nitric oxide NO, which are generated either by metabolism from ordinary redox reactions, mainly in mitochondria, or through exposure to exogenous stimuli-like infectious and inflammatory agents, pollutants, UV radiation, or alcohol (1, 2). When produced, ROS have a very short half-life, and they can react near the site where they developed. They interact and damage various cellular compounds such as DNA, proteins, carbohydrates, or lipids and occasionally launch chain reaction leading to destruction of new macromolecules (3, 4). In addition, it has been demonstrated *in vitro* that ROS at low doses participate in many physiological processes such as signal transduction, cell differentiation, apoptosis, and modulation of transcription factors (5–7).

From prokaryotes to primates, cells have developed different kinds of reparative or defensive systems to combat toxic processes of ROS. These defense systems include antioxidant enzymes such as superoxide dismutases, catalase, glutathione peroxidases, and a quite recently found, rapidly growing family of peroxiredoxins (7, 8). Although for example catalase is located only in peroxisomes and decomposes hydrogen peroxide to molecular oxygen and water, peroxiredoxins are present in various cellular compartments and reduce peroxides to the corresponding alcohol (or water), just like the other peroxidases (9–11). However, Prx family has some special features compared with the other peroxidases. They act both as the peroxidase and the cosubstrate because when reducing H_2O_2 , Prxs themselves are oxidized (10). Then Prxs are reduced mainly by thioredoxin, except for Prx VI, the electron donor of which is not known yet. Instead of having heme or selenocysteine, as with many other peroxidases, peroxiredoxins have cysteine(s) as their active site. In Prxs I–IV, which have two conserved cysteines and belong therefore to 2-Cys subgroup, the NH_2 -terminal Cys-SH group has been shown to be the primary site of oxidation and when oxidized, it rapidly reacts with another conserved cysteine in COOH terminus to form an intermolecular disulfide bond (12). Prx I and II are cytosolic proteins, whereas Prx III is present in mitochondria and Prx IV in endoplasmic reticulum and lysosomes but is also secreted to extracellular space (7, 13). Peroxiredoxin VI has only one conserved cysteine, and it is the only member of 1-Cys peroxiredoxin subgroup and is found as the highest concentrations in lung tissue (14–16). Prx V has been classified to atypical 2-Cys

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³ The abbreviations used are: ROS, reactive oxygen species; TNM, tumor-node-metastasis; Prx, peroxiredoxin.

subgroup and its catalytic site represents a special case among the Prx isoforms, and it has been speculated that Prx V could be more effective against ROS compared with other Prxs (17, 18). Moreover, Prx V is subcellularly located to peroxisomes and mitochondria to places where protection against ROS is mostly needed.

There is fastly growing evidence that oxidative stress is important not only for normal cell physiology but also for many pathological processes like atherosclerosis, neurodegenerative diseases, allergies, and cancer (3, 19–21). ROS participate in carcinogenesis in all its stages, *e.g.*, initiation, promotion, and progression (3). Elevated O_2^- levels have been measured in breast cancer, and $^{\bullet}OH$ modification has been discovered to rise up to 17-fold over normal breast tissue in invasive ductal carcinoma (1, 22). It is still uncertain whether this is attributable to increased DNA damage by ROS or diminished DNA repair enzyme activity. There is also epidemiological evidence between oxidative stress and cancer; the protective role of antioxidants against cancer development has been demonstrated (23). Also, high intake of transition metals such as iron, which enhances the production of ROS, has been shown to accelerate tumor induction (24). Nevertheless, the connection between peroxiredoxins and breast cancer has not yet been properly studied (25).

In this study, we examined a large set of breast carcinomas for the immunohistochemical expression of peroxiredoxin isoforms I–VI in breast cancer tissue. The results were correlated with known clinical and biochemical parameters of the tumors such as TNM stage, estrogen and progesterone receptor status, proliferation, and survival.

MATERIALS AND METHODS

Study Material. Originally, 642 breast cancer samples and three nonneoplastic breast samples were obtained from the archives of the Department of Pathology, Oulu University Hospital, during the years 1979–2000. The specimens had been fixed in formalin and embedded in paraffin blocks. From each sample, one representative tumor region was chosen and included to multitissue microarray blocks. Microarray sample diameter was 1300 μm ; blocks were created with Beecher Instruments Manual Tissue Arrayer (Beecher Instruments, Silver Spring, MD). Nonneoplastic control biopsies were processed as individual slides. The material consisted of ductal ($n = 451$), lobular ($n = 92$), and other histological types of carcinoma ($n = 40$), including medullary, mucinous, tubular, and papillary carcinoma (26). Clinical data such as tumor size, presence of metastases, and patient survival were obtained from hospital records. There were 98 patients having tumor size T_1 according to TNM classification, 186 tumors in T_2 category, 46 in T_3 , and 31 tumors in T_4 category. Regional lymph node metastases were present in 206 patients and 187 of these cases was classified as N_1 , 12 as N_2 , and 7 as N_3 ($n = 395$). There were 20 patients having distant metastases according to hospital data ($n = 361$). Grading was distributed as follows ($n = 375$): grade I, 36; grade II, 191; and grade III, 148.

Immunohistochemistry. The polyclonal Prx antibodies have been described previously (7, 14, 27, 28). Specimens were first sectioned (thickness, 4 μm), then deparaffinized in xylene

and rehydrated through descending ethanol series. Then they were immersed in 10 mM citric acid monohydrate (pH 6.0) for 10 min, boiled in a microwave oven at 850 W for 2 min and at 350 W for 8 min. After that, primary antibodies were incubated on the slides for 1 h with a dilution of 1:1500 for Prx I, 1:1000 for Prx II, 1:500 for Prx III, 1:1000 for Prx IV, and 1:2000 for Prxs V and VI. The immunostaining was done using the Histostain-Plus Bulk Kit (Zymed Laboratories, Inc., South San Francisco, CA). The same procedure was used to generate negative control sections except that primary antibodies were replaced by PBS and serum isotype controls (Zymed Laboratories, Inc.).

The immunostaining results were evaluated semiquantitatively by dividing the staining reaction into four categories: – = no immunostaining present; + = weak immunostaining; ++ = moderate immunostaining; and +++ = strong immunostaining.

Cell proliferation was studied with a monoclonal mouse antihuman Ki-67 antibody (Zymed Laboratories Inc.). The dilution used was 1:50. The immunostaining was performed as described above, except that avidin-biotin-peroxidase complex method was used and 3,3'-diaminobenzidine was as the chromogen. The results were evaluated as percentage of positive cells of the whole cell population.

The presence or abundance of the estrogen and progesterone receptors was also studied immunohistochemically as above. The dilution for both was 1:100 (Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom). In each case, the percentage of positively stained nuclei was evaluated and then a percentage of positively stained tumor cells for estrogen and progesterone receptors were obtained. CerBb2-immunostaining was performed as Prxs, and a mouse monoclonal antibody was used for the immunostaining (Novocastra Laboratories Ltd.). Dilution used was 1:500.

Statistical Analysis. SPSS 10.1.4 for Windows (Chicago, IL) was used for statistical analysis. The significance of the associations was determined using Fisher's exact probability test, *t* test, and Cox multivariate regression analysis. In survival analysis, Kaplan-Meier curve was used, and the significance was measured by the log-rank, Breslow, and Tarone-Ware tests. Probability values $P \leq 0.05$ were considered significant.

RESULTS

All of the Prx isoforms could be shown in the majority of breast cancer specimens by using isoform-specific antibodies (Fig. 1). Because of exhaustion of the blocks, detachment of samples, and occurrence of nonrepresentative areas in the punch samples, the originally larger study material decreased to <500 cases. In nonmalignant breast tissue, all Prx isoforms showed lower levels compared with those of carcinomatous tissue, moreover, Prx IV was entirely undetectable in healthy tissue. In nonneoplastic tissue, highest Prx levels were observed in equal terms in acinar and ductal cells; cells of larger ducts were more negative for all Prx isoforms.

In our tumorous lesions, Prx I was present in 83.2% of cases ($n = 475$), Prx II in 59.0% ($n = 458$), where only one case showed strong positivity, and Prx III in 89.0% of cases ($n = 446$). The most intensive staining was shown by Prx IV; 93.7%

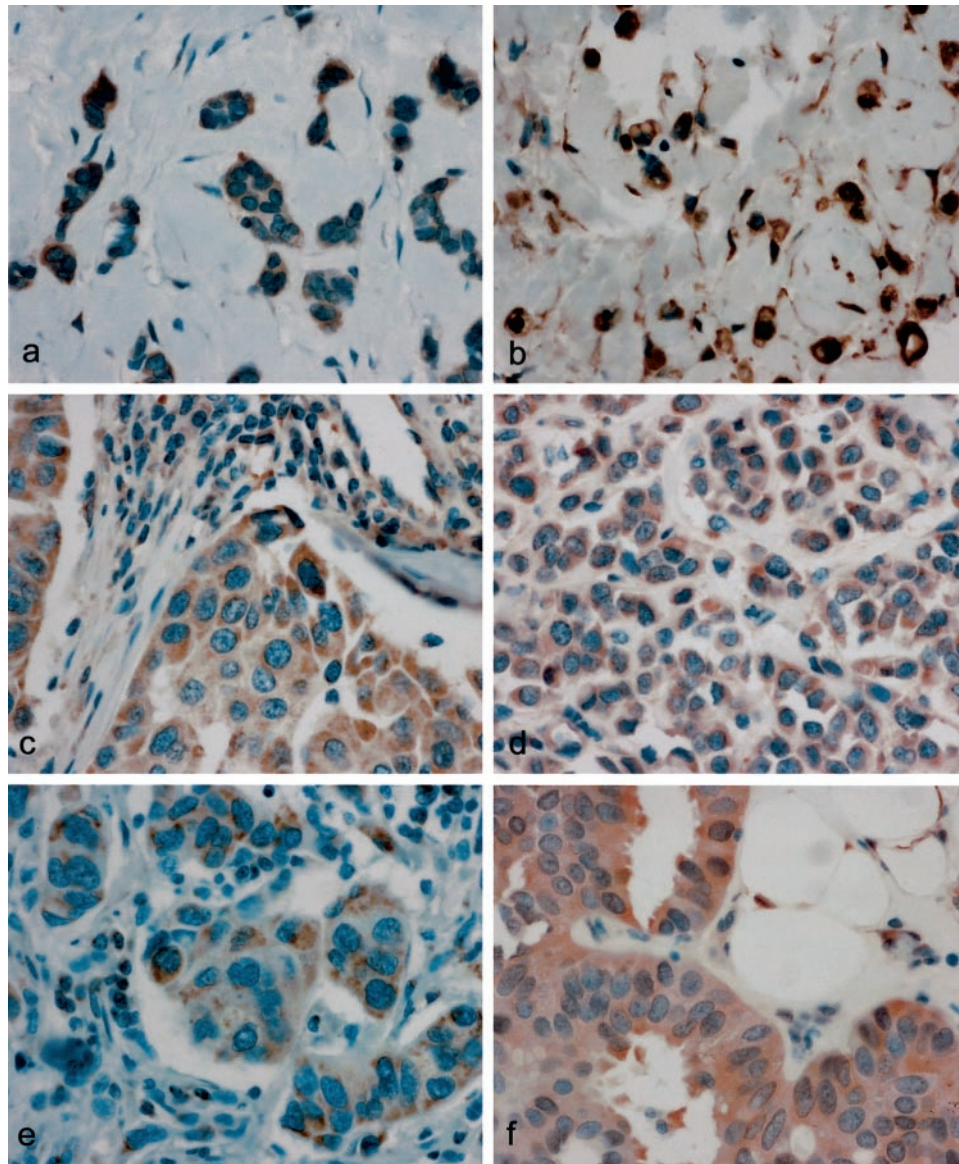


Fig. 1 A panel showing Prx subtype expression in breast carcinoma. Prx I (a), Prx II (b), Prx III (c), Prx IV (d), Prx V (e) and Prx VI (f).

were positive ($n = 447$) and 48.3% of specimens were at least moderately stained. Prx V-positive staining was seen in 79.8% of samples ($n = 481$), more than half (52.6%) at least moderately stained. Prx VI could be shown in 52.6% of cases ($n = 380$), but no cases stained strongly for this subtype. The distribution of different Prx isoform expressions is shown in Table 1.

When comparing Prxs to each other or to tumor parameters, cell differentiation, receptor status, or survival, Prx immunostaining was divided to two groups: 0 = no staining or weak staining and 1 = moderate or strong staining present. T categories were subclassified to either small (T_1 , tumor ≤ 2 cm in greatest dimension) or large tumor size (T_2 – T_4 , >2 cm) and N categories as lymph node negative (N_0) or lymph node positive (N_1 – N_3) groups. Grading was distributed into two subgroups: 0 = well or moderately differentiated and 1 = poorly differentiated.

Prx I was the only peroxiredoxin isoform that did not have significant association with any clinicopathological parameter studied. Neither Prx II showed any statistical association with the tumor size or the presence of either lymph node or other metastases, but according to histopathological grading, at least moderate expression tended to be associated with poorly differentiated tumors, although not significantly ($P = 0.07$). However, Prxs III ($P = 0.03$), IV ($P = 0.03$), and V ($P = 0.03$) showed significantly increased expression in poorly differentiated tumors.

We discovered that tumor size associates with the augmented extent of staining of Prx V, alone among the Prx isoforms. When Prx V expression was at least moderate, there was significantly increased risk to tumor's size being T_2 or more (>2 cm in greatest dimension; $P = 0.05$). However, no relationship between other Prxs expression, and tumor size was

Table 1 Results of immunostainings with antibodies to different Prx isoforms in breast carcinomas

	–	+	++	+++
Prx I	80 (16.8%)	285 (60.0%)	95 (20.0%)	15 (3.2%)
Prx II	188 (41.0%)	238 (52.0%)	31 (6.8%)	1 (0.2%)
Prx III	49 (11.0%)	180 (40.4%)	158 (35.4%)	59 (13.2%)
Prx IV	28 (6.3%)	203 (45.4%)	173 (38.7%)	43 (9.6%)
Prx V	97 (20.2%)	131 (27.2%)	156 (32.4%)	97 (20.2%)
Prx VI	180 (47.4%)	173 (45.5%)	27 (7.1%)	0 (0%)

Table 2 P in different parameters studied in breast carcinomas

	T > 1	N > 0	M	ER ^a	PR	Grade > 2	Ki-67
Prx I	0.76364	0.35282	0.22903	0.84481	0.39846	0.25259	0.380
Prx II	0.31715	0.61929	0.453163	0.42647	0.3939	0.070393	0.089
Prx III	0.09190	0.29822	0.067725	<i>0.03271^b</i>	<i>0.02006</i>	<i>0.033216</i>	0.957
Prx IV	0.35996	0.087693	0.209967	0.24637	<i>0.00908</i>	<i>0.027155</i>	0.165
Prx V	<i>0.05082</i>	<i>0.03836</i>	0.111919	0.46692	0.17665	<i>0.030970</i>	0.939
Prx VI	0.89864	0.56882	0.409504	0.27366	<i>0.03606</i>	0.200482	0.704

^a ER, estrogen receptor status; PR, progesterone receptor status; Ki-67, proliferation index.

^b Italicized numbers indicate statistically significant value.

observed. Prx V was the only isoform to have increased expression in the lymph node metastasis-positive cases ($P = 0.04$). We found no significant associations between the presence of distant metastases and Prx staining.

There was a significant relationship between the expression of Prx III and Prx IV ($P = 0.00003$) and between Prx III and Prx VI ($P = 0.002$). In addition, the connection between Prx III and Prx V was near significant ($P = 0.07$). No associations between cell proliferation and Prx immunostaining could be found, although there were near-significant associations between Prx II and Ki-67 ($P = 0.09$). Neither was proto-oncogene *CerbB2* overexpression related to any Prx expression.

When investigating association between Prx isoforms and hormone receptor status, we observed that augmented expression of Prx III was linked with increased presence of both estrogen ($P = 0.03$) and progesterone ($P = 0.02$) receptors. Stronger Prx IV-immunostaining associated with the positivity of progesterone receptors ($P = 0.009$) but not with estrogen receptors. The presence of Prx VI showed also positive association with progesterone receptors the P being 0.04 but not with estrogen receptors. We found no association between the staining of Prx isoforms I, II, and V and the existence of either hormone receptor. The associations between peroxiredoxin I–VI expression and studied clinicopathological parameters are shown in Table 2.

The association between the expression of Prxs and patient survival was also studied. When the specimens were divided in two groups as above, the presence of Prx isoforms III ($P = 0.002$) and IV ($P = 0.02$) seemed to be associated with a better prognostic value when using the log-rank test. Also, Breslow and Tarone-Ware tests showed a significantly positive association between prognosis and Prx staining. Patients' mean survival rate was 140 months when Prx III immunostaining was weakest, and it increased to 186 months when staining was moderate or strong. Corresponding numbers for Prx IV were 143 and 159 months. There were no big differences when dividing staining to

Prx-positive and Prx-negative groups, higher level of Prxs III ($P = 0.0005$, log-rank test) and IV ($P = 0.01$, log-rank test) still showed better survival than absence of these isoforms, however, presence of Prx V was now in connection with a shorter survival ($P = 0.04$). When all Prx expressions were summed together (weak or no expression = 0; moderate or strong = 1), stronger Prx total activity had no effect on patients' life expectancy ($P = 0.094$, log-rank; $P = 0.1008$, Breslow; $P = 0.073$, Tarone-Ware). In Cox regression analysis, no Prx showed any significant independent prognostic value, neither was the age of the patients in association with Prx expression. Figs. 2–4 show survival in relation to Prx expression.

DISCUSSION

During the very last years, some investigations on Prxs in different types of cancer have been carried out, but to our knowledge, this is the first study where the expression of all of the six Prx isoforms has been compared with clinicopathological parameters in breast cancer. Prx I has been demonstrated to be overexpressed in follicular thyroid neoplasms and thyroiditis (29), malignant mesothelioma (11), lung cancer (30), and also in breast cancer (25). In addition, Prx II, III, V, and VI levels were increased in malignant mesothelioma (11), and Prxs II and III were overexpressed in breast cancer in relation to normal tissue (25). These studies are consistent with our findings that all Prxs appeared to be more expressed in carcinomas than in our control breast tissues. Percentages of Prx I–III expression in our study correlated quite well with those of the previous breast cancer study, although our material was considerably larger, containing >400 studied specimens compared with 24 specimens of the previous study (25). We observed that in breast cancer Prxs could be divided clearly to two groups according to their expression; the staining of Prxs II and VI was negative almost in half of cases, whereas isoforms I, III, IV, and V showed stronger expression. Comparison of these results with mesothelioma

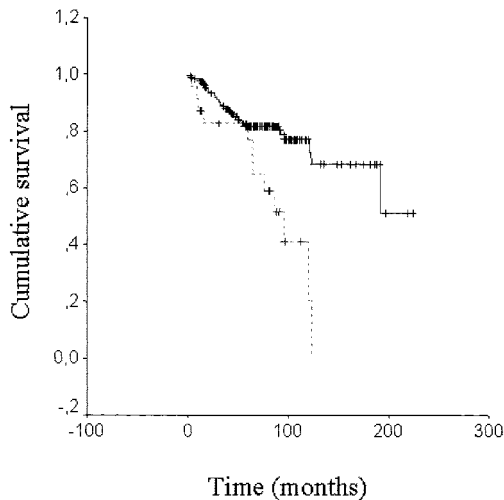


Fig. 2 A Kaplan-Meier curve showing patient survival in relation to positive or negative Prx III immunostaining. Patients with immunopositivity (unbroken line) have the best prognosis ($P = 0.0005$). + indicates the censored cases.

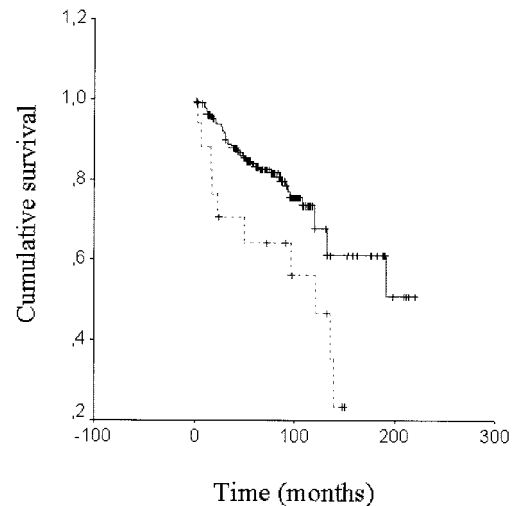


Fig. 3 A Kaplan-Meier curve showing patient survival in relation to positive or negative Prx IV immunostaining. Patients with immunopositivity (unbroken line) have the best prognosis ($P = 0.01$). + indicates the censored cases.

suggests that Prxs may show cancer type-specific difference in the expression of different Prx subtypes.

In previous studies, TNM classification and pathological changes have been attempted to connect with Prx I expression in breast and other tumors (25, 29, 31). In oral squamous cell carcinoma, Yanagawa *et al.* (31) found low Prx I expression levels to be associated with larger tumor masses, lymph node metastases, and poorly differentiated tumors. Nevertheless, we didn't find any correlation with any clinicopathological features and Prx I expression in breast carcinoma, which is also in line with the study of Noh *et al.* (25). Instead, we found that expression of Prxs III, IV, and V was significantly stronger when tumors were poorly differentiated or separately with Prx V when tumors were larger or had lymph node metastases. It has previously been demonstrated *in vitro* that Prx genes I–IV are overexpressed when H_2O_2 concentration in cells is elevated (32), and, on the other hand, peroxisomes and mitochondria have been reported to be the two major intracellular sources of ROS, including H_2O_2 (33). These observations are in line with our results that specifically expressions of Prx III–V, which are subcellularly located near these places, were pronounced in most malignant tumors. Prx III is the only isoform that is located only in mitochondria, Prx IV is present in lysosomes and extracellularly, but it is also found in the proximity of mitochondria, whereas Prx V is compartmentalized to both peroxisomes and mitochondria (9, 11, 12). Moreover Prx III has been localized particularly in the vicinity of mitochondrial degeneration, and it has been hypothesized that this could be because of induction of Prx III by oxidative stress (11). Taken together, this suggests that in malignant breast tumors, production of H_2O_2 increases especially in peroxisomes and mitochondria, and cells respond to impending oxidative stress by increasing Prx levels near these areas.

Noh *et al.* (25) have previously presented that overexpression of Prxs I–III in breast cancer could be explained by the

antiapoptotic and proliferative effects of these proteins. Therefore, another possible explanation for increased Prx levels in larger tumors would be that they are a result of Prxs' antiapoptotic features that provide growth advantage to tumor cells. There are several studies on antiapoptotic effects of Prxs I and II (34, 35), and it is apparent that ROS cause apoptosis (36). Thus, it is probable that also other Prx isoforms are able to inhibit H_2O_2 -mediated physiological apoptosis, cause abnormal proliferation, and thereby may lead to tumorigenesis. However, in our material, only expression of Prx V was associated with tumor size and furthermore, we did not find any relationship between cell proliferation and Prx expression.

Increased expression of Prxs I and II has been previously marginally and Prx V significantly connected to longer patient survival in malignant mesothelioma (11). In line with this, we found that the expression of Prx III and IV were associated with a better prognosis of the patients. Prxs did not, however, have independent prognostic significance. Prx III tended to have increased expression in cells with positive progesterone and estrogen receptor status and Prx IV overexpressed in progesterone receptor-positive cells. As is well known, positive hormone receptor status correlates with longer survival. This might be one reason for the association of these Prxs with a better survival, but it still remains unclear why increased Prx III and IV expressions are associated with the presence of hormone receptors. In addition, Prx VI was also overexpressed in progesterone receptor-positive tumors, but it was not associated with any other parameters, including survival. Connections between the extent of staining of Prx III and Prxs IV and VI can probably be explained by tendency of these isoforms to overexpress in progesterone receptor-positive cases, corresponding association between Prx III and Prx VI has been previously found also in malignant mesothelioma (11).

By contrast, increased expression of Prx V was associated with larger tumor size, positive lymph node status, poor differ-

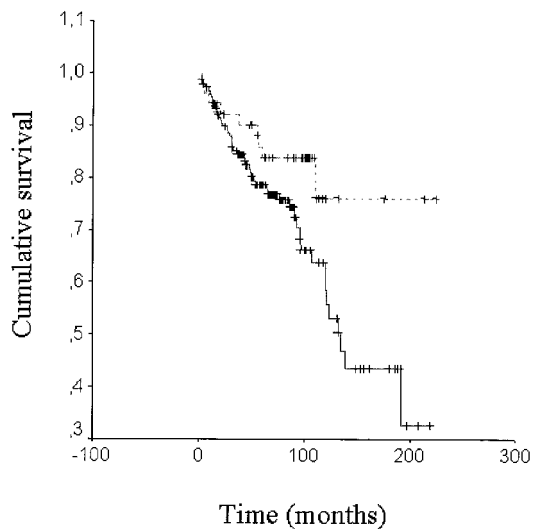


Fig. 4 A Kaplan-Meier curve showing patient survival in relation to positive or negative Prx V immunostaining. Patients with Prx V-immunonegativity (dashed line) have the best prognosis ($P = 0.04$). + indicates the censored cases.

entiation, and a shorter patient survival (mean survival declined from 185 to 140 months) when staining was divided in either positive or negative. Logically, with Prx III and IV, expression of Prx V was independent from hormone receptor status, and this gives support to our hypothesis that longer survival in Prx III and IV overexpressive cases may be secondary to their association with hormone receptors, not necessarily suggesting a direct role between Prx expression and survival. Relationship between hormone receptor status and Prxs I–III has been studied earlier in a small set of breast cancers without finding significant associations (25).

In summary, our study demonstrated expression of all Prx isoforms in breast cancer tissue. It has been previously suggested that high Prx levels in cancer may be because of proliferative and antiapoptotic functions that may participate in tumorigenesis. Nevertheless, we found that especially mitochondrial and peroxisomal Prx isoforms, Prxs III–V, were overexpressed in the most malignant tumors and this, together with our other results showing no correlation between Prx and cell proliferation, suggests the induction of these proteins by oxidative stress. In addition, ubiquitous nature of Prxs, divergent subcellular locations, and overexpression in variety of diseases related to ROS all support high physiological importance and protective function of Prxs, although overwhelmed by elevated H_2O_2 levels of breast cancer. Additional investigations are needed also to reveal mechanisms how increased Prx III and IV levels are connected with positive hormone receptor status and probably thereby longer survival.

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