Elevated Serum 8-OHdG Is Associated with Poor Prognosis in Epithelial Ovarian Cancer

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Abstract. *Background: 8-Hydroxydeoxyguanosine (8-OHdG) is a marker of oxidative stress in DNA. This study was undertaken to reveal whether serum 8-OHdG could be a prognostic factor in epithelial ovarian carcinoma (EOC). Patients and Methods: Preoperative serum 8-OHdG levels were examined by enzyme-linked immunosorbent assay in 84 stage I–IV EOC patients. Immunohistochemical (IHC) 8-OHdG expression was determined in 78 of these patients. Results: Strong 8-OHdG immunostaining predicted poor survival. High serum 8-OHdG (>140 pg/ml) was associated with poor ovarian cancer-specific survival ($p<0.05$) in patients with grade 1-2 EOC ($p<0.05$), but was not observed among the grade 3 patients. High 8-OHdG levels both in the serum and in the tumour tissue was associated with traditional factors of poor prognosis and serous histology. Conclusion: Both serum and IHC 8-OHdG assessment may serve as prognostic tools in EOC and the role of oxidative stress as a carcinogenic factor in ovarian cancer pathogenesis is also suggested.*

Ovarian cancer is one of the main causes of cancer-related death in women. Seventy percent of ovarian carcinomas are diagnosed at an advanced stage, most patients relapse within two years and five-year survival at its best is only 40 %. Evidence suggests that ovulation increases the levels of inflammatory agents, which can further lead to mutations in DNA (1-4). Ovulation creates a void on the ovarian surface which leads to the wound-healing process, with increased levels of inflammatory mediators and reactive oxygen species (ROS) (1, 3). ROS are important factors in carcinogenesis (5) and are usually formed by either the incomplete reduction of oxygen during cellular respiration, or following exposure to external agents such as ultraviolet light, ionizing radiation or redox-state modifying drugs. Because of their unpaired electrons, ROS molecules are very unstable and react easily with other molecules. They can interact directly with DNA and they can oxidize lipids and proteins, generating intermediates that react with DNA, a cascade with the potential to cause DNA mutations when antioxidant defences are overwhelmed (5). The hydroxyl radical (•OH) is extremely unstable and reacts rapidly with other molecules. When it attacks DNA, 8-hydroxydeoxyguanosine (8-OHdG) can be formed.

8-OHdG is one of the most studied markers of DNA damage and changes in tissue expression and serum levels of 8-OHdG have been shown to be associated with the prognosis of several carcinomas (6-10). The expression of 8-OHdG has been investigated by immunohistochemistry (IHC) in various carcinomas, but serum 8-OHdG levels in only a few cancer types. High serum 8-OHdG levels have been found to be a predictor of short survival in non-small cell lung cancer patients (10) and have also been found in children with acute leukaemia (11). In colorectal carcinomas 8-OHdG and nitrotyrosine IHC expression levels have been shown to be significantly higher in malignant tissues compared with adenomas or non-tumourous tissues of the colon (12, 13).

Women with a high genetic risk of ovarian cancer have been shown to be more likely to express multiple inclusion cysts in their ovaries (4). As a marker of oxidative stress, 8-OHdG levels have been shown to be elevated in ovarian surface cells in postovulatory follicles of humans and sheep (1, 3). High 8-OHdG levels have also been found in the epithelia of pre- and postovulatory follicles in egg-laying hens (3).

DNA repair enzymes prevent the accumulation of damaged DNA and antioxidants protect cells against free radicals. Antioxidative enzymes include superoxide dismutase (SOD) enzymes, catalase, glutathione peroxidases (GPx), peroxiredoxins (Prx) and thioredoxins (Trx). The antioxidant enzyme Prx I was shown to be highly over-expressed in serous ovarian carcinoma compared to borderline tumours and correlated significantly with poor

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overall survival (14). Similarly in our previous studies, induction of antioxidant Prx isoenzymes IV, V and VI in borderline ovarian tumours and ovarian carcinomas and their association with poor prognosis were observed (6, 15).

In epithelial ovarian cancer (EOC), we previously showed the association of high tissue expression of 8-OHdG and poor prognosis. This study is the first in which systemic oxidative stress in patients with EOC has been evaluated by measuring serum 8-OHdG levels to determine the correlation between serum and tissue 8-OHdG expression and to assess the value of serum 8-OHdG as a prognostic factor in EOC.

Patients and Methods

Patients. The study population consisted of 84 patients with ovarian cancer, diagnosed and treated from 1997-2005 at the Gynaecological Oncology Unit, Oulu University Hospital. Serum samples were available from all the patients and tumour samples from 78. The patients were treated by means of debulking surgery (with optimal or suboptimal results) and with platinum- and taxane-containing chemotherapy. Optimal surgery was defined as no macroscopic residual disease. The patient characteristics are shown in Table I. Blood samples were collected on the day before surgery and tumour samples during primary surgery. Serum samples were stored at –70˚C until use. The tissue samples were fixed in neutral formalin, embedded in paraffin blocks and stored at the Department of Pathology, Oulu University Hospital. Histological diagnoses of the tumours were determined according to the criteria of the most recent WHO classification of ovarian cancer (16).

Enzyme-linked immunosorbent assay (ELISA). Serum levels of 8-OHdG were measured by using an ELISA (Highly Sensitive 8-OHdG Check ELISA kit; Gentaur Europe, Brussels, Belgium). Assays were carried out by following the manufacturer's instructions, with a few modifications. First all the serum samples were pre-processed using Millipore Microcon filters (Millipore, Billerica, MA, USA). The filters were dampened with 100 ml of distilled water and then centrifuged at 14,000 ×*g* for 5 min. Then the filters were turned around and centrifuged for another 5 min to remove any remaining water. The damp filters were moved to new tubes and 200 ml of serum sample was added into each tube and then centrifuged for 30 min at 14,000 ×*g*. The primary antibody was reconstituted with the primary antibody solution then 50 ml of sample or standard were added to the wells of a well plate each in duplicate before adding 50 ml of reconstituted primary antibody to each well. The plate was shaken and covered with adhesive strip and then incubated at 4˚C for overnight. After incubation, the contents of the wells were poured off and each well was washed with 250 ml of washing solution three times. Secondary antibody was subsequently reconstituted with the secondary antibody solution; 100 ml of reconstituted secondary antibody was added to each well. The plate was then shaken, covered with adhesive strip and incubated for 1 h at room temperature. Washing was repeated at the end of the incubation period. After that, a substrate solution was prepared and 100 ml was added to each well and the plate was shaken. The plate was incubated in the dark for 15 min at room temperature then 100 ml of reaction terminating solution was added to each well and the plate was shaken. Absorbance was measured at 450 nm in a plate reader and a standard curve was used to determine the level of 8-OHdG in the samples. Duplicates of each sample were assayed and also the outermost wells of the microtitre plates were used. Forty samples were assayed in one plate. If the measured values in duplicate samples differed by more than 10%, the assay was repeated.

Immunohistochemistry (IHC). Four-micron-thick sections were cut from a representative paraffin block and placed on SuperFrostPlus glass slides (Menzel-gläser, Braunschweig, Germany). The sections were first de-paraffinized in xylene and rehydrated in a descending series of ethanol concentrations, incubated in 10 mM citrate buffer (pH 6.0), boiled in a microwave oven for 10 min, and cooled at room temperature. The slides were then immersed in 3% hydrogen peroxide in methanol for 15 min to consume the endogenous peroxide. The sections were incubated with a 1:125 primary antibody dilution against 8-OHdG (mouse monoclonal 8-OHdG antibody, Gentaur, Europe) overnight at 4˚C. Both ELISA kit and immunostainings were based on the same 8-OHdG antibody (N45.1).

Immunostaining was carried out using a biotinylated secondary antibody 1:400 dilution with an avidin–biotin–peroxidase complex (Dakopatts, Glostrup, Denmark). Aminoethyl carbazole (Zymed Laboratories Inc., South San Francisco, CA, USA) was used as a chromogen. Immersed in 2% ammonia water, Meyer's haematoxylin was used for counterstaining and finally, the sections were mounted with Immu-Mount (Shandon, Pittsburgh, PA, USA). Negative controls were prepared using the same procedure except that the primary antibodies were replaced with PBS or serum isotype controls (Zymed Laboratories Inc.).

Immunoreactivity in the samples was assessed semi-quantitatively by assessing both the staining intensity in the nuclei of the tumour cells and the proportion of positively stained nuclei in the tumour cells. The immunoreactivity was evaluated by three independent investigators (MP, SK, PK), who divided the staining reactions into four groups: –, no staining intensity and no positive or only a few positive cells; +, weak staining intensity (>20-49% of positive cells); ++, moderate staining intensity (>50-89% of positive cells); +++, strong staining intensity (>90% of positive cells). If the evaluation of immunoreactivity differed between investigators, the sample was re-evaluated and consensus was reached. The investigators were blinded to the clinical data. For statistical analysis, the immunoreactivity results were separated into two groups: –/+ and ++/+++. Tumour grade was separated into groups of 1-2 and 3, and stages into I-II and III-IV.

Statistical analyses. SPSS 17.0 for Windows (IBM Corporation, Somers, NY, USA) and R-language (R Foundation for Statistical Computing, Vienna, Austria) were used for statistical analysis. The significance of associations was defined using the Mann–Whitney *U*-test and Pearson's Chi-square test; the reported values are two-sided. Survival was analysed by means of Kaplan–Meier curves with log-rank, Tarone–Ware and Breslow tests. Cox multivariate regression analysis was used for multivariate analysis. In the survival analysis, only confirmed death caused by ovarian cancer was considered as an event. Probability values <0.05 were considered significant. The statistician (AS) confirmed the data analysis.

Table I. *Patient and tumour characteristics. *Optimal surgery, patients with no macroscopic residual tumour.*

Characteristics	Number of patients (n=84)	%
Age (years)	58 (32-81)	
Histology		
Serous	63	75.0
Mucinous	7	8.3
Endometrioid	11	13.1
Clear cell	2	2.4
Other	1	1.2
Stage		
I	13	15.5
II	1	1.2
III	51	60.7
IV	17	20.2
Unknown	2	2.4
Grade		
1	11	13.1
2	25	29.8
3	47	56.0
Unknown	1	1.2
Surgery*		
Optimal	28	33.3
Suboptimal	56	66.7

Results

The median concentration of serum 8-OHdG was 176.4 pg/ml (mean 198.6 pg/ml, range 61.7-502.8 pg/ml, standard deviation 80.2 pg/ml). The serum 8-OHdG level 140 pg/ml was recognized as an optimal cut-off level and the serum values above that level correlated to poor ovarian cancer-specific survival (log-rank test, p=0.049; Breslow test, p=0.038; Tarone–Ware test, p=0.041). The patients with serum 8-OHdG concentrations above 140 pg/ml had a mean ovarian cancer-specific survival of 64.5 months, while patients with lower 8-OHdG levels had a mean ovarian cancer-specific survival of 95.9 months (Figure 1A).

Interestingly, the association was even more obvious among the patients with grade 1-2 disease (log-rank test, p=0.032; Breslow test, p=0.040; Tarone–Ware test, p=0.034) (Figure 1B) but an association was not observed in the patients with high-grade EOC. Thirty-six women had grade 1-2 EOC, among whom nine had serum 8-OHdG levels below 140 pg/ml and none of these patients died during the follow-up. In contrast, 11 out of 27 (41.7%) of those women with grade 1-2 cancer and serum 8-OHdG concentrations above 140 pg/ml died from EOC during the follow-up. In multivariate Cox regression analysis, residual disease after surgery was the only independent prognostic factor.

According to the Mann-Whitney test, a high serum 8-OHdG level was associated with advanced stage (p=0.021), serous histology (p=0.002) and suboptimal surgery (p=0.007).

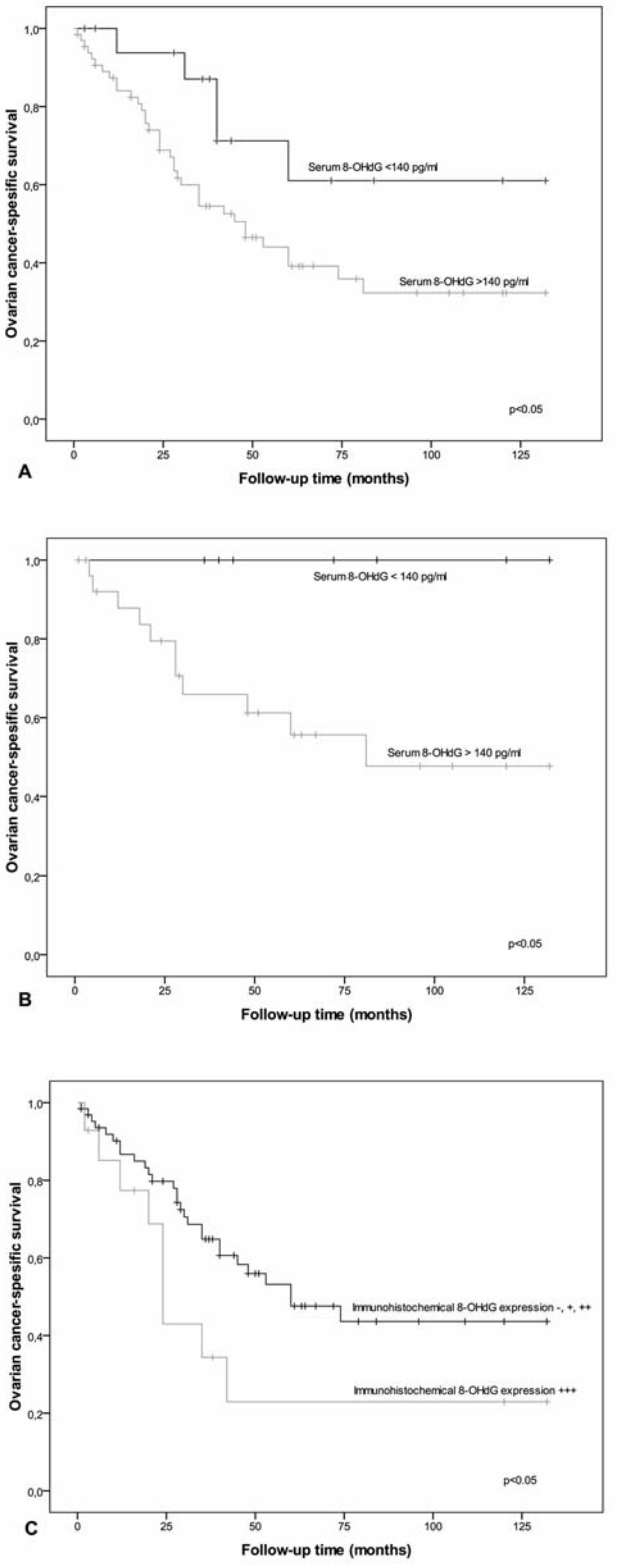

Figure 1. *Kaplan–Meier curves showing ovarian cancer-specific survival. A: Serum 8-OHdG, all patients. B: Serum 8-OHdG, patients with grade 1-2 disease. C: Immunohistochemical 8-OHdG staining. Crosses indicate censored cases.*

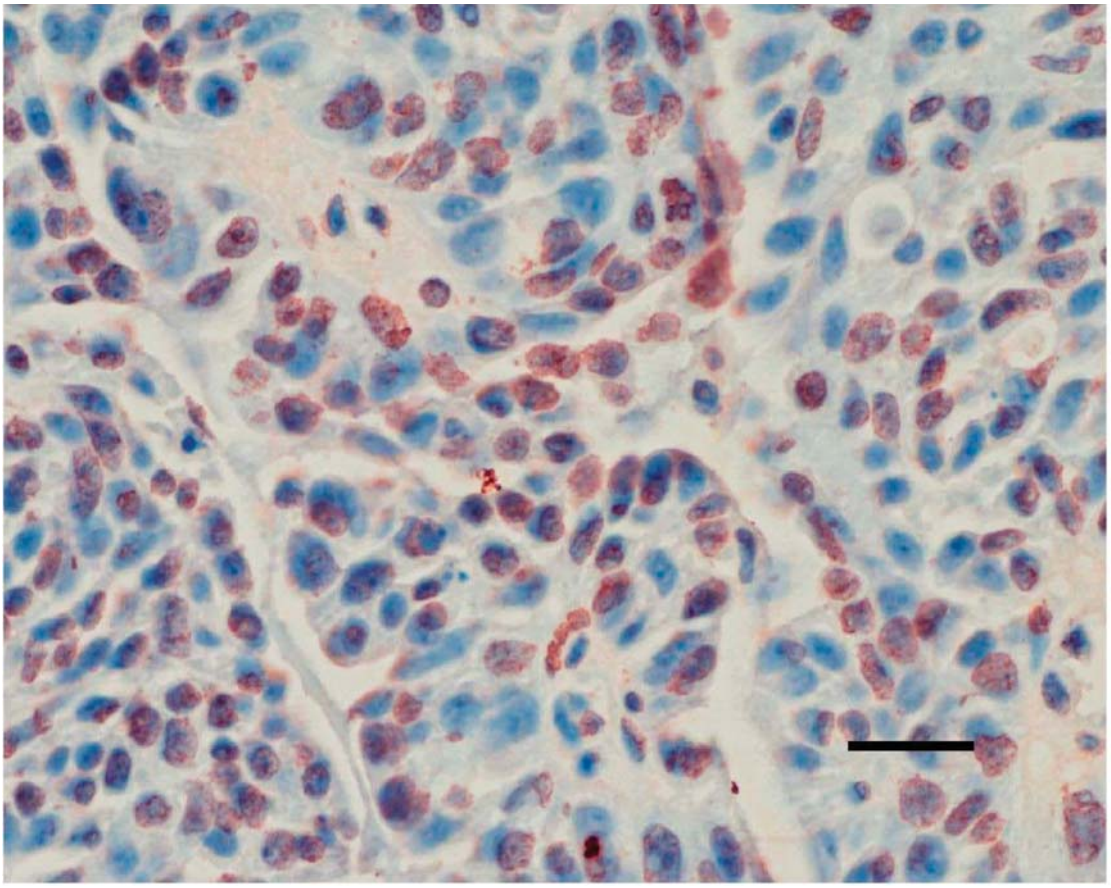

Figure 2. *8-OHdG immunoreactivity in moderately differentiated (grade 2) serous ovarian adenocarcinoma. Bar=50 μm.*

The 8-OHdG immunostaining was located mainly in the nuclei (Figure 2). Forty one percent of the samples were evaluated as negative, 24.4% as +, 16.7% as ++ and 17.9% as +++. IHC 8-OHdG expression in the serous tumours was significantly greater than in the other histological groups (p=0.03). Strong IHC staining of 8-OHdG also correlated with higher grade (p=0.041) and had a trend to be correlated with suboptimal surgery (p=0.094). Highly positive (+++) 8-OHdG IHC expression was associated with poor survival (log-rank test, p=0.053; Breslow test, p=0.045; Tarone–Ware test, p=0.043) (Figure 1C). There was no correlation between serum 8-OHdG levels and tissue 8-OHdG expression.

Discussion

High serum 8-OHdG levels were associated with poor survival in the EOC patients and especially for grade 1-2 disease. However, probably due to the rather limited sample size, only residual disease after surgery had independent prognostic relevance. Higher levels of serum 8-OHdG were found in the patients with serous ovarian carcinoma compared with tumours of other histological types. The serum 8-OHdG concentrations were also noticeably higher in stage III-IV carcinomas compared with more local tumours. Furthermore, preoperative levels of serum 8-OHdG highly significantly predicted suboptimal surgery outcome. Strong 8-OHdG IHC expression in the tumour tissue correlated to poor prognosis and confirmed the results of our previous study (6). The serum levels of 8-OHdG did not correlate significantly with the IHC 8-OHdG expression in the ovarian cancer cells and it is likely that, *e.g.* oxidative bursts of neutrophils, tumour necrosis and certain inflammatory conditions, may affect serum 8-OHdG concentrations.

The mutagenic properties of 8-OHdG have been clearly demonstrated in various experimental models and mutations associated with 8-OHdG are likely to reflect total ROS-derived damage in DNA (17). DNA repair enzyme knockout mice, which are at an increased risk of malignant diseases also displayed increased levels of ROS (18). In our previous study, oxidative stress levels were equal in benign and borderline ovarian tumours, but far less than in ovarian cancer (15). 8-OHdG immunopositivity was observed in

approximately 20% of cases of benign and borderline tumours (15), whereas in the current study 59.0% of the ovarian carcinomas were 8-OHdG-positive. The current study with a larger number of patients was in line with our previous study (6) showing that high IHC 8-OHdG expression in ovarian cancer was associated with poor prognosis and differentiation, higher stage and non-optimal surgical outcome.

Although high serum levels of 8-OHdG have been associated with poor prognosis, in breast cancer, low levels of serum 8-OHdG have rather surprisingly been found to be an independent prognostic factor of poor survival (9). This probably highlights some cancer specificity in antioxidant defence mechanisms and 8-OHdG-specific DNA repair systems. The differences could also be partially explained by the dissimilarities in clinical behaviour between different cancer types. For example, ovarian and lung cancer have often metastasized at the time of diagnosis, and leukaemia is considered to be systemic disease, whereas breast cancer at the time of diagnosis is usually locoregional.

Data on the prognostic value of IHC expression of 8-OHdG in various carcinomas are rapidly increasing. The present results suggest that serum 8-OHdG assessment may serve as a prognostic tool, especially in grade 1-2 EOC, and for this patient group might serve as an additional marker for targeting adjuvant treatment and surveillance. The results also confirm high IHC 8-OHdG expression as a marker of poor prognosis in EOC. If validated in further studies, the preoperative measurements of serum 8-OHdG levels could well have more clinical value than IHC analyses in the assessment of primary operability and adjuvant treatment for ovarian cancer patients.

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