Down-regulation of 8-Hydroxydeoxyguanosine and Peroxiredoxin II in the Pathogenesis of Endometriosis-associated Ovarian Cancer

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Abstract. *Aim: To evaluate the roles of oxidative stress marker 8-hydroxydeoxyguanosine (8-OHdG) and peroxiredoxin (PRX) antioxidants in the development of endometriosis and endometriosis-associated ovarian cancer (EAC). Materials and Methods: Tissue expressions of 8-OHdG, PRX II and PRX IV were determined immunohistochemically in tissue from 22 women with benign endometriosis (BE) and 33 women with EAC, among whom endometriosis and cancer tissues were analyzed separately. Results: When all three groups were compared simultaneously, EAC tumor cells had significantly weaker nuclear 8-OHdG and PRX II expression ($p<0.05$ and $p<0.01$, respectively) and significantly weaker cytoplasmic 8-OHdG expression ($p<0.01$) than EAC endometriosis and BE epithelial cells. This same trend was also observed when groups were compared pair-wise. Conclusion: Nuclear PRX II and 8-OHdG were down-regulated in EAC tumorous tissue compared with BE and EAC endometriotic tissue, suggesting a role of oxidative stress in the pathogenesis of EAC.*

Endometriosis is a disease where endometrial tissue grows outside the uterus, causing pain and infertility. Endometriosis is reported to occur in 10% of women of a reproductive age and in 30-50% of infertile women (1, 2). There have been studies concerning the role of oxidative stress on the development of endometriosis (3, 4). Good evidence exists that oxidative stress is increased in patients with endometriosis compared with healthy women (5-9) and that antioxidant defense is also induced in these women (3, 10-12).

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Reactive oxygen species (ROS), such as the hydroxyl radical (•OH), hydrogen peroxide (H_2O_2) and the superoxide radical (O2•–) are mainly products of normal cellular metabolism, but they can also come from exogenous sources. They have prominent physiological functions, but as a result of their reactive nature, ROS, especially •OH, can react with proteins, lipids and especially with DNA, forming a potential source of pro-carcinogenic mutations in the genome (13). ROS are very difficult to assay directly because of their short lives. When •OH interacts with either nuclear or mitochondrial DNA, 8-hydroxydeoxyguanosine (8-OHdG) is formed. 8-OHdG is the most widely used biomarker of oxidative DNA damage, in addition, it has been proven to be pro-mutagenic and it can also be considered as a biomarker of carcinogenesis (14, 15). Cells have a number of defense mechanisms protecting them from the toxic effects of ROS, the most important being antioxidant enzymes, such as peroxiredoxins (PRXs) (16). The PRX family consists of six (I–VI) different proteins, the main function of which is to reduce alkyl hydroperoxides and H_2O_2 (17). They are widespread throughout the intracellular space and are highly important members of cell redox state-regulating enzymes (18). PRX II and VI are profoundly induced during ovarian carcinogenesis and lack of either PRX II or VI leads to increased oxidative stress and detrimental effects in knock-out mouse models (19-21). Therefore, we focused on examine the role of these PRX isoforms in this study.

The results of recent studies have suggested that endometriosis is a neoplastic disease and therefore it can be assumed to have premalignant potential (22-24). In particular, endometrioid and clear cell-carcinomas have been shown to have the ability to develop from endometriosis (25). The mechanisms behind the progression of benign endometriosis to carcinoma remain largely unknown (22). Recent data suggest that the underlying etiology could be centered on oxidative stress, inflammation or hormonal changes in cells in endometriosis (22, 26, 27), but some genetic mutations have also been identified (28).

In this study, we immunohistochemically determined the expression of 8-OHdG, PRX II and PRX VI in cancerous and endometriotic tissue from patients with endometriosis-associated ovarian cancer (EAC) and in endometriotic tissue from patients with benign conditions. We compared the expression of these markers with each other to investigate the role of oxidative stress in the development of endometriosis and EAC.

Materials and Methods

The study population comprised 22 patients with benign endometriosis (BE) and 33 women with EAC. Of these, 17 patients with cancer had endometrioid and 16 had clear cell histology. Tissue samples from patients operated upon between 1999 and 2009 were acquired from the archives of the Department of Pathology, Oulu University Hospital. When possible, paraffin-embedded samples from cancer patients were used that contained both malignant and endometriotic tissue. Otherwise, two separate samples were chosen to represent the two types of tissue. Both cancerous and endometriotic tissue were obtained in all but one case. Because of the exhaustion of representative paraffin blocks, PRX II and PRX VI were stained in only 13 cases of BE. Clinical data were collected from the records of Oulu University Hospital. Histological diagnoses of the tumors were determined according to the criteria of the 2003 WHO classification of ovarian cancer (29).

Novocastra Novolink Polymer Detection Systems Kit (Leica Microsystems, Wezlar, Germany) was used for all cases. The tissue specimens had been fixed in 10% phosphate-buffered neutral formalin and embedded in paraffin. For immunohistochemistry with anti-8-OHdG, anti-PRX II and anti-PRX VI, 4-μm-thick sections were cut, deparaffinized in xylene, rehydrated in a series of graded ethanol solutions and washed in 0.01 M phosphate-buffered saline (PBS). To predigest the sections, they were placed in a microwave oven and boiled in 10 mM citric acid monohydrate for 10 minutes and then cooled at room temperature. Subsequently, endogenous peroxide was removed by placing the slides in 3% hydrogen peroxide in methanol for 15 minutes. The sections were then incubated with primary antibodies overnight. The primary antibodies used were mouse monoclonal 8-oxodG antibody 1:100 dilution (Japan Institute for the Control of Aging, Fukuroi, Japan), rabbit polyclonal PRX II antibody 1:50 dilution (Ab Frontier, Seoul, Korea) and rabbit polyclonal PRX VI antibody 1:2000 dilution (Ab Frontier, Seoul, Korea) for 8-OHdG, PRX II and PRX VI, respectively. The sections were then incubated with ready-to-use Post Primary Block (Leica Microsystems, Wezlar, Germany) for 30 minutes. Aminoethyl carbazole (Zymed Laboratories Inc., South San Francisco, CA, USA) was used as a chromogen in all cases. Finally, the samples were counterstained in Meyer's hematoxylin and then mounted with Immu-Mount (Shandon, Pittsburgh, PA, USA). Representative images of positive immunostainings of the markers used in our study in all patient groups are presented in Figure 1.

The intensity of staining and the proportion of cells positive for 8-OHdG, PRX II and PRX VI were evaluated separately in nuclei of cancer cells, cytoplasm of cancer cells, nuclei of epithelial cells of endometriosis, cytoplasm of epithelial cells of endometriosis and nuclei of stromal cells of endometriosis. The specimens were evaluated by two readers, with a multihead microscope, which were blinded to the clinical data. Staining reactions were divided into three categories: –, negative immunostaining (<10% of cells showing positivity); +, weak/moderate immunostaining (10-70% of cells showing positivity); and ++, strong immunostaining (>70% of cells showing positivity). Grade and stage of cancer were divided into two groups for statistical analyses: grade 1 and grade 2-3; stage was sub-classified as local (stage I-II) or advanced (III-IV) disease. All clear cell carcinomas were considered to be grade 3.

The significance of associations between immunohistochemical expression and clinicopathological characteristics was analyzed by using the chi-square test and Fisher's two-sided exact test. The software used was SPSS 16.0 for Windows. Probability values of <0.05 were considered significant.

Results

8-OHdG, PRX II and PRX VI in BE and EAC. Differences in the expression of markers were analyzed between all distinct groups and also separately between pairs. When all three groups of tissues were taken into account, 8-OHdG immunostaining was significantly weaker in cancer cells than in endometriosis epithelial cells (nuclear $p<0.05$ and cytoplasmic $p<0.01$). In addition, nuclear PRX II was weaker in cancer cells compared with endometriosis epithelial cells ($p<0.01$). These differences are shown in Table I and Figure 2.

When compared in pairs, 8-OHdG immunostaining was significantly weaker in cancer cells than in endometriosis epithelial cells in EAC (nuclear $p<0.01$; cytoplasmic $p<0.01$). Likewise, cancer cells had significantly weaker cytoplasmic 8-OHdG expression when compared with epithelial cells in BE ($p<0.01$). PRX II immunostaining was weaker in the cytoplasm of cancer cells than in the cytoplasm of endometriosis epithelial cells in EAC ($p<0.05$). In addition, nuclear expression of PRX II was weaker in cancer cells than in epithelial cells in BE ($p<0.01$). Furthermore, PRX VI immunostaining was weaker in nuclei and cytoplasm of cancer cells when compared with endometriosis epithelial cells in EAC (nuclear $p<0.05$, cytoplasmic $p<0.01$) and stronger in the cytoplasm of cancer cells when compared with the cytoplasm of epithelial cells in BE ($p<0.05$).

8-OHdG and PRX in different EAC groups. Cytoplasmic 8-OHdG exhibited greater expression in low-grade tumors. None of the grade 1 tumors were negative for cytoplasmic 8-OHdG, whereas 72.2% of grade 2-3 tumors were 8-OHdG-negative ($p<0.01$). Endometrioid carcinomas exhibited greater cytoplasmic 8-OHdG expression than did clear cell carcinomas ($p<0.01$). Differences in cytoplasmic 8-OHdG expression between different grades and types of histology are illustrated in Figure 3. Nuclear PRX VI was overexpressed in low-grade tumors ($p<0.05$). Cancer stage did not have any effect on the expression of 8-OHdG, PRX II or PRX VI in tumor tissue. In addition, the expression of 8-

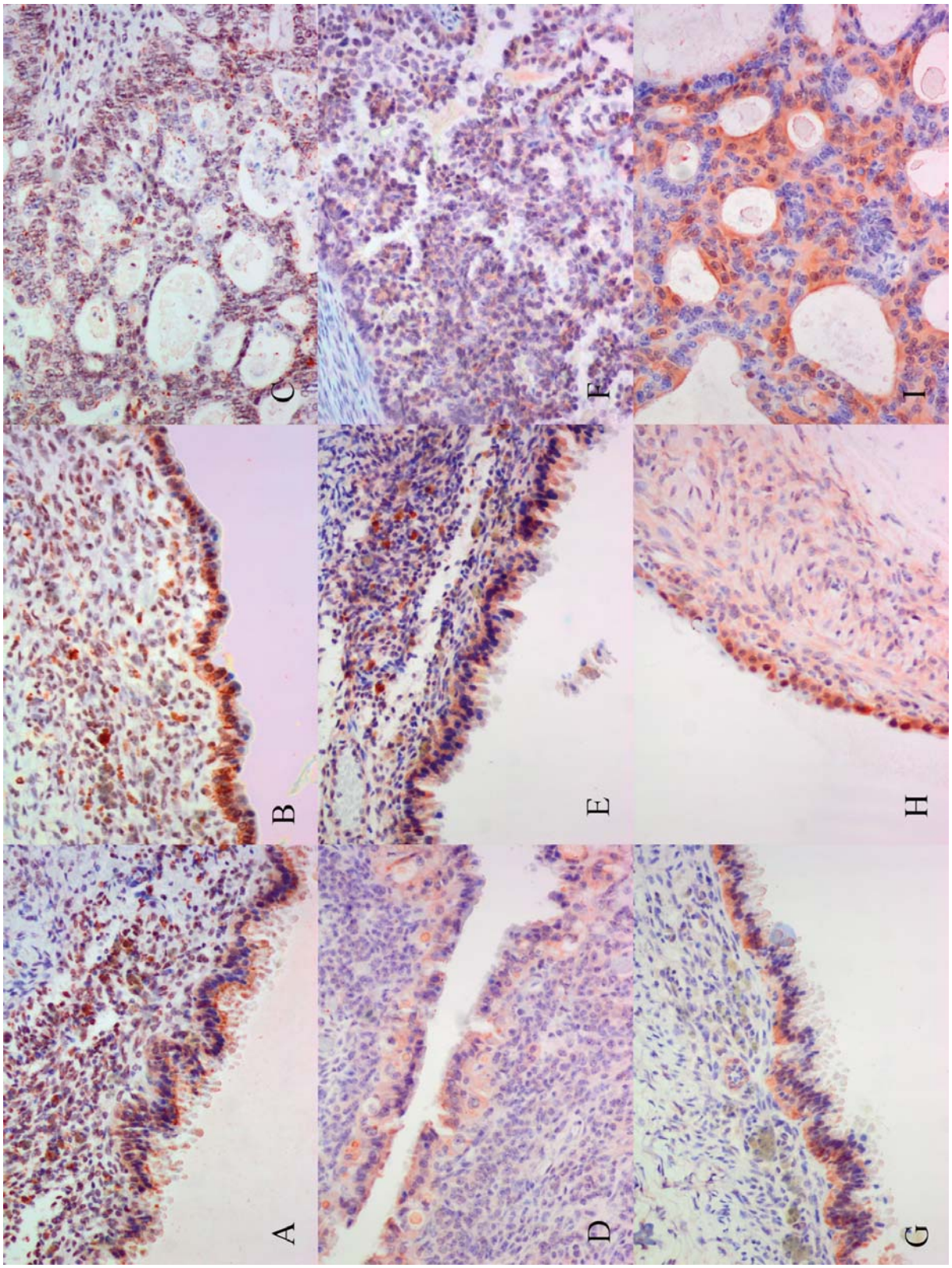

Figure 1. *The immunohistochemical panel demonstrates the staining reaction pattern for 8-hydroxydeoxyguanosine (8-OHdG), peroxiredoxin (PRX) II and PRX VI in all patient groups. Positive 8-OHdG expression in benign endometriosis (BE) (A), and endometriosis (B) and tumor in endometriosis-associated ovarian cancer (C). Positive PRX II expression in BE (D), and in endometriosis (E), and tumor in EAC (F). Positive PRX VI expression in BE (G), and endometriosis (H) and tumor in EAC (I). Original magnification, ×200.*

Table I. *Distribution of nuclear and cytoplasmic 8-hydroxydeoxyguanosine (8-OHdG), peroxiredoxin (PRX) II and PRX VI immunohistochemical expressions in endometriosis-associated ovarian cancer tumor and endometriosis cells and in cells in benign endometriosis. p-Values were calculated with Chi-square test between all three groups.*

	Endometriosis-associated ovarian cancer		Benign endometriosis in (%)	*p*-Value
	Tumor in (%)	Endometriosis in (%)		
Nuclear 8-OHdG				<0.05
–	6 (18.2)	3 (9.4)	1 (4.5)	
+	21 (63.6)	11 (34.4)	13 (59.1)	
++	6 (18.2)	18 (56.3)	8 (36.4)	
Cytoplasmic 8-OHdG				<0.01
–	18 (54.5)	5 (15.6)	0 (0.0)	
+	13 (39.4)	12 (37.5)	9 (40.9)	
++	2 (6.1)	15 (46.9)	13 (59.1)	
Nuclear PRX II				<0.01
–	25 (75.8)	15 (46.9)	3 (23.1)	
+	7 (21.2)	14 (43.8)	10 (76.9)	
++	1 (3.0)	3 (9.4)	0 (0.0)	
Cytoplasmic PRX II				0.084
–	2 (6.1)	0 (0.0)	0 (0.0)	
+	25 (75.8)	17 (53.1)	9 (69.2)	
++	6 (18.2)	15 (46.9)	4 (30.8)	
Nuclear PRX VI				0.093
–	18 (54.5)	9 (28.1)	6 (46.2)	
+	15 (45.5)	23 (71.9)	7 (53.8)	
++	0 (0.0)	0 (0.0)	0 (0.0)	
Cytoplasmic PRX VI				<0.01
–	1 (3.0)	0 (0.0)	4 (30.8)	
+	29 (87.9)	19 (59.4)	7 (53.8)	
++	3 (9.1)	13 (40.6)	2 (15.4)	

OHdG, PRX II and PRX VI was independent of histology, stage or grade of cancer in cases of endometriosis in EAC.

Discussion

In the present study, we report that endometriosis in EAC and BE tissue exhibited stronger expression of the oxidative stress marker 8-OHdG and the antioxidant enzyme PRX II than carcinoma tissue in EAC.

According to the results of recent studies, endometriosis can be considered a neoplastic disease and to have malignant potential (22-24, 30). The evidence has also linked endometriosis strongly to the development of epithelial ovarian cancer, especially to the clear cell and endometrioid subtypes. ROS-derived oxidative stress may be one of the key factors in the pathogenesis of endometriosis and EAC (3, 22, 23, 30). Several investigators have reported enhanced lipid peroxidation in peritoneal fluid of patients with endometriosis compared with controls (3, 31, 32), whereas antioxidant enzymes, mainly superoxide dismutases and glutathione peroxidase, appear to be reduced in peritoneal fluid of such patients (3, 12). Some evidence also suggests that in endometriosis, the tissue expression of superoxide dismutases is greater than in healthy endometrium (10, 11). Finally, it seems that there is increased ROS production in endometriotic tissue *in vitro*, leading to accelerated proliferation that favors the spread of the disease (7).

The presence of free iron has been suggested to be a particularly important source of oxidative stress as regards the malignant behavior of endometriosis (22, 33). The reaction between iron and H_2O_2 (Fenton reaction) produces the most reactive free radical, the hydroxyl radical (•OH). This radical is able to damage all four types of DNA base, and the hydrolytic product of guanine is 8-OHdG. Yamaguchi *et al*. analyzed the contents of cystic fluid of endometriotic cysts and reported elevated levels of free iron, 8-OHdG, lactose dehydrogenase, potential antioxidant and lipid peroxidase compared with non-endometriotic cysts (34). They hypothesized that the contents of endometriotic cysts create a stressful microenvironment that exposes epithelial cells to constant oxidative stress which may initiate carcinogenesis. They also suggested that carcinomas arising from the etiology mentioned above would be more resistant to the surrounding oxidative stress. From our data, we

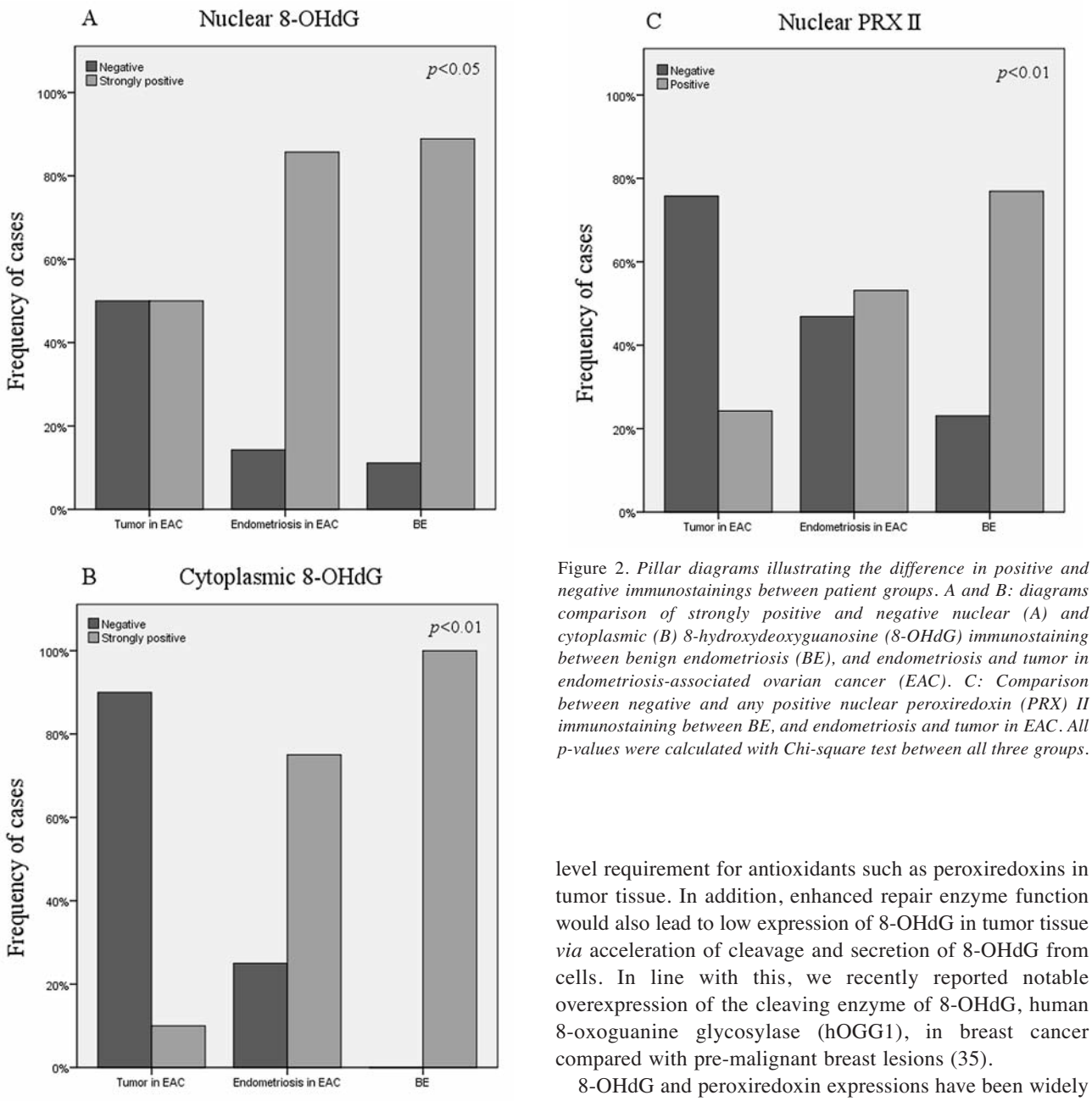

Figure 2. *Pillar diagrams illustrating the difference in positive and negative immunostainings between patient groups. A and B: diagrams comparison of strongly positive and negative nuclear (A) and cytoplasmic (B) 8-hydroxydeoxyguanosine (8-OHdG) immunostaining between benign endometriosis (BE), and endometriosis and tumor in endometriosis-associated ovarian cancer (EAC). C: Comparison between negative and any positive nuclear peroxiredoxin (PRX) II immunostaining between BE, and endometriosis and tumor in EAC. All p-values were calculated with Chi-square test between all three groups.*

observed high hydroxyl radical-derived oxidative stress and high antioxidant activity in endometriotic tissue in EAC and BE compared with cancer. Our findings concerning the high level of oxidative damage in endometriotic tissue are in line with the results of former studies and could be explained by the hypothesis offered by Yamaguchi *et al*. (34).

Persistent oxidative stress in the early stages of a malignant process may lead to the induction of DNA repair enzymes in cancer tissue. This would be reflected as a low-level requirement for antioxidants such as peroxiredoxins in tumor tissue. In addition, enhanced repair enzyme function would also lead to low expression of 8-OHdG in tumor tissue *via* acceleration of cleavage and secretion of 8-OHdG from cells. In line with this, we recently reported notable overexpression of the cleaving enzyme of 8-OHdG, human 8-oxoguanine glycosylase (hOGG1), in breast cancer compared with pre-malignant breast lesions (35).

8-OHdG and peroxiredoxin expressions have been widely examined in other malignancies, and in most of these studies they seem to have significant prognostic relevance (17, 36-42). However, there are only a few studies that have focused on 8-OHdG and peroxiredoxins in ovarian cancer. Sanchez *et al*. reported significantly increased levels of 8-OHdG in ovarian cancer tissue compared with healthy ovarian tissue (43). Assessment of 8-OHdG was carried out by using high-performance liquid chromatography and most of the study material consisted of serous and mucinous cancer types. Our group has previously reported that high immuno-histochemical expression of 8-OHdG and elevated concentrations of serum 8-OHdG correlated to poor survival

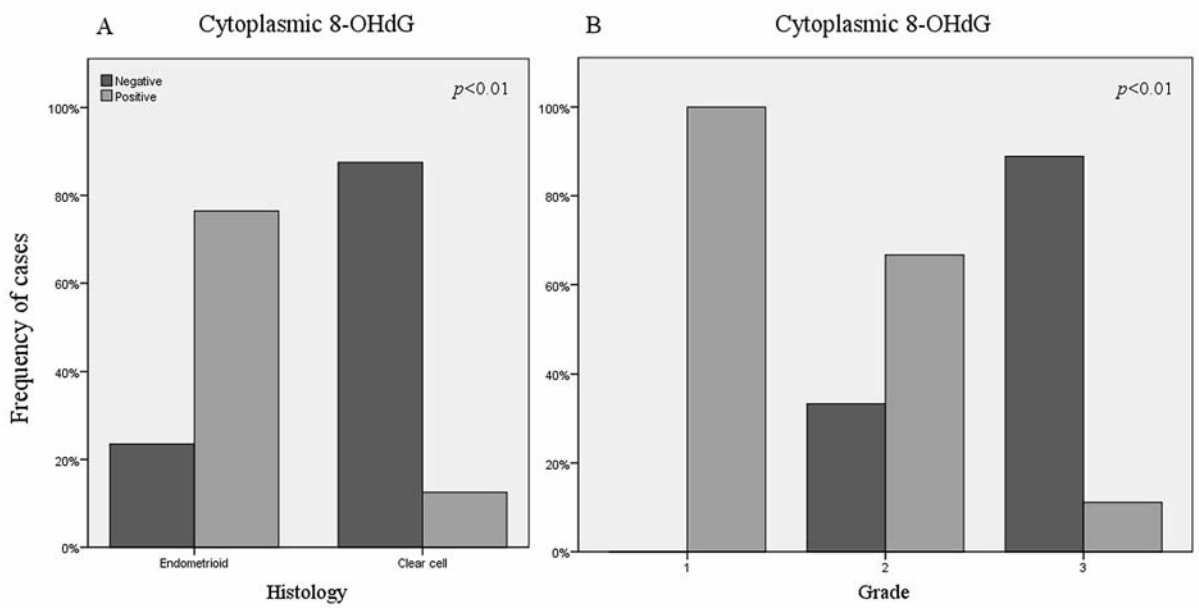

Figure 3. *Pillar diagrams comparing negative and positive cytoplasmic 8-hydroxydeoxyguanosine (8-OHdG) immunostaining between endometrioid and clear cell cancer and between different histological grade in both tumor types. p-Values were calculated with Chi-square test.*

and worse differentiation in ovarian carcinoma and that 8-OHdG was overexpressed in serous ovarian carcinomas compared with other types (44, 45). We have also discovered that levels of peroxiredoxins, especially PRX II and VI, were significantly higher in borderline than in benign ovarian tumors (19). The great majority of patients in these three studies had serous cancer histology. The results of these previous studies confirm the nature of endometriosis as a premalignant lesion but the ekspression of oxidative markes seem to be different in serous ovarian cancers than seen in this study. This suggests a different etiology of EAC, which usually has a favorable prognosis and is found at earlier stages than other epithelial ovarian cancer types (46, 47). Recently, Banz *et al*. discovered that the molecular signature of EAC differs significantly from other epithelial ovarian cancers, which further strengthens the suggestion that these two diseases arise from completely different etiologies (48).

To conclude, ovarian endometrioid and clear cell carcinomas exhibit down-regulated PRX II expression compared with their associated endometriosis and epithelial cells in BE. 8-OHdG as a marker of oxidative stress-derived DNA damage is also more profoundly present in cancer-associated endometriosis and epithelial cells in BE than in carcinomas, which might be linked to iron overload of endometriotic cysts. Ovarian carcinomas arising from these oxidatively stressful environments may therefore become naturally more resistant to oxidative stress by either enhancing their DNA repair functions or promoting antioxidant defenses other than peroxiredoxins. The results of the current study also strengthen the hypothesis for a difference in the pathogenesis of EAC cancer compared with other epithelial ovarian cancer subtypes.

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