

Biomarker evidence of DNA oxidation in lung cancer patients: association of urinary 8-hydroxy-2'-deoxyguanosine excretion with radiotherapy, chemotherapy, and response to treatment

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Abstract Ratios of urinary 8-hydroxy-2'-deoxyguanosine to urinary creatinine (8-OHdG/creatinine) have been considered as a good biological indicator of DNA oxidation. Urinary 8-OHdG/creatinine levels of lung cancer patients were evaluated by enzyme-linked immunosorbent assay using a monoclonal antibody N45.1 during radiotherapy and chemotherapy. An increase in urinary 8-OHdG/creatinine was found in non-small-cell carcinoma (non-SCC) patients during the course of radiotherapy. SCC patients showed higher levels of urinary 8-OHdG/creatinine than the controls. Furthermore, SCC patients with complete or partial response to the chemotherapy showed a significant decrease in urinary 8-OHdG/creatinine while patients with no change or progressive disease showed an increase.

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Key words: Oxidative DNA damage; Lung cancer; Radiotherapy; Chemotherapy; 8-Hydroxy-2'-deoxyguanosine; Monoclonal antibody

1. Introduction

Radiotherapy and chemotherapy are the primary treatments for non-operable lung cancer [1]. Free radical formation is the key mechanism responsible for producing cytotoxicity in the irradiated cells. Reactive oxygen species generated by ionizing radiation react with guanine bases in DNA to form 8-hydroxyguanine (also as 8-oxo-guanine) [2–6], which has been proposed as a key biomarker of oxidative DNA damage. It is established that either hydroxyl radical, singlet oxygen or photodynamic action is responsible for the formation of 8-OHdG (reviewed in [7]). The oxidized DNA is continuously repaired, and the excised deoxyribonucleosides are excreted in the urine [8–11]. Anthracyclines such as doxorubicin, which are used in combination chemotherapy against

small-cell carcinoma of lung [1], exert their cytotoxicity also via reactive oxygen species [12].

Recently, the concept of 'persistent oxidative stress in cancer' has been hypothesized to partly explain the characteristics of tumor biology such as activated transcription factors and proto-oncogenes, genomic instability, chemotherapy-resistance, invasion and metastasis [13]. Indeed, increasing evidence suggests that malignant cells produce a large amount of hydrogen peroxide [14] and have high levels of oxidized DNA lesions (reviewed in [13]).

Previous animal and in vitro studies have shown increased levels of 8-OHdG in DNA after irradiation [4,5]. There are, however, few clinical studies on this issue. A recent study reported an increase in 8-OHdG content in lymphocytes DNA of cancer patients during exposure to therapeutic doses of ionizing radiation [15]. The levels of urinary 8-OHdG has been indicated to be affected by smoking [16] and metabolic rate [8]. Tagesson et al. recently reported that high levels of urinary 8-OHdG were found in patients subjected to whole body irradiation for bone marrow transplantation of leukemia and lymphoma patients by the use of automated coupled-column high performance liquid chromatography (HPLC) [17].

Recently, we have developed a monoclonal antibody specific for 8-OHdG (N45.1), and constructed an enzyme-linked immunosorbent assay (ELISA) system for 8-OHdG determination [18,19]. An easier measurement of urinary 8-OHdG is possible by this method than HPLC-based methods. In the present study, we for the first time evaluated the changes in urinary 8-OHdG excretion during clinical course of radiotherapy and anthracycline-based chemotherapy for lung cancer patients. In addition, we compared urinary 8-OHdG levels between 37 lung cancer patients and 52 controls which were matched for age, sex and smoking habit.

2. Materials and methods

2.1. Patients and controls

The study was conducted in two Finnish Central Hospitals within the same county. 37 cancer patients and 52 controls entered the study. Patients were previously untreated persons having histologically verified lung cancer. None of the patients had received any high-dose vitamin supplementation during the 3 months prior to the study. The controls were smokers or ex-smokers with no evidence of cancer, living in the same county. The mean age was 65.9 (range 43–82) for

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Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; SCC, small-cell carcinoma; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; Gy, gray; CR, complete response; PR, partial response; NC, no change; PD, progressive disease; ANOVA, an analysis of variance; LSD, least significance difference

the patients and 60.3 (range 45–87) for the controls. The lifetime cigarette consumption was expressed as pack-years (i.e. packs smoked per day × years smoked). The Ethical Committees of both hospitals approved the study protocol and an informed consent form was given by all the participants of the study.

The study consisted of three groups: (1) 16 patients (10 squamous cell carcinoma, 2 adenocarcinoma, 1 small-cell carcinoma, 1 anaplastic carcinoma and 2 histologically unclassified carcinoma), receiving complete cure-aimed radiotherapy, provided urine samples before the therapy, after the total cumulative doses of 2, 10 and 30 Gy, and two months after the last dose of radiotherapy; urine was collected from 0 a.m. to 6 a.m. the following day after each cumulative dose. Most of the patients were hospitalized throughout the course of radiotherapy and received standard hospital diet; (2) 12 patients having small-cell carcinoma of lung (clinical data of the patients are published in Table 1 of reference [12]) received a standard dose of vincristine (1.5 mg/m², maximal dose each time 2.0 mg), doxorubicin (adriamycin; 50 mg/m²) and cyclophosphamide (750 mg/m²) iv on day 1. The first urine samples were collected before the chemotherapy on day 1 from 0 a.m. to 6 a.m., and the second one before the chemotherapy on day 22 from 0 a.m. to 6 a.m. when the patients came to receive the second course of treatment. The patients were hospitalized for two days before giving urine samples; (3) a cross-sectional study was done including the preceding two groups of patients and 9 new untreated lung cancer patients (6 squamous cell carcinoma, 2 adenocarcinoma and 1 histologically unclassified carcinoma) with 52 matched control persons.

2.2. Evaluation of the clinical response to the therapy

Response to the therapy was evaluated by the attending oncologist according to the criteria of World Health Organization [20]. The patients were divided into two groups according to their response as follows: complete or partial response group, and no change or progressive disease group. Complete response (CR) was defined as the disappearance of all known disease determined by two observations not less than two weeks apart; partial response (PR) as a 50% or more decrease in the total tumor mass of those lesions measured during two observations not less than four weeks apart; no change (NC) as cases where a 50% decrease in the total tumor size could not be established, but there also had not been a 25% or greater increase in the size of one or more measurable lesions; progressive disease (PD) as a 25% or greater increase in the size of one or more measurable lesions or the appearance of new lesions.

2.3. Urine samples

Urine samples were obtained from each individual at the first morning voiding after approximately 6 hours' collection (generally 0 h to 6 h), and stored at -80°C until analyses.

2.4. Determination of urinary 8-hydroxy-2'-deoxyguanosine by ELISA

Urine samples were centrifuged at 10000 × g for 10 min and the supernatant after proper dilution was used for the determination with a competitive ELISA kit (8-OHdG check, Japan Institute for the Control of Aging, Fukuroi, Shizuoka). The determination range was 0.64–2000 ng/ml. The specificity of the monoclonal antibody N45.1 used in the competitive ELISA kit has been established [18,19]. The values for urinary 8-OHdG determined by conventional HPLC method and the ELISA method had a fairly good correlation (Ochi, H. and Osawa, T., unpublished data). Urinary creatinine was determined by a kit (Creatinine-test, Wako, Osaka). Data were shown as the urinary [8-OHdG (ng/ml)/creatinine (mg/ml)] ratio.

2.5. Statistical analysis

An analysis of variance (ANOVA) with repeated measurements was applied to evaluate the following three items: (1) differences in 8-OHdG/creatinine between each responding group (CR, PR, NC and PD) of patients; (2) changes in 8-OHdG/creatinine during the treatment period; and (3) interaction between the group and the time-course of treatment. In the chemotherapy group, the changes in 8-OHdG/creatinine were calculated, and the differences between responders (CR/PR) and non-responders (NC/PD) were tested using an unpaired *t*-test. In the cross-sectional study, an ANOVA was used to compare the overall lung cancer patients with their matched controls. Fisher's least significance difference (LSD) method was used in post hoc comparisons to test the influence of small-cell carcinoma of lung on 8-OHdG/creatinine.

3. Results

The creatinine values in each patient of chemotherapy group were intensively followed through the course since the WHO criteria of side effects was also reported elsewhere [12]. There were no significant changes in the values. Irradiation of lung cancer does not usually affect renal function since less than 1% of the total dose is scattered to the distance where kidney is located [21]. Tumor disappearance in non-small-cell carcinoma is so slow that tumor lysis syndrome does not occur.

The results of the changes in urinary 8-OHdG/creatinine level after chemotherapy of small-cell carcinoma patients are shown in Fig. 1. There was no significant difference in the amount of 8-OHdG between the two groups (PR/CR versus NC/PD) before the chemotherapy. A repeated measure ANOVA showed no significant changes over time for all the small-cell carcinoma patients combined (data not shown). The temporal pattern for urine 8-OHdG, however, was markedly different between the two response groups (group × time interaction, $p=0.007$). The patients showing PR/CR to the therapy had a mean decrease of 13.6 in urinary 8-OHdG/creatinine while those with NC/PD showed a mean increase of 10.9. The change in urinary 8-OHdG/creatinine before and after the chemotherapy between the two responding groups was statistically significant ($p=0.007$, unpaired *t*-test).

The changes in urinary 8-OHdG/creatinine in the radiotherapy group are shown in Fig. 2. The patients were divided into two groups on the basis of response to the therapy. There was an increasing trend (10 and 30 Gy) in the values during the course of radiotherapy. Two months after the radiotherapy, urinary 8-OHdG/creatinine returned to the baseline levels. A repeated measures ANOVA found a significant difference between the response groups (PR/CR versus NC/PD, $p=0.02$) and a significant time effect ($p=0.006$). The patients showing no response to the therapy revealed more pronounced increase in urinary 8-OHdG/creatinine at 10 and 30 Gy than the re-

Table 1
Demographic data and urinary 8-OHdG/creatinine levels of lung cancer patients before treatment and of matched controls

	Lung cancer patients ($N=37$)	Controls ($N=52$)
Gender (male/female)	82/18 (%)	90/10 (%)
Age	66 ± 10	60 ± 11
Smoking (pack-years)	28 ± 17	31 ± 19
Urinary 8-OHdG/Creatinine (ng/mg)		
overall	22.6 ± 13.0	19.4 ± 8.5
in SCC ($N=14$)	27.2 ± 17.4*	
in non-SCC ($N=23$)	19.8 ± 8.6	

*Significantly higher ($p < 0.05$, Fisher's least significance difference method) compared to the matched controls and to non-SCC patients. SCC, small-cell carcinoma (means ± SD).

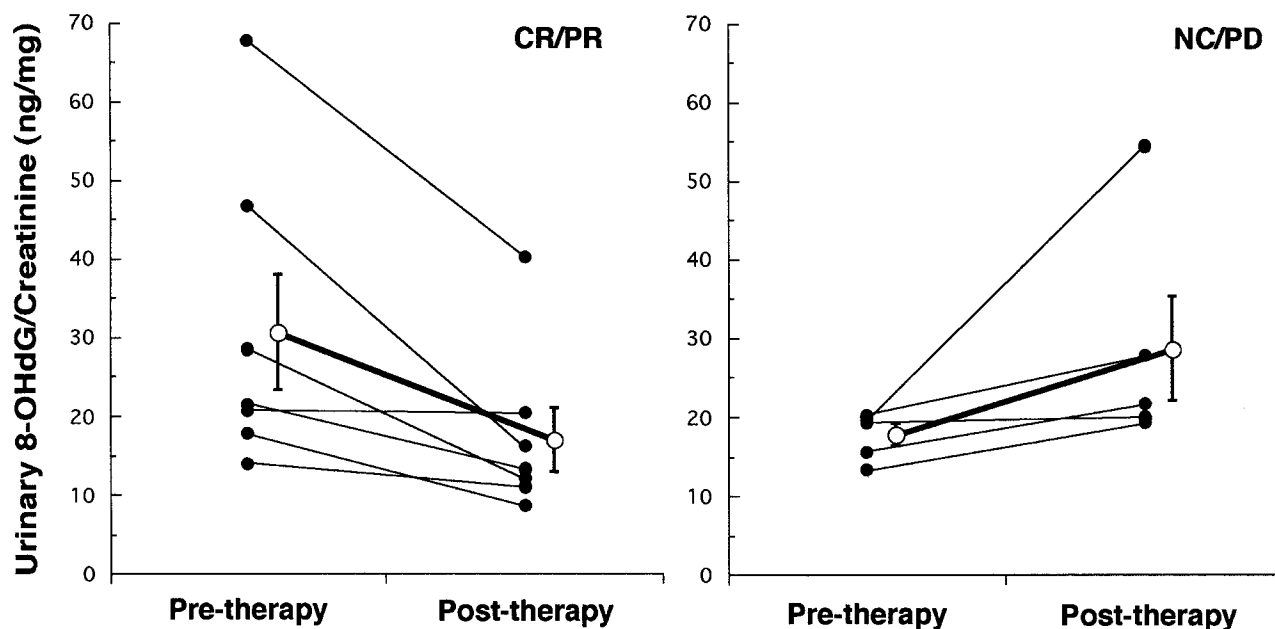


Fig. 1. Urinary 8-OHdG/creatinine (ng/mg) in patients of small-cell carcinoma of lung before and after the chemotherapy. Individual paired data and means \pm SEM are shown. CR, complete remission; PR, partial remission; NC, no change; PD, progressive disease. Refer materials and methods section and results section for detail.

sponding group (group \times time interaction, $p = 0.08$). Three of the patients with PD died before the final measurement.

In the cross-sectional study, there was no significant difference between the cancer group and their matched controls in urinary 8-OHdG/creatinine level (22.6 ± 13 versus 19.4 ± 8.5 ; means \pm SD) as shown in Table 1. The group of small-cell carcinoma patients ($N = 14$) had, however, significantly higher values than the control group (27.2 ± 17.4 versus 19.4 ± 8.5 ; $p < 0.05$, Fisher's LSD test) or than the non-small-cell carcinoma lung cancer patients (19.8 ± 8.6 ; $p < 0.05$, Fisher's LSD test). Non-small-cell carcinoma lung cancer patients showed no significant difference in urinary 8-OHdG/creatinine excretion from the matched controls.

In the cross-sectional study, the lung cancer patients were slightly older than the healthy controls (66 versus 60 years), but there were no significant differences between the groups with regard to gender or smoking habits. None of these variables correlated with the baseline urinary 8-OHdG/creatinine level in the present study.

4. Discussion

The major findings in our study are that (1) oxidative DNA damage, as expressed by urinary 8-OHdG/creatinine level, increased during the course of radiotherapy in lung cancer patients with no change/progressive disease response, (2) small-cell carcinoma patients had higher levels of urinary 8-OHdG/creatinine before therapy than control subjects or non-small-cell carcinoma lung cancer patients, (3) significant increase in urinary 8-OHdG/creatinine was observed after chemotherapy in small-cell carcinoma patients with no change/progressive disease response whereas significant decrease in urinary 8-OHdG/creatinine was found in small-cell carcinoma patients with complete remission/partial remission response.

Previous experimental studies have indicated increased lev-

els of 8-OHdG in cells and tissues after irradiation [4,5] and this has recently been confirmed in clinical situations as well [15,17]. The present report is the first to show an increase in urinary 8-OHdG in lung cancer treatment. The maximal urinary excretion of 8-OHdG/creatinine was observed after total cumulative doses of 10 and 30 Gy. It is crucial to note that the patients showing radiation therapy-resistance showed more pronounced increase in their values than did the responding ones. These results are likely to be affected by the decrease in the tumor mass via radiation-induced necrosis. Our data suggests that tumor necrosis is not the cause of increase in urinary 8-OHdG/creatinine levels. However, this has to be carefully elucidated.

We found no difference in urinary 8-OHdG/creatinine excretion between the overall lung cancer patients participated in the present study and their matched controls. The small-cell carcinoma patients, however, had significantly higher levels of urinary 8-OHdG/creatinine than the controls. Small-cell carcinoma of lung is a highly malignant systemic disease characterized by rapid and wide spread dissemination of the tumor cells at the time of diagnosis. We have previously reported a decreased plasma peroxyl radical trapping capacity in small-cell carcinoma patients [22]. Thus, it appears that lung cancer of this histologic type is associated with an increased oxidative stress that is most likely to be due to the systemic nature of the disease.

Small-cell carcinoma of lung is highly sensitive to chemotherapy and most of the patients respond to the initial treatment. In 30–40% of the cases, CR will be obtained. Survival beyond 5 years is, however, achieved in only 10–20% of the patients with limited disease and 3–5% of the patients with extensive disease [1,23]. Seven patients in our study showed initial CR or PR to the therapy. The levels of 8-OHdG/creatinine clearly decreased compared to the baseline in these patients. We believe that this reduction reflects the decrease

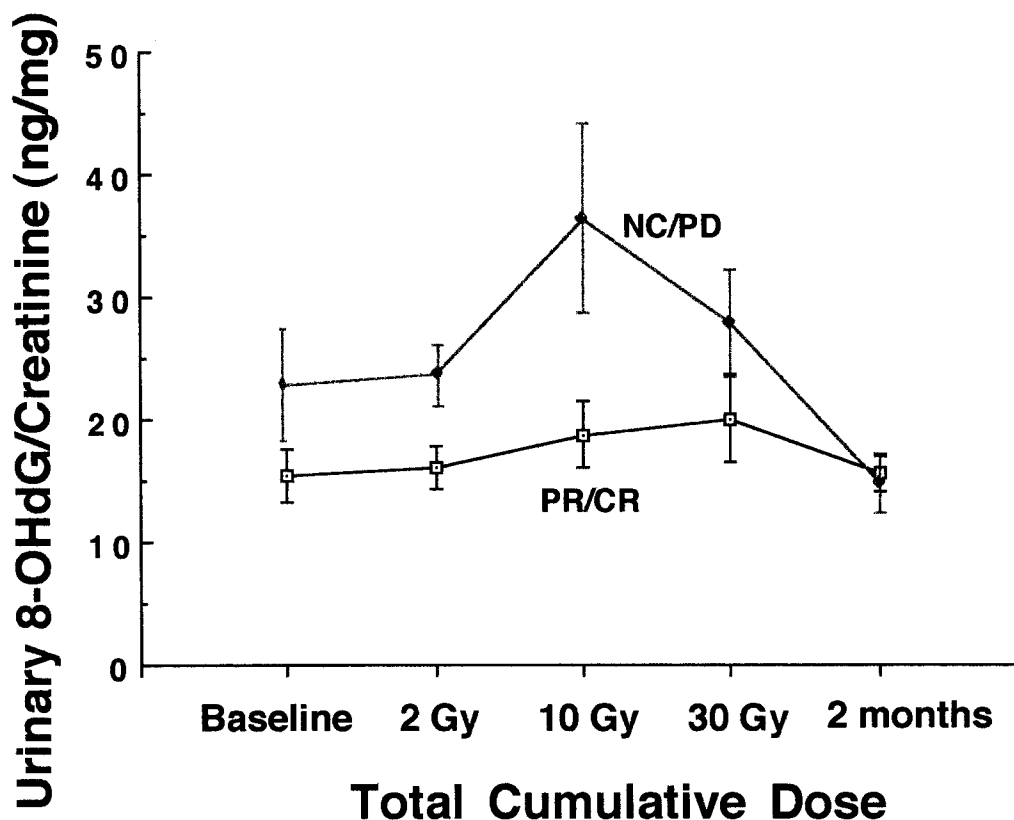


Fig. 2. Urinary 8-OHdG/creatinine (ng/mg) in patients of non-small-cell carcinoma of lung before, during and after the radiotherapy (means \pm SEM; PR/CR, $N=9$; NC/PD, $N=7$). Refer materials and methods section and results section for detail.

in the tumor mass and, thus, a relief of oxidative stress as well. In contrast, patients with PD or NC response showed a significant increase in urinary 8-OHdG/creatinine levels.

Urinary 8-OHdG has been proposed as a highly sensitive index of oxidative stress, but various individual factors such as smoking [16,24] have been reported to possibly affect its levels. We analyzed the data in consideration of the effect of smoking. Diet is unlikely to be a confounding factor here because most of the patients were hospitalized during the therapy and thus received a standard hospital diet. Furthermore, it was shown in animal experiments that urinary 8-OHdG levels were not affected by the nucleic acid content of the diet though urinary 8-hydroxyguanine levels were affected [8].

The ELISA method we have used in the present experiment depends on the specificity of monoclonal antibody N45.1 for 8-OHdG since this antibody recognizes both the modified base and deoxyribose structure [19]. The ELISA method is superior to HPLC methods in the following aspects: (1) no need for any expensive equipment except an ELISA reader; (2) reduced analyzing time; (3) low running cost; and (4) small urine sample volume required. This method will be useful in a variety of experiments and clinical settings.

5. Conclusion

Our original findings warrant further clinical prospective studies especially in small-cell carcinoma of lung. It is tempting to speculate that a follow-up of urinary 8-OHdG/creatinine might in the future be a useful tool to evaluate the re-

sponse to therapy and help in selecting the patients who are most likely to benefit from the treatments. This potential use is quite timely considering the heated discussion in Great Britain just now about totally quitting small-cell carcinoma treatments due to their poor cost-benefit ratio in public health care.

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