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Oxidative stress-induced antioxidant enzyme expression is an early phenomenon in ovarian carcinogenesis

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ABSTRACT

Oxidative stress and antioxidant enzymes have been widely investigated in various carcinomas. However, there is only some information about their role in ovarian carcinogenesis or in ovarian carcinomas *in vivo*. We studied immunohistochemical nuclear and/or cytoplasmic expression of oxidative stress markers 8-hydroxydeoxyguanosine (8-OHdG) and nitrotyrosine, as well as major antioxidative enzymes peroxiredoxins (PRDX) I–VI and thioredoxin (TXN) in ovarian tumours. The material consisted of 20 benign (10 serous, 10 mucinous) and 51 borderline (33 serous, 18 mucinous) epithelial ovarian tumours. The markers of oxidative stress, 8-OHdG and nitrotyrosine, were seen already in benign tumours (in 20% and 45% of the tumours, respectively) and their expression patterns were similar in benign and borderline tumours. The levels of PRDX II, III, IV, V and VI were significantly higher in borderline than in benign tumours ($p < 0.02$ for all). Specifically for PRDX II (for both nuclear and cytoplasmic expression, $p < 0.00005$) and PRDX VI (for cytoplasmic expression, $p = 0.0003$ and for nuclear expression, $p = 0.0005$) the difference between benign and borderline tumours was remarkable. In general, serous benign and borderline tumours expressed higher antioxidant enzyme levels than mucinous ones. Nuclear TXN was expressed more strongly in benign than in borderline tumours ($p = 0.003$). Oxidative stress occurs already in benign ovarian tumours and the levels are comparable to borderline tumours. However, some of the antioxidant enzymes, especially PRDX II and VI, are more profoundly induced in borderline ovarian tumours, reflecting their possible role as cancer preventers. This difference could also offer a potential tool for differential diagnosis between benign and borderline epithelial ovarian tumours.

1. Introduction

Reactive oxygen species (ROS) are defined as molecules with one or more unpaired electrons in atomic or molecular orbitals. Several studies have shown that ROS are an important factor in carcinogenesis.[1] ROS are formed from either the incomplete reduction of oxygen during cellular respiration or following exposure to external agents such as light, ionising radiation, or some redox drugs. Because of the unpaired electrons, ROS molecules are very unstable and react easily

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with other molecules. ROS can interact directly with DNA or they can oxidise lipids or proteins generating intermediates that react with DNA causing mutations.[1]

Superoxide radical ($O_2^{\cdot-}$) is the most common radical in aerobic cells and it is produced mostly in mitochondria through an electron transport chain when electrons leak out and react with molecular oxygen.[1,2] Hydrogen peroxide (H_2O_2) is usually formed from $O_2^{\cdot-}$ by the catalytic activity of superoxide dismutases (SOD). Hydroxyl radical (·OH) can be formed from H_2O_2 when it reacts with Fe^{2+}. ·OH is extremely unstable and reacts fast with other molecules. When ·OH attacks DNA, 8-hydroxydeoxyguanosine (8-OHdG) can be formed. 8-OHdG is the most widely-used marker of oxidative DNA damage and it is easy to detect and reliable to measure immunohistochemically. The increased levels of 8-OHdG levels have been shown in several carcinomas compared to non-malignant tissue.[2]

DNA repair enzymes prevent the accumulation of damaged DNA. Antioxidants, on the other hand, protect the cells from free radicals. Antioxidant enzymes include SOD enzymes, catalase, glutathione perixidases (GPx), peroxiredoxins (PRDX) and thioredoxins (TXN). The PRDX family (I–VI) is considered one of the most important cell redox state-regulating enzymes and these isoenzymes are widely distributed through subcellularly, in contrast to the most other antioxidant enzymes. The production of PRDXs increases when oxidative stress occurs.[3] PRDXs also modulate intracellular signalling cascades that deploy H_2O_2 as a second messenger molecule and regulate cell proliferation.[2] TXN provides a defense against oxidative stress by reducing peroxides such as H_2O_2, but it also controls the reduced intracellular redox environment, cellular growth and controls apoptosis.[4]

Oxidative stress markers (PRDX or TXN expressions) have been shown to correlate with poor prognosis in ovarian cancers in our preliminary data.[5] The aim of this study was to analyse the role of oxidative stress and major antioxidative enzymes in early ovarian carcinogenesis.

2. Materials and methods

The study consisted of 71 patients with ovarian tumours. Diagnoses were made during the years 2000–2007 and the treatments were centralised in the Gynecological Oncology Unit in Oulu University Hospital. All the patients had surgery and six of them were also treated with cytotoxic agents. The samples were collected on primary operations before any adjuvant treatments. The tumours had been fixed in neutral formalin and further embedded in paraffin blocks and stored in the Department of Pathology of Oulu University Hospital. The histological diagnoses of the tumours were determined according to the criteria of the recent WHO classification of borderline ovarian tumours.[6] The material consisted of 10 benign serous and 10 benign mucinous ovarian tumours, 33 serous borderline and 18 mucinous borderline tumours. Among the borderline tumours, there were 47 stage I tumours, one stage II tumour, two stage III tumours and one stage IV tumour. Only one patient died for subsequent ovarian cancer. The study was approved by the Local Ethics Committee (53/2008).

2.1. *Immunohistochemistry*

Four-micron thick sections were cut from a representative paraffin block and placed on SuperFrostPlus glass slides (Menzel-gläser, Germany). The sections were first de-paraffinised in xylene and re-hydrated in a descending ethanol series, incubated in 10 mM citrate buffer (pH 6.0), boiled in a microwave oven for 10 min, and cooled properly at room temperature before adding the primary antibody. Negative controls were prepared using the same procedure except that the primary antibodies were replaced by PBS and serum isotype controls (Zymed Laboratories, Inc.).

Immunoreactivity in the samples was assessed semi-quantitatively by grading both the staining intensity in the tumour cells and the number of positively-stained tumour cells. The immunoreactivity was evaluated by three independent investigators (M.P., S.K., P.K.) by dividing the staining reaction into four groups: 0 = no staining intensity and no positive or only few positive cells; 1 = weak staining intensity (>20–49%); 2 = moderate staining intensity (>50–89%); and 3 = strong staining intensity (>90%). The immunoreactivity investigation was blinded for clinical data. Both cytoplasmic and nuclear stainings were separately assessed with the

Table 1 – Details of antigens and antibodies used.

Antigen	Antibody	Dilution	Immuno-staining method	Source of primary antibody
PRDX I	Rabbit polyclonal PRDX I antibody	1:1500	Histostain-Plus Bulk Kit	Labfrontier, York, United Kingdom
PRDX II	Rabbit polyclonal PRDX II antibody	1:1000	Histostain-Plus Bulk Kit	”
PRDX III	Rabbit polyclonal PRDX III antibody	1:750	Histostain-Plus Bulk Kit	”
PRDX IV	Rabbit polyclonal PRDX IV antibody	1:750	Histostain-Plus Bulk Kit	”
PRDX V	Rabbit polyclonal PRDX V antibody	1:2000	Histostain-Plus Bulk Kit	”
PRDX VI	Rabbit polyclonal PRDX VI antibody	1:2000	Histostain-Plus Bulk Kit	”
TXN	Goat polyclonal anti-human TXN antibody	1:200	A biotinylated secondary anti-goat antibody; avidin–biotin–peroxidase complex	American Diagnostica, Greenwich, CT
8-OHdG	Mouse monoclonal 8-OHdG antibody	1:100	Dako Envision Kit	Gentaur, Brussels, Belgium
Nitrotyrosine	Rabbit polyclonal nitrotyrosine antibody	1:100	Histostain-Plus Bulk Kit	Upstate, NY, USA

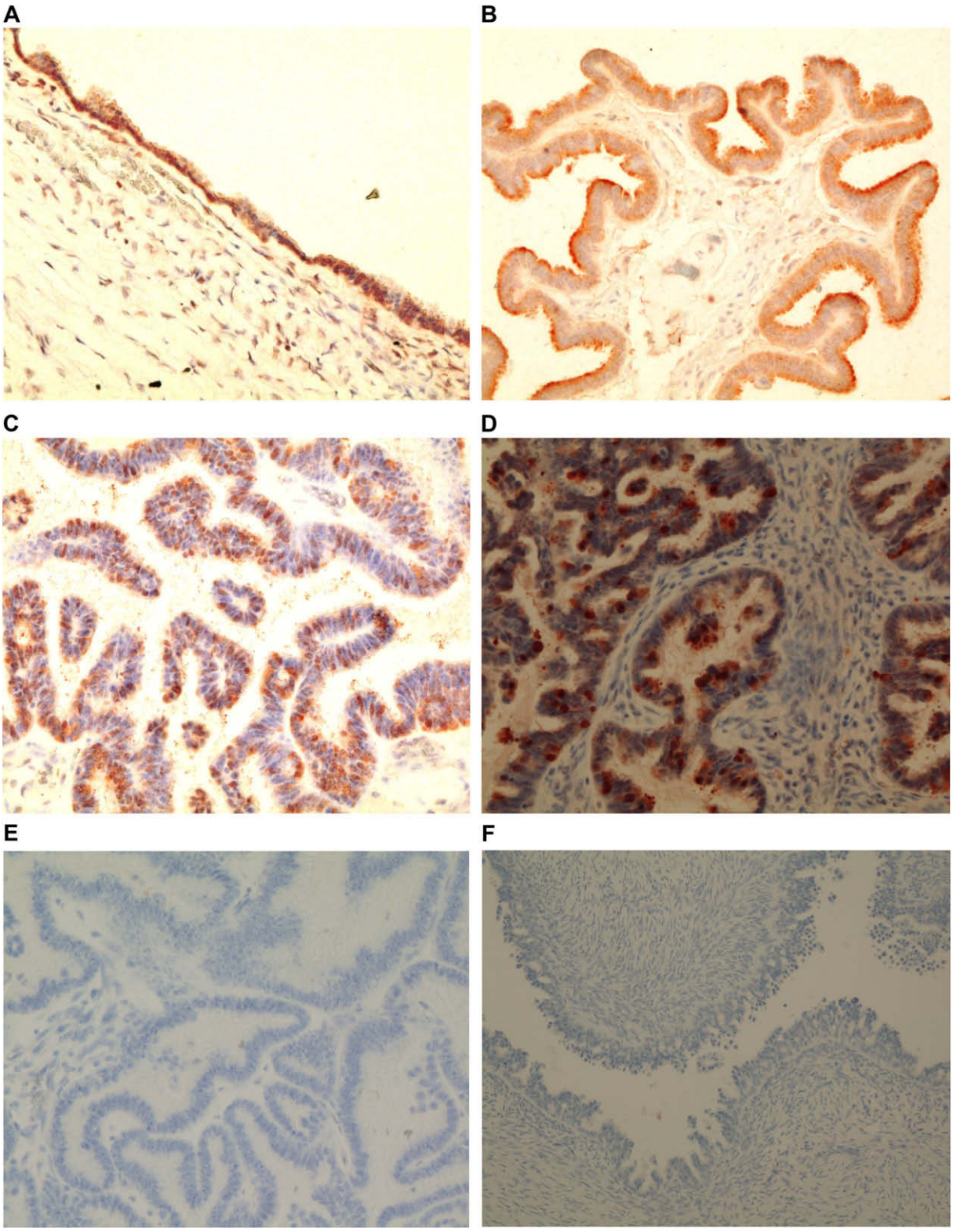

Fig. 1 – Moderate to strong 8-OHdG positive nuclei can be observed in benign ovarian epithelium (A). Strong cytoplasmic immunostaining for PRDX V is seen in mucinous borderline epithelium, nuclear immunostaining is also found (B). Granular nuclear PRDX VI immunostaining is found in borderline ovarian epithelium (C). Strong TXN expression is seen in some nuclei of mucinous borderline tumour. Stromal reaction is negative and cytoplasm is immunopositive in places (D). Negative control stainings for 8-OHdG (E) (mouse isotype control) and PRDX VI (F) (rabbit isotype control) are also demonstrated.

PRDX and TXN antibodies, except for PRDX III, IV and nitrotyrosine cytoplasmic staining and for 8-OHdG only nuclear staining was evaluated (Table 1).

2.2. Statistical analysis

SPSS 16.0 for Windows (Chicago, IL, USA) was used for statistical analysis. The significance of the associations was determined using two-sided Fisher's exact probability test. The probability values of $p < 0.05$ were considered significant.

3. Results

3.1. Oxidative stress in ovarian carcinogenesis

Twenty percentage of the benign and borderline tumours expressed 8-OHdG (Figs. 1 and 2, Table 2). Most of those samples stained weakly and none showed a strong expression. 8-OHdG and nitrotyrosine immunostaining associated with each other in benign tumours ($p = 0.0002$), but not in borderline tumours.

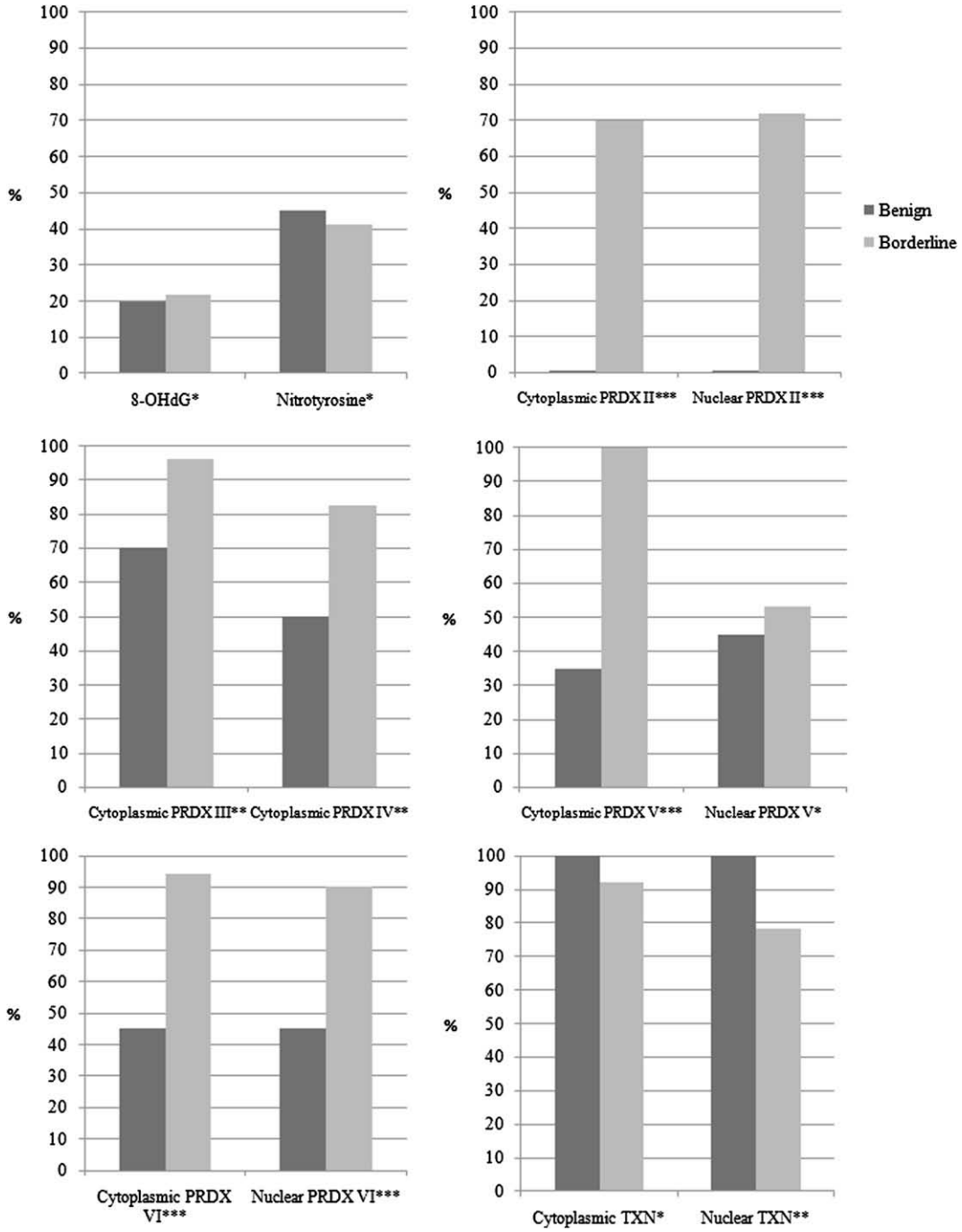

Fig. 2 – The staining pattern of 8-OHdG, nitrotyrosine, PRDXs II, III, V, VI and TXN. *Statistically non-significant difference between benign and borderline tumours; **$p < 0.05$ between benign and borderline tumours; and ***$p < 0.005$ between benign and borderline tumours.

Table 2 – The distribution of immunostaining in benign and borderline tumours, percentage is given in brackets.

	Benign tumours			Borderline tumours		
	Negative	+	++ or +++	Negative	+	++ or +++
PRDX I, cytoplasmic	20 (100)	0	0	46 (92)	3 (6)	1 (2)
PRDX I, nuclear	20 (100)	0	0	50 (100)	0	0
PRDX II, cytoplasmic	20 (100)	0	0	15 (30)	17 (34)	18 (36)
PRDX II, nuclear	20 (100)	0	0	14 (28)	25 (50)	11 (22)
PRDX III, cytoplasmic	6 (30)	6 (30)	8 (40)	2 (4)	22 (43)	27 (53)
PRDX IV, cytoplasmic	10 (50)	3 (15)	7 (35)	9 (18)	24 (47)	18 (35)
PRDX V, cytoplasmic	6 (30)	8 (40)	6 (30)	0	22 (43)	29 (57)
PRDX V, nuclear	13 (65)	5 (25)	2 (10)	24 (47)	24 (47)	3 (6)
PRDX VI, cytoplasmic	11 (55)	3 (15)	6 (30)	3 (6)	20 (40)	28 (55)
PRDX VI, nuclear	11 (55)	7 (35)	2 (10)	5 (10)	26 (51)	20 (39)
TXN, cytoplasmic	0	7 (35)	13 (65)	4 (8)	18 (35)	29 (57)
TXN, nuclear	0	9 (45)	11(55)	11 (22)	31 (61)	9 (18)
8-OHdG	16 (80)	3 (15)	1 (5)	40 (78)	8 (16)	3 (6)
Nitrotyrosine	11 (55)	6 (30)	3 (15)	30 (59)	17 (33)	4 (8)

Nitrotyrosine expression was found in 45% of benign and 41.1% of borderline tumours. There were no statistical differences in the oxidative stress marker levels between the benign and borderline tumours or between the mucinous and serous histological types. However, there was a trend of higher nitrotyrosine expression in mucinous than in serous benign tumours ($p = 0.07$) (Table 3).

3.2. *Antioxidative enzymes in ovarian carcinogenesis*

PRDX expression in general was stronger in borderline tumours than in benign tumours. In benign tumours there was neither cytoplasmic nor nuclear PRDX I or PRDX II expression. However, 8% of the borderline tumours expressed cytoplasmic PRDX I. Most of the borderline tumours showed at least weak PRDX II expression and the difference was statistically highly significant when set against PRDX II expression in benign tumours both in cytoplasm ($p = 0.000002$) and in nuclei ($p = 0.000001$).

Table 3 – Differences of studied antibodies between benign and borderline tumours and corresponding significances. NS = no statistical significance.

Studied antibody	*p*-Value	Higher expression
8-OHdG	NS	
Nitrotyrosine	NS	
PRDX I, cytoplasmic	NS	
PRDX I, nuclear	NS	
PRDX II, cytoplasmic	0.000004	Borderline
PRDX II, nuclear	0.000002	Borderline
PRDX III, cytoplasmic	0.02	Borderline
PRDX IV, cytoplasmic	0.02	Borderline
PRDX V, cytoplasmic	0.0006	Borderline
PRDX V, nuclear	NS	
PRDX VI, cytoplasmic	0.0003	Borderline
PRDX VI, nuclear	0.0005	Borderline
TXN, cytoplasmic	NS	
TXN, nuclear	0.003	Benign

In the benign tumours, PRDX III and IV cytoplasmic expression was seen in 70% and 50% of the samples, respectively. In the borderline tumours, the expression of these antioxidant enzymes was over 80%. The difference between the PRDX III and IV expression levels observed in the benign or borderline tumours were statistically significant (for PRDX III, $p = 0.02$ and PRDX IV, $p = 0.02$). These PRDX isotypes were located subcellularly only in cytoplasm.

With regard to PRDX V and VI, a substantial amount of samples did not show any expression in benign tumours whereas most of the borderline tumours were positive for these isotypes (the *p*-values between benign and borderline tumours for cytoplasmic PRDX V and PRDX VI were 0.0006 and 0.0003, respectively). The nuclear expression of both PRDX V and PRDX VI was lower but the same statistically significant higher expression in borderline tumours was observed for PRDX VI ($p = 0.0005$).

TXN was expressed strongly in the benign and borderline tumours. All the benign samples stained at least weakly positively, whereas in the borderline tumours 92.2% expressed cytoplasmic TXN and 78.4% nuclear TXN. There was no difference in the cytoplasmic TXN expression between benign or borderline tumours, whereas nuclear TXN expression was higher in benign tumours ($p = 0.03$).

The cytoplasmic expression of PRDX I ($p = 0.04$), PRDX III ($p = 0.002$), PRDX IV ($p = 0.008$), PRDX V ($p = 0.005$), PRDX VI ($p = 0.00004$) and TXN ($p = 0.04$) were significantly higher in the serous than in mucinous borderline tumours. Also in the benign tumours the cytoplasmic expression of PRDX III ($p = 0.01$), PRDX IV ($p = 0.03$), PRDX V ($p = 0.03$), PRDX VI ($p = 0.01$) and TXN ($p = 0.03$) was higher in the serous tumours than in the mucinous ones.

3.3. *Association between oxidative stress and antioxidant enzymes*

In the benign tumours, 8-OHdG associated significantly with cytoplasmic PRDX III ($p = 0.02$) expression and with nuclear PRDX V ($p = 0.01$) and VI ($p = 0.006$) expression (Table 4). Nitrotyrosine expression associated with cytoplasmic PRDX

Table 4 – Associations between 8-OHdG and PRDX enzymes in benign and borderline ovarian epithelial tumours and corresponding significances. NS = no statistical significance.

	Benign 8-OHdG	Borderline 8-OHdG
PRDX I, cytoplasmic	N/A	0.002
PRDX I, nuclear	N/A	N/A
PRDX II, cytoplasmic	N/A	NS
PRDX II, nuclear	N/A	NS
PRDX III, cytoplasmic	0.02	0.002
PRDX IV, cytoplasmic	NS	NS
PRDX V, cytoplasmic	NS	NS
PRDX V, nuclear	0.01	NS
PRDX VI, cytoplasmic	NS	NS
PRDX VI, nuclear	0.006	NS

III ($p = 0.06$), PRDX IV ($p = 0.01$) and V ($p = 0.007$) levels and with nuclear PRDX V ($p = 0.01$) and VI ($p = 0.003$) expression. There was a highly significant correlation between 8-OHdG and nitrotyrosine ($p = 0.0002$). In the borderline tumours, 8-OHdG associated with an elevated expression of cytoplasmic PRDX I ($p = 0.002$) and PRDX III ($p = 0.002$) and nitrotyrosine associated with a higher cytoplasmic PRDX I expression ($p = 0.01$).

4. Discussion

We report here on oxidative stress derived DNA mutations and tyrosine nitration already in benign ovarian tissue. This is the first study to show ROS derived oxidative damage both in the nuclei and in cytoplasm of benign and borderline ovarian tumours. Our results also demonstrate that oxidative stress occurs in ovarian epithelial cells, and is not, e.g. derived solely from macrophages which are also a potential source of ROS. The oxidative stress levels were equal in the benign and borderline tumours, 8-OHdG positivity was observed in approximately 20% and nitrotyrosine in approximately 45% of both benign and borderline samples. Nevertheless, levels were considerably lower compared to invasive ovarian carcinomas in our previous study, which was performed in the same laboratory with identical methods.[5] In that study, 88.5% of samples were 8-OHdG positive and 82.3% nitrotyrosine positive, reflecting high ROS derived damage both in DNA and cytoplasm in invasive ovarian carcinomas. It appears that generation of free radicals in non-invasive tissues is relatively low and the antioxidant levels are in balance with generation of free radicals until progression to invasive disease. In fact, a certain background level of ROS production is necessary for the proper function of ROS mediated cellular signalling cascades and mild oxidative stress appears to be the indispensable consequence of physiological ROS metabolism in benign ovarian tissue. In addition, it has been proposed that it makes more energetic sense for cells to stand low oxidative stress background damage than maintain an excess antioxidant defense.[7]

The mutagenic properties of 8-OHdG have been clearly demonstrated in various experimental models and 8-OHdG mutations are likely to reflect total ROS derived damage in DNA.[8] According to our previous study, in ovarian carcinomas 8-OHdG was associated with poor prognosis and differentiation, a higher stage and non-optimal surgical outcome.[5] High serum 8-OHdG levels were a predictor of short survival in resected non-small-cell lung cancer patients.[9] Regarding colorectal carcinomas, there are several reports that 8-OHdG and nitrotyrosine levels are significantly higher in malignant tissue compared to adenomas or normal colon tissues.[10,11] We have earlier reported a significant increase of nitrotyrosine levels from benign breast hyperplasias to ductal carcinoma in situ and further to invasive T1N0 breast carcinomas.[12] Two other groups have recently found high inducible NOS (iNOS) levels to be an important predictor of aggressive disease and poor outcome in ovarian cancer.[13,14] The current results are thus in line with these previous observations and it may be suggested that oxidative and nitrosative stress plays a highly carcinogenic role also in ovarian cancer *in vivo*.

PRDX and TXN overexpression has been observed in many carcinomas and there seems to be cancer-specific PRDX and TXN expression models. In *in vitro* studies most PRDX enzymes seem to induce easily under oxidative stress.[2,3,15]

According to our previous study in ovarian carcinomas cytoplasmic PRDX IV is associated with a better prognosis, whereas cytoplasmic PRDX V and VI are associated with a higher stage,[5] reflecting their different roles in carcinogenesis. In the current study the PRDX II–VI expression was significantly higher in the borderline than benign tumours and most of the PRDX isoenzymes correlated with oxidative stress markers 8-OHdG and nitrotyrosine. It seems that the induction of PRDX is sufficient to prevent the carcinogenic consequences of oxidative stress since oxidative stress levels were comparable in benign and borderline tissues. On the other hand, induction of PRDX in borderline tumours may prevent oxidative stress derived apoptosis allowing the accumulation of DNA-damages with the possible promotion of carcinogenesis. PRDX enzymes are thought to be tumour preventive in physiological situations, e.g. loss of PRDX I in mice leads to premature death from cancer.[16] As antioxidants, PRDXs detoxify peroxides and also peroxynitrite from cancer cells and as high levels prevent apoptosis, induce proliferation and may support tumour maintenance.[2]

Both high cytoplasmic and nuclear TXN expression associated with decreased disease-free survival and more aggressive disease phenotype in breast carcinomas.[17] TXN is an essential growth factor in cancer cells and promotes tumour angiogenesis and also activates many transcription factors, such as NF-κB, AP-1and SP-1.[4] In the current study, a higher nuclear TXN expression was more frequent in benign than in borderline tumours. This is in contrast with the trend observed with PRDX expression and suggests that TXN is not as effectively re-reduced and induced under oxidative stress as PRDX isoenzymes. The determination of thioredoxin reductase expression or its activity would give more information for this hypothesis. On the other hand, TXN is known to have diverse roles in cytoplasm and nucleus; in cytoplasm its primary function is antioxidative, whereas in nucleus it mainly regulates transcription factors.[4] Decreased nuclear TXN expression in borderline tumours could therefore lead to an inappropriate regulation of above mentioned transcription factors. Like PRDXs, also cytoplasmic TXN was expressed

significantly more frequently in serous than in mucinous tumours.

To summarise, there are signs of oxidative stress already in benign ovarian tumours, which are essentially at the same levels as in borderline tumours. Different PRDX subtypes seem to have different roles in ovarian carcinogenesis. The highly significant difference seen in the expression of PRDX II and VI in benign and borderline ovarian tumours may also be applicable in differential diagnosis between these tumour types.

Conflict of interest statement

All authors disclose any financial and personal relationships with other people or organisations that could inappropriately influence the study.

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