Increased Peroxiredoxin 6 Expression Predicts Biochemical Recurrence in Prostate Cancer Patients After Radical Prostatectomy

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Abstract. *Background/Aim: Elevated levels of oxidative stress biomarkers have been shown to associate with more aggressive behavior in malignancies. The aim of the present study was to determine the relationship between the expression of peroxiredoxins (Prx) and sulfiredoxin (Srx) in localized prostate cancer (PC) with clinicopathological parameters and outcome after radical prostatectomy (RP). Materials and Methods: Samples of 240 RP patients were analyzed for Prx1, 2, 5 and 6 and Srx expression by immunohistochemistry and the results were correlated with clinicopathological data, biochemical recurrence-free survival (BFS), prostate cancer-specific survival (PCS) and overall survival (OS). Results: Augmented Prx2 and Prx6 expression was associated with several conventional prognostic factors. Increased Prx2 and Prx6 expression predicted for shortened BFS (p=0.027 and p=0.020) and worse OS (p=0.045 and p=0.033). In the multivariate analysis, Prx6 expression was an independent predictor of BFS (p=0.030). Conclusion: Elevated Prx6 expression associates with a worse prognosis after RP for clinically localized PC.*

Currently, a large number of prostate cancer (PC) cases are diagnosed at an early stage. Many patients have low-risk disease that exerts no impact on life expectancy. After radical prostatectomy (RP), the decision between follow-up and adjuvant therapies can be challenging, if the evaluation is limited to clinical and pathological prognosis factors, such as PSA value, pT class and Gleason score. Recently, attempts have been made to more accurately predict the aggressiveness of PC. Research has revealed mechanisms linked with progression and regulation of growth of PC. The up-regulated metabolic pathways may reveal detectable molecules that could serve as biomarkers for cancer risk assessment in the future. However, despite promising findings, biomarkers accurate enough for use in clinical practise are still lacking (1, 2).

Reactive oxygen species (ROS) are continuously generated during aerobic respiration under physiological conditions. In addition, exogenous stressors, such as radiation and environmental agents, can produce elevated levels of ROS consequently leading to carcinogenesis. Peroxiredoxins (Prx) are enzymes that protect the cell against oxidative stress by reducing hydrogen peroxide and alkyl hydroperoxides to the corresponding alcohol or water. The Prx family is divided into six isoforms (Prx1-6) all of which are found in the cytosol with some also being located in specific cell organelles. Prxs need to be oxidized back in order to restore their reducing properties; this occurs in a reaction catalysed usually by thioredoxins. Under conditions of extreme oxidation, thioredoxins cannot perform the reversing reaction and then hyperoxidized Prxs are able to be converted into their active form by sulfiredoxins (Srx) which support protecting function of Prxs against ROS (3, 4).

Increased expression of Prxs has been demonstrated to be linked with aggressive behavior of several cancers, such as hepatocellular cancer, gall bladder carcinoma, renal cell cancer, breast cancer and ovarian cancer (5-9). In the case of PC, augmented Prx1-6 activity has been detected in PC samples in comparison with benign tissue (10-14). There are also studies conducted in cell cultures showing activation of Prx2, Prx3 and Prx4 mediated pathways leading to the

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progression of PC (14-16). In addition, the association between increased Prx3 expression and shortened biochemical recurrence-free survival (BFS) has been demonstrated in the studies carried-out using tissue bank material (13). However, the prognostic value of Prx expression has not been explored in a clinical PC patient population.

There exist a few publications describing Srx expression in tumors. High Srx expression has been found in lung tumors and skin cancer, but there do not seem to be any reports concerning PC (17, 18).

The present study evaluated the prognostic value of the expression levels of Prx 1, 2, 5 and 6 and Srx in PC patients treated with radical prostatectomy (RP). The expression of Prxs and Srx was compared not only with conventional clinicopathological determinants but also with the outcome of patients with a retrospective study design.

Materials and Methods

Patients. A total of 240 PC patients were treated with radical prostatectomy (RP) in Kuopio University Hospital, Finland between 1987 and 2009. All the tumors were localized according to clinical staging procedures including digital rectal examination, transrectal ultrasonography, serum PSA and bone scans when needed. None of the patients received neoadjuvant hormone therapy. The follow-up was conducted 2, 6 and 12 months later and then according to clinical practice. The monitoring data were gathered from laboratory database and patient records. Biochemical recurrence (BCR) was defined as PSA elevation of 0.2 ng/ml or more (19). This research was approved by the Research Ethical Committee of Kuopio University Hospital. All the procedures have been performed in compliance with institutional guidelines of Kuopio University Hospital and University of Eastern Finland.

Table I. *Demographic data of the patients, n=240.*

Characteristics	n (%)
Median age, years (SD)	63.0 (5.5)
Median follow up, years (range)	11.7 (3.3-25.8)
Median PSA at diagnosis, ng/ml (SD)	8.1 (12.3)
PSA* ng/ml	
<10	144 (61.0)
≥10	92 (39.0)
pT category	
2	160 (66.7)
3a	49 (20.4)
3b	31 (12.9)
Gleason score	
2-6	154 (64.2)
7-10	86 (35.8)
Capsule invasion	
No	157 (65.4)
Yes	83 (34.6)
Surgical marginal status	
Negative	141 (58.8)
Positive	99 (41.2)
BCR	
Yes	109 (45.4)
No	131 (54.6)
Mortality	
Alive	189 (78.8)
Dead	51 (21.2)
Cause of death	
Prostate cancer	19 (37.3)
Other	32 (62.7)

SD, Standard deviation; PSA, prostate specific antigen; pT, pathological stage; BCR, biochemical recurrence. *PSA value missing in four cases.

Histopathological analyses. The tissue samples had been fixed in neutral formalin and embedded in paraffin. Two pathologists (YS, VK) re-evaluated all the samples for pT class, Gleason score, surgical marginal status and capsule invasion blinded to clinical data and conducted a consensus assessment in the each case. The TNM-classification was conducted according to UICC guidelines and Gleason score according to the ISUP 2005 modification (20, 21). The samples for the immunohistochemical analyses were obtained from four representative regions of the PC tissues and these were integrated into multitissue microarray blocks with Beecher Instruments Manual Tissue Arrayer (Beecher Instruments, Silver Spring, MD, USA). The microarray sample diameter was 1,300 μm.

Immunohistochemistry. The immunohistochemical procedure was conducted as follows. Four-micron thick sections were cut from the microarray blocks. The sections were then de-paraffinised in xylene and rehydrated in descending ethanol series. For antigen retrieval, the sections were incubated in 10 mM citrate buffer (pH 6.0) in a microwave oven for 2 min at 850 W followed by 8 min at 350 W. Endogenous peroxidase activity was blocked by incubation in 0.1% hydrogen peroxide in absolute methanol for 10 min. Polyclonal rabbit anti-human peroxiredoxin antibodies (LabFrontier, York, UK) were used with a dilution of 1:1500 for Prx1, 1:1000 for Prx2, 1:2000 for Prx5 and Prx6 and 1:500 for Srx. The primary antibodies for Prxs and Srx were revealed using the Histostain-Plus Kit (Zymed Laboratories Inc, South San Francisco, CA, USA).

In the samples, the expression of Prx1, Prx2, Prx5, Prx6 and Srx was partly nuclear but mainly cytoplasmic (Figure 1). The immunoreactivity for the five biomarkers was initially analyzed in tumor cells as follows; Prx1 and Prx2: 0%=negative, 1-5%=weak positive, 6-50%=moderately positive, 51-100%=strong positive; Prx5, Prx6 and Srx: 0-5%=negative, 6-50%=weak positive, 51-100%=strong positive. The mean value of the sum from the four malignant areas was considered as the representative score. The data were then divided into two groups; 0-50%=negative and 51-100%=positive. Two pathologists (YS, VK) performed the evaluation blinded to the clinical data and a consensus assessment was agreed in each case.

Statistical analyses. The statistical analyses were performed with the SPSS 19.0 program package (SPSS Inc., Chicago, IL, USA). The Chi-square test was used to determine the association between clinicopathological prognostic factors and expression of the oxidative stress markers. Biochemical recurrence free survival (BFS), prostate cancer specific survival (PCS) and overall survival (OS) were analyzed by the Kaplan Meier method. The univariate and multivariate analysis was assessed with Cox's method. *p*-Values <0.05 were considered statistically significant.

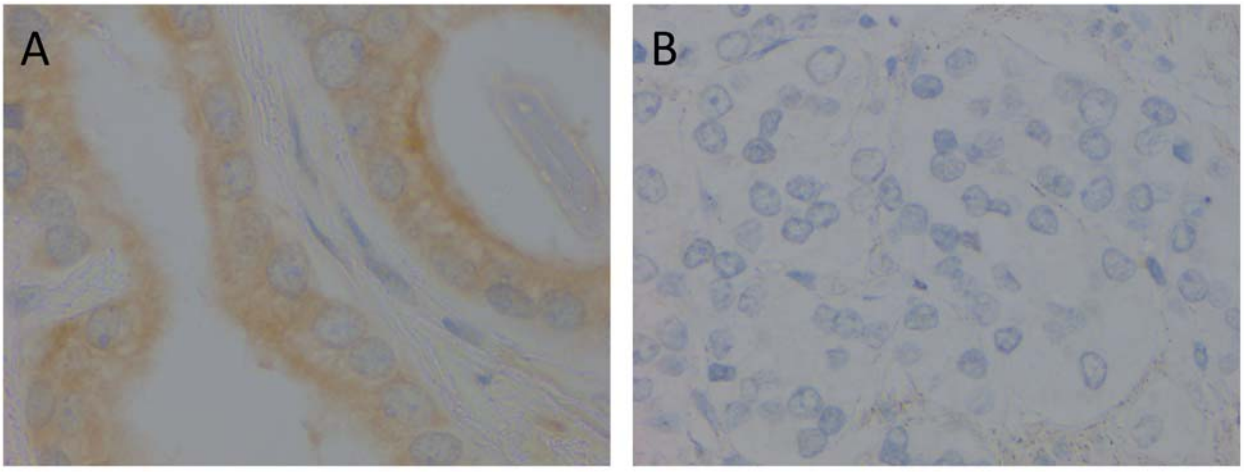

Figure 1. *Immunohistochemical detection of Prx6 expression in prostate adenocarcinoma (PC) tissues. A: PC sample displaying cytoplasmic Prx6 positivity (original magnification, x630). B: Negative expression of Prx6 in PC tissue (original magnification, x400).*

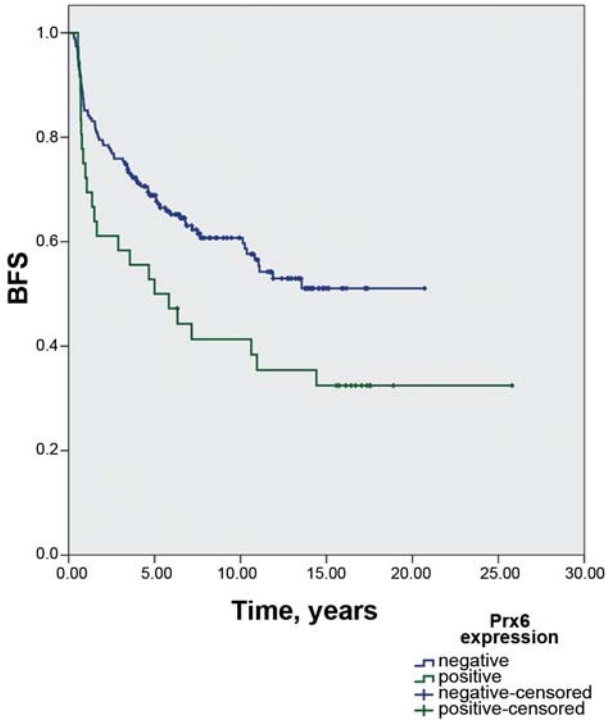

Figure 2. *Kaplan-Meier curve demonstrating the association between Prx6 expression and biochemical recurrence free survival (BFS) (Log-rank, p=0.020).*

Results

Clinical and histopathological data. Patients' demographic characteristics are summarized in Table I. All tumors of the 240 patients belonged to pT categories 2, 3a and 3b according to histopathological analyses. The median observation period was 11.7 (3.3-25.8) years. A total of 109 (45.4 %) of the patients experienced the BCR during follow-up time. Fifty-one (21.3 %) died with PC being the cause of death in 19 (37.3 %) cases and other causes in 32 (62.7 %) cases.

Several clinicopathological prognosis factors were related with positive Prx expression as follows: Positive Prx1 expression associated with capsule invasion (p=0.001); Prx2 with pT class (p=0.037), positive surgical margin (p=0.003), capsule invasion (p<0.001), BCR (p=0.043), PCS (p=0.036) and OS (p<0.001); Prx5 with OS (p=0.001); Prx6 with pT class (p=0.006), capsule invasion (p=0.009), BCR (p=0.004), PCS (p<0.001) and OS (p<0.001). Furthermore, positive Srx expression displayed an inverse association with a high PSA level at diagnosis (p=0.001) and OS (p=0.026) (Table II).

Survival analysis. In the Kaplan Meier analysis, the shortened BFS was associated with positive Prx2 (p=0.027) and Prx6 (p= 0.020) expression (Figure 2). Positive surgical marginal status (p<0.001), high Gleason score (p<0.001) and pT class (p<0.001) also revealed an association with shortened BFS. Positive expression of Prx6 (p=0.037) was related to PCS, as well as with important clinicopathological factors capsule invasion (p=0.008), high Gleason score (p<0.001) and pT class (p<0.001). Positive Prx2 (p=0.045) and Prx6 expression (p=0.033) predicted worse OS. The association with worse OS was also demonstrated with high Gleason score (p<0.001), pT class (p<0.001) and higher PSA levels at diagnosis (p=0.037).

In the multivariate analysis, positive Prx6 expression (p=0.030), pT class (p=0.020), positive surgical marginal status (p=0.025) and Gleason score (p<0.001) were independent predictors of BFS, when factors (Prx2 and Prx6

Table II. *Association between clinical and pathological prognosis factors and the expression of peroxiredoxins and sulfiredoxin in prostate cancer samples.*

	Prx1 (n=233)			Prx2 (n=233)			Prx5 (n=232)			Prx6 (n=231)			Srx (n=229)		
Variables	-	+	*p*-Value	-	+	*p*-Value	-	+	*p*-Value	-	+	*p*-Value	-	+	*p*-Value
pT															
pT2	52	104	ns.	78	78	0.037	124	31	ns.	134	21	0.006	50	104	ns.
pT3a	13	35		22	26		37	11		43	5		17	29	
pT3b	6	23		7	22		18	11		18	10		16	13	
PSA at[†] diagnosis															
<10	47	93	ns.	68	72	ns.	114	26	ns.	125	15	ns.	39	99	0.001[#]
≥10	24	65		39	50		63	25		70	17		43	44	
Gleason score															
2-6	47	101	ns.	69	79	ns.	115	32	ns.	120	27	ns.	53	92	ns.
7-10	24	61		38	47		64	21		75	9		30	54	
Surgical margin															
Negative	49	90	ns.	75	64	0.003	111	27	ns.	120	17	ns.	47	91	ns.
Positive	22	72		32	62		68	26		75	19		36	55	
Capsule invasion															
No	58	96	0.001	85	69	<0.001	123	30	ns.	136	17	0.009	52	101	ns.
Yes	13	66		22	57		56	23		59	19		31	45	
BCR															
No	42	85	ns.	66	61	0.043	102	25	ns.	115	12	0.004	40	86	ns.
Yes	29	77		41	65		77	28		80	24		43	60	
PCS															
Alive	70	145	na.	103	112	0.036	168	46	ns.	187	27	<0.001	76	136	ns.
Dead	1	17		4	14		11	7		8	9		7	10	
OS															
Alive	60	124	ns.	96	88	<0.001	150	33	0.001	168	15	<0.001	59	122	0.026[#]
Dead	11	38		11	38		29	20		27	21		24	24	

Prx, Peroxiredoxin; Srx, sulfiredoxin; -, negative; +, positive, pT, pathological stage; PSA, prostate specific antigen; na, non-applicable; ns, non-significant; BCR, biochemical recurrence; PCS, prostate cancer specific survival; OS, overall survival. [†]PSA value missing in four cases, [#]Inverse association.

expression, surgical marginal status, Gleason score and pT class) were included into the analysis (Table III). None of the analyzed biomarkers had any independent predictive value for PCS or OS according to multivariate analysis.

Discussion

Nowadays, most men suffering from PC have a slow-growing tumor with excellent progression-free prognosis and only a small proportion of PC patients are at a risk of suffering metastatic and life-threatening disease (22). In addition to clinicopathological parameters, nomograms have been developed in attempts to estimate the aggressiveness of PC at the diagnosis or after provision of curative treatment. The accuracy of these conventional tools as predicting BCR is still as low as 70-80% (23). Modern research of PC has revealed several biomolecules which have been claimed to be potential indicators of tumor growth and invasion process (24). However, there are still no biomarkers suitable and sufficiently reliable for clinical use (25).

A recent study demonstrated that increased expression of Prxs was linked with conventional prognosticators of PC with the clearest association was found in the case Prx2 and Prx6. In agreement with our findings, Basu *et al.* reported that Prx2 and Prx6 expression associated with worse clinicopathological prognostic factors in tissue bank material (13). In addition, the association with Prx2 expression and progression of PC has been revealed earlier also in castration-resistant PC cell lines (15). Furthermore, a link between PC progression and the activity of Prx6 has been detected in the animal models of PC (26).

The results reported herein demonstrate for the first time that elevated Prx6 expression can independently predict increased risk for BCR in clinical PC patients with a localized tumor. In previous research, augmented Prx6 expression has also been found to be associated with worse survival of renal cell cancer and breast cancer patients (7, 27). In this context, it is important to note that Prx6 exhibited prognostic value in our material of PC patients with long life expectancy

Table III. *Cox proportional hazards regression models in estimating biochemical recurrence-free survival.*

Variable	Hazard ratio	95 % Confidence interval	*p*-Value
Univariate analysis			
pT (pT2, pT3a, pT3b)	1.854	1.455-2.364	<0.001
PSA at diagnosis (<10 *vs.* ≥10)	1.394	0.951-2.045	ns.
Gleason score (2-6 *vs.* 7-10)	2.168	1.484-3.165	<0.001
Surgical margin (negative *vs.* positive)	2.215	1.517-3.236	<0.001
Capsule invasion (no *vs.* yes)	1.430	0.971-2.106	ns.
Prx1 expression (negative *vs.* positive)	1.202	0.784-1.842	ns.
Prx2 expression (negative *vs.* positive)	1.549	1.047-2.292	0.028
Prx5 expression (negative *vs.* positive)	1.277	0.827-1.973	ns.
Prx6 expression (negative *vs.* positive)	1.712	1.082-2.709	0.022
Srx expression (negative *vs.* positive)	0.778	0.526-1.152	ns.
Multivariate analysis			
pT (pT2, pT3a, pT3b)	1.402	1.054-1.864	0.020
Gleason score (2-6 *vs.* 7-10)	2.071	1.380-3.107	<0.001
Surgical margin (negative *vs.* positive)	1.640	1.064-2.530	0.025
Prx2 expression (negative *vs.* positive)	1.029	0.659-1.606	ns.
Prx6 expression (negative *vs.* positive)	1.756	1.055-2.922	0.030

pT, Pathological stage; PSA, prostate specific antigen; ns, non-significant; Prx, peroxiredoxin; Srx, sulfiredoxin.

compared to those with more aggressive malignancies, since the elevation in the PSA levels is the first sign of clinical progression in PC (28). Although Prx6 appears to be promising indicator of PC progression according to our results, a prospective study design will be needed to explore the value of Prx6 in cancer risk evaluation in clinical practice.

We also found Prx2 and Prx6 expression to predict worse OS, but these markers failed to remain as independent predictors in the multivariate analysis. This finding is in line with the observations that Prx enzymes play crucial roles in combatting oxidative stress to promote cell survival (29). On the other hand, progression of the organ-confined PC is slow and it might be challenging to confirm the independent prognostic value of biomarkers in a survival analysis.

Based on the finding that Srx reduces hyperoxidized Prx enzymes into their active state, one could postulate that Srx expression would be high in conjunction with increased expression of these enzymes. Interestingly, we observed an inverse association between Srx expression and clinical factors such as high PSA at diagnosis and OS. The relevance of this finding remains unclear based on the present clinically orientated study.

In summary, we revealed that the expression of Prx enzymes associated with the traditional clinicopathological prognosticators and augmented Prx6 expression could predict BFS in PC patients treated with radical prostatectomy. In the future, Prx6 might serve as a candidate biomarker for cancer risk assessment in PC patients with localized disease.

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