Peroxiredoxin 6 Serum Levels and Risk of Neutropenic Infections in Diffuse Large B-cell Lymphoma

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Abstract. *Background/Aim:* Diffuse large B-cell lymphoma (DLBCL) is an aggressive malignancy where antioxidant enzyme peroxiredoxin 6 (Prx6) has previously been associated with adverse outcomes. Its systemic effects in DLBCL are unknown. *Materials and Methods:* This study included 53 patients with DLBCL, five patients with primary central nervous system lymphoma (PCNSL) and 20 healthy controls. The expression of Prx6 was evaluated immunohistochemically in DLBCL tissue samples and compared to its expression in blood serum. *Results:* Prx6 expression was the highest in healthy controls, followed by DLBCL patients and PCNSL patients. Febrile neutropenic infection after the first treatment course was associated with low pre-treatment Prx6 serum levels (<14 ng/ml) ($p=0.025$, $OR=8.615$, 95% confidence interval=1.032-71.933). Serum levels of Prx6 recovered after treatment ($p=0.006$). *Conclusion:* Patients with low Prx6 levels might be more prone to treatment-related adverse effects through elevated levels of oxidative stress.

Diffuse large B-cell lymphoma (DLBCL) is the most common form of non-Hodgkin lymphoma, accounting for 30-40% of all cases. DLBCL is a group of different diseases that displays considerable variability regarding clinical

presentation and prognosis (1). Oxidative stress markers such as 8-hydroxydeoxyguanosine (8-OHdG) and antioxidant enzymes, thioredoxin and glutamate-cysteine ligase seem to have a prognostic relevance in certain types of DLBCL where they are strongly expressed compared to more indolent types of lymphoma (2, 3). Peroxiredoxins (Prxs) are a group of six antioxidant enzymes that reduce hydrogen peroxide. In a recent retrospective immunohistochemical study, strong cytoplasmic intensity of peroxiredoxin 6 (Prx6) in DLBCL was shown to be associated with adverse outcomes in patients, while other Prxs (Prxs 1, 2, 3 and 5) were not associated with disease presentation or prognosis (4).

In the present study, we immunohistochemically assessed 8-OHdG and Prx6 expression in tumour tissue samples and compared the results with their expression in patients' serum to explore their systemic effects. In addition, healthy controls and patients with primary central nervous system lymphoma (PCNSL) were included, in order to evaluate differences between CNS lymphoma and systemic disease.

Patients and Methods

Patients. A total of 58 patients who had diagnostic blood serum samples available were retrospectively selected for the study. Study subjects comprised 53 patients with DLBCL, five patients with PCNSL and 20 healthy controls. Blood levels of Prx6 and 8-OHdG were measured using ELISA in all participants. Patient serum samples were obtained at the time of diagnosis and again at a median of 9 months, with a range of 1-38 months, after the end of treatment ($n=24$). Routine diagnostic biopsy samples were immunohisto-chemically stained for Prx6 and 8-OHdG. Five patients with DLBCL were lacking a representative biopsy sample.

Patients were treated at the Oulu University Hospital in 2003-2017. Clinical data were collected from hospital records. Primary treatment regimens of patients with systemic disease consisted of R-CHOP

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(rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone), or R-CHOP-like-regimens R-CHOEP (R-CHOP with etoposide) or R-CEOP (rituximab, cyclophosphamide, epirubicin, vincristine, and prednisolone). PCNSL patients were treated with the Bonn regimen or blood brain barrier-based therapy (5). This study was approved by the Ethics committee of Northern Ostrobothnia Hospital District (11/2014) and was conducted in accordance with The World Medical Association Declaration of Helsinki ethical principles.

Immunohistochemistry and ELISA. Representative tumour areas were fixed in formalin and embedded in paraffin. Three μm sections were cut from the paraffin blocks and placed on SuperFrostPlus glass slides (Menzel-Gläser, Braunschweig, Germany). The sections were deparaffinised in a clearing agent, Histo-Clear (National Diagnostics, Atlanta, GA, USA), and rehydrated in descending ethanol series. Antigen retrieval for each series was performed in a microwave oven using a 10 mM sodium citrate buffer, pH 6. The sections were cooled to room temperature for 20 min and then incubated in a 3% H_2O_2 solution for five min to block the endogenous peroxidase activity. The staining was continued using the Dako REAL™ EnVision™ Detection System (Dako Denmark A/S, Glostrup, DK) in accordance with the manufacturer's instructions. Primary antibody incubations were carried out in a humidity chamber at room temperature for Prxd6 (LF-MA0018, AbFrontier, Seoul, Republic of Korea) for 30 min using a 1:6000 dilution, and for 8-OHdG (MOG-100P, JaiCA, Nikken SEIL Co., Ltd., Shizuoka, Japan) for 1 h using a 1:100 dilution. Diaminobenzidine (DAB) was used to detect the immunoreaction and the immunostaining of nuclei was performed with Mayer's haematoxylin (Reagen, Toivola, Finland). Finally, the sections were dehydrated and mounted with Histomount (National Diagnostics, Atlanta, GA, USA). All the washes between different steps were performed with phosphate buffered saline (PBS) including 0.05% Tween-20.

The ELISA kits used were LF-EK0206 (AbFrontier, Seoul, Republic of Korea) for Prx6 and the Highly Sensitive 8-OHdG Check kit, KOG-HS10E (JaiCA, Shizuoka, Japan) for 8-OHdG. ELISAs were performed in accordance with the manufacturer's instructions.

The analysis of the immunohistochemical staining results was performed by three experienced haematopathologists (J Böhm, KM Haapasaari and HR Teppo) blinded to the clinical data. The staining intensity in cancer cells was recorded separately in the nuclei, cytoplasm and cell membranes. The intensity was categorised as low, moderate or strong.

Statistical analysis. IBM SPSS Statistics V23 for Windows (IBM, Armonk, NY, USA) was used for the statistical analysis. Tests included the Student's *t*-test, Fisher's exact test, Pearson Chi-Square test, Mann-Whitney *U*-test, Kruskal-Wallis test, Spearman correlation and the Wilcoxon Signed Rank Test. Overall survival (OS) was calculated from the date of diagnosis to the date of death or last follow-up date. Disease-specific survival (DSS) was calculated from the date of diagnosis to the date of lymphoma-specific death or last follow-up and relapse-free survival (RFS) was calculated from the time of diagnosis to the time of relapse. *p*-Values of <0.05 were considered statistically significant.

Results

Patient demographics. Patient demographics are presented in Table I. The mean age of the patients was 58.2 years with

Table I. Patient demographics at the time of diagnosis.

	n	%
Gender		
Male	32	55
Female	26	45
DLBCL subtype		
NOS	46	79
T-cell rich	6	10
EBV DLBCL NOS	1	2
PCNSL	5	9
B symptoms		
None	26	45
Yes	26	45
Data missing	6	10
Age		
≤60	29	50
>60	29	50
Serum lactate dehydrogenase		
Normal	23	40
Elevated	33	57
Data missing	2	3
Stage		
I-II	30	52
II-IV	27	46
Data missing	1	2
WHO performance status		
0-1	38	65
>1	19	33
Data missing	1	2
Extranodal affusion		
0-1	39	67
>1	13	23
Data missing	6	10
IPI		
0-2	30	52
3-5	22	38
Data missing	6	10
Leukocyte count		
Low	1	2
Normal	33	57
High	23	39
Data missing	1	2
Neutrophil count		
Low	2	4
Normal	36	62
High	17	29
Data missing	3	5
Lymphocyte count		
Low	19	33
Normal	29	50
Data missing	10	17
C-reactive protein		
Normal	31	43
High	26	45
Data missing	1	2
Erythrocyte sedimentation rate		
Normal	31	53
High	23	40
Data missing	4	7
Neutropenic infection after the first treatment		
No	40	69
Yes	17	29
Data missing	1	2

Figure 1. Representative staining from four different DLBCL patients. Clockwise from top left: weak cytoplasmic Prx6 expression, strong cytoplasmic Prx6 expression, strong nuclear 8-OHdG expression and weak nuclear 8-OHdG expression.

a range of 35-89 years, and the mean age of controls was 43.1 years with a range of 26-59 years ($p < 0.001$, Student's *t*-test). Controls had equal sex representation. The median patient follow-up time was 41.5 months. There were 16 deaths, of which ten were disease-specific. Following primary treatment, 53 patients (91.4%) achieved complete remission and five patients (8.6%) had progressive disease. A total of 12 patients (20.7%) experienced a relapse. Overall, 28 patients presented with germinal centre phenotype (GC) and 14 with non-GC phenotype; this information was missing from four patients (6). There were also six T-cell rich BCL patients and one EBV+ DLBCL NOS patient. All PCNSL patients represented stage IV disease while the DLBCL patients had roughly equal presentation of all stages ($p = 0.014$, Fisher's exact test). There were 17 (29.3%) neutropenic infections after the first treatment cycle.

Median blood serum albumin in the diagnostic samples was 45 g/l among controls and 42 g/l among lymphoma patients ($p = 0.038$, Mann-Whitney *U*-test). Low albumin levels were associated with B-symptoms ($p = 0.007$), age > 60 years ($p = 0.011$), poor WHO performance status ($p = 0.002$), IPI index > 2 ($p = 0.019$), progressive disease ($p = 0.020$), anaemia ($p = 0.040$), and low neutrophil count ($p = 0.042$).

Immunohistochemical analysis. Cytoplasmic Prx6 expression was observed in all DLBCL tissue samples. The median expression in malignant cells was 80%. There was no nuclear expression. 8-OHdG was detected in most nuclei with 80% median expression. Low to moderate cytoplasmic expression was seen in approximately half of the samples (Figure 1). Prx6 expression was found to be moderate or strong in all tissue samples of PCNSL. Cytoplasmic

Figure 2. Median serum levels of Prx6 (a) and 8-OHdG (b) among DLBCL patients, PCNSL patients and controls. *p*-Values represent Kruskal–Wallis test.

expression of 8-OHdG was found in 80% of PCNSL samples compared to 57.4% of DLBCL samples ($p=0.399$, Pearson Chi-Square test).

Serum samples. Distribution of Prx6 and 8-OHdG in blood serum samples was skewed. Pre-treatment median serum levels of Prx6 and 8-OHdG differed between the groups (Figure 2). Median serum levels of Prx6 were 10.69 ng/ml in DLBCL patients, 8.10 ng/ml in PCNSL patients and 20.94 ng/ml in controls ($p<0.001$, Kruskal–Wallis test). Median serum levels of 8-OHdG were 3.35 ng/ml in DLBCL patients, 2.93 ng/ml in PCNSL patients and 2.95 ng/ml in controls ($p=0.048$, Kruskal–Wallis test). When a cut-off value of 60 years was used for the whole population (controls were all younger), age showed a statistically significant association ($p=0.038$, Mann–Whitney *U*-test) with Prx6 levels.

Based on a ROC-curve, 14 ng/ml of Prx6 and 6 ng/ml of 8-OHdG were used as cut-off values in the analysis of all clinical variables. These were the best values in discerning controls from patients. There was a significant connection between the presence of a febrile neutropenic infection after the first treatment course and low pre-treatment Prx6 serum levels (<14 ng/ml) ($p=0.025$, Fisher’s exact test) (Figure 3). 38.1% of patients with Prx6 serum levels under 14 ng/ml had a neutropenic fever compared to only 6.7% of patients with higher values [OR=8.615 (1.032-71.933)]. High 8-OHdG serum levels were seen in 22.6% of the DLBCL patients and

in none of the PCNSL patients ($p=0.573$, Fisher’s exact test). High Prx6 and 8-OHdG serum levels were associated with each other ($p=0.025$, Fisher’s exact test). At a median of nine months after treatment, Prx6 levels among patients were 19.55 ng/ml ($n=24$) compared to an initial 10.69 ng/ml ($n=58$) ($p=0.006$, Wilcoxon Signed Rank Test). There were no significant associations with survival or differences in chemotherapy received between groups.

Correlations of serum samples and immunohistochemistry. There was a statistically significant negative association between strong nuclear 8-OHdG expression and the presence of 8-OHdG in the cytoplasm ($p=0.036$, Pearson Chi-Square test) and between strong nuclear 8-OHdG expression and low 8-OHdG serum levels ($p=0.015$, Mann–Whitney *U*-test). Strong cytoplasmic Prx6 expression was also non-significantly associated with lower 8-OHdG serum levels ($p=0.060$, Mann–Whitney *U*-test). There was no significant correlation between the other study parameters (Table II).

Correlations between immunohistochemical expression and clinical data. Strong cytoplasmic Prx6 expression was associated with extranodal DLBCL involvement of more than one site ($p=0.039$, Fisher’s exact test) and a high IPI score (3-5) ($p=0.035$, Fisher’s exact test). There were no other correlations between the results of the immunohistochemical analysis and disease presentation.

Table II. Correlations of serum Prx6 and 8-OHdG levels and their immunohistochemical expression. + stands for positive association and – negative association.

	Prx6 cytoplasm (strong)	8-OHdG serum levels (ng/ml)	8-OHdG present in cytoplasm	8-OHdG nucleus (strong)
Prx6 serum levels (ng/ml)	+	+	+	–
	$p=0.680^*$	$p=0.532^{**}$	$p=0.195^*$	$p=0.774^*$
Prx6 cytoplasm (strong)		–	+	+
		$p=0.060^*$	$p=0.522^{***}$	$p=0.110^{***}$
8-OHdG serum levels (ng/ml)			–	–
			$p=0.461^*$	$p=0.015^*$
8-OHdG present in cytoplasm				–
				$p=0.036^{***}$

*Mann–Whitney *U*-test; **Spearman correlation; ***Pearson Chi-Square test.

Figure 3. The number of patients with or without first course neutropenic infection according to Prx6 serum levels. *p*-Value was calculated using Fisher's exact test.

Discussion

In this study, it is shown for the first time that patients presenting with DLBCL and PCNSL have low levels of circulating immunoreactive Prx6 when compared to healthy controls. Moreover, low serum levels (Prx6 <14 ng/ml) were related to neutropenic infections following the first treatment. Strong cytoplasmic Prx6 expression was also associated with the presence of IPI factors, while in a previous study there was no such association (4).

Prxs are a ubiquitously expressed conserved family of antioxidant peroxidases that also appear to have various other functions (7, 8). Prx6 differs from the rest of the Prx-group by using glutathione as a reducing agent. It also has a phospholipase A2 (PLA2) activity relevant to lung surfactant metabolism (9). Prx6 is overexpressed in many types of neoplasms (10-15) and cancer cells have been shown to benefit separately from peroxidase and phospholipase activities. As cancer cells often suffer from hypoxia and oxidative stress, extensive antioxidant machinery and overexpression of Prx6 can be a feature promoting growth and survival and invasiveness (9).

Prx6 has a major role in phospholipid turnover. It can reduce oxidative insults in phospholipids, thus, restoring the integrity of plasma and organelle membranes and avoiding cell death. It is located in the cytosol, where it readily binds to peroxidised phospholipids, but it also functions in acidic lysosomes where Prx6 can bind and hydrolyse native phospholipids (9). The low levels of serum Prx6 and high tissue levels in our patient cohort could therefore be explained by the high proliferation rate of lymphoma cells that critically need phospholipid turnover (16).

Prx6 seems to be strongly expressed in DLBCL and PCNSL. Strong Prx6 expression in the cytoplasm appears to be related with higher levels of 8-OHdG in the nucleus, which is in agreement with a role in the elimination of reactive oxygen species and prevention of DNA damage. 8-OHdG itself did not correlate with any clinical disease parameters but it has previously been associated with extranodal involvement and high IPI score in DLBCL (2). We have previously shown that Prx6 is associated with worse outcomes in DLBCL (4). We could not demonstrate the prognostic impact of lymphoma cell Prx6 expression, which is probably due to the small sample size analysed in this study. Prx6 expression was, however, associated with adverse clinical features.

Elevated Prx6 serum levels have been reported in patients with breast cancer (17), lung cancer (18) and ovarian

carcinoma (19). Autoantibodies against Prx6 have been found in patients with lung cancer (18), gastric cancer (20) and oesophageal squamous cell carcinoma (21). In contrast to other types of malignancies, low serum levels of Prx6 in DLBCL and PCNSL patients compared to controls was found. Lower serum levels of Prx6 may reflect different disease biology and redox balance in lymphoma compared to other cancer types. The source of serum Prx6 has not been clearly defined. Expression of Prx6 in various tissues has been shown to rise in marine animal models following bacterial stimuli (22, 23). The same effect has been seen in mouse bone marrow-derived macrophages following stimulation with interferon gamma and lipopolysaccharide (24). One study demonstrated translocation of Prx6 to the plasma membrane during neutrophil activation and that this is required for optimal function of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (25). Other Prxs have also been found in body liquids and shown to modulate inflammation by interacting with toll like receptors and cyclophilin A (7).

Under oxidative stress, Prx6 expression is induced by nuclear factor erythroid 2 related factor 2 (Nrf2) that binds to an antioxidant response element upstream from the *PRDX6* gene (9). Nrf2 is known to be one of the principal regulators of the whole antioxidant system and to have anti-inflammatory and cytoprotective effects. Recent studies have shown Nrf2 to be an important regulator of innate immunity (26). Therefore, Prx6 may have an immunological function in addition to its other properties.

Several chemotherapeutics cause DNA damage by inducing the production of reactive oxygen species. Together with Prx6's immunological effects, we hypothesized that serum antioxidant capacity might interfere with the host's vulnerability to therapy-related side effects. Because of the retrospective nature of the study, the only side effect that was reliably compiled from hospital records was the incidence of therapy-related infections. In line with this, a strong association between the incidence of neutropenic infections after the first cycle and low serum Prx6 level was found. Currently there are no good biological predictors for infection risk and serum Prx6 shows some potential in lymphoma patients, but this needs to be verified in a prospective clinical setting. This finding also implies that improving hosts' antioxidant capacity might protect the patient from chemotherapy-induced adverse effects. Low albumin levels have been associated with multiple adverse clinical features and it is believed to reflect blood thiol pool and antioxidant capacities (27). In the current study however, albumin was not associated with neutropenic infections and did not correlate with serum levels of Prx6 or 8-OHdG. Thus, blood serum albumin and Prx6 appear to have different functions and Prx6 might be more sensitive to predict therapy-related side effects.

Weaknesses of this study include its retrospective nature, the small sample size in all groups and the age disparity between

patients and controls. Since there are no Prx6 serum reference values, age seems to be a significant factor affecting serum concentrations, as has previously been seen. This work supports the notion of Prx6 being associated with adverse clinical features in DLBCL and possibly in PCNSL. Interestingly, the pre-treatment serum levels of Prx6 could be potentially used to evaluate the treatment-related risk of neutropenic infection. This finding should be confirmed in a larger prospective study.

Conflicts of Interest

The Authors state no conflicts of interest regarding this study.

Authors' Contributions

Kari and Kuusisto designed and wrote the study and analysed data, participated in clinical data and biopsy sample collection. Böhm analysed immunohistochemistry. Haapasaari participated in biopsy sample collection and analysed immunohistochemistry. Teppo assisted with statistical analysis and analysed immunohistochemistry. Karihtala designed the study and assisted with statistical analysis. Hakalahti designed the study. Turpeenniemi-Hujanen analysed data and designed the study. Kuittinen designed and wrote the study and analysed data. All authors reviewed and edited the manuscript.

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