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RESEARCH ARTICLE

Examination of The Relationship Between DNA Damage and Inflammation in Primary Non-Small Cell Lung Cancer Patients

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Examination of the Relationship between DNA Damage and Inflammation in Primary Non-Small Cell Lung Cancer Patients

Summary

Reactive oxygen species secreted by inflammatory cells can damage DNA by several mechanisms and so contribute to progression of carcinogenesis. Recent studies suggest that chronic inflammation plays an important role in the development of lung cancer. Our aim in this study was to investigate the relationship between 8-hydroxy-2-deoxyguanosine (8OHdG), as a marker of oxidative DNA damage, and C-reactive protein (CRP), as a marker of chronic inflammation, in non-small cell lung cancer. 8OHdG was measured by the gas chromatography – mass spectrometry (GC-MS) method in 24-hour urine samples and C-reactive protein (CRP) was measured by the nephelometer in serum of patients with primary non-small cell lung cancer (n=21) and healthy controls (n=20). The levels of CRP and 8OHdG were significantly higher in patients than those in the controls (p<0.0001 and p<0.005, respectively). There was significantly positive correlation between the levels of 8OHdG and CRP (r=0.636, p=0.002) in patient group. The correlation was significantly higher in current smoker patients and advanced cancer patients (r=0.830, p=0.0001 and r=0.744, p=0.002, respectively). Our results suggest that reactive oxygen species secreted by inflammatory cells may contribute to DNA damage in progressing of lung carcinogenesis.

Key Words: *C-reactive protein, 8OHdG, lung cancer.*

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Primer Küçük Hücreli olmayan Akciğer Kanserli Hastalarda DNA Hasarı ve İnflamasyon Arasındaki İlişkinin İncelenmesi

Özet

İnflamatuvar hücreler tarafından salgılanan reaktif oksijen türleri çeşitli mekanizmalarla DNA'ya hasar verebilir ve böylece karsinogenezin ilerlemesine katkıda bulunur. Son çalışmalar, akciğer kanseri gelişmesinde kronik inflamasyonun önemli bir rolü olduğunu öne sürmektedir. Bu çalışmada amacımız, küçük hücreli olmayan akciğer kanserinde oksidatif DNA hasarının bir belirteci olarak 8-hidroksi-2-deoksiguanozin (8OHdG) ve kronik inflamasyonun bir belirteci olarak C-reaktif protein (CRP) arasındaki ilişkiyi incelemekti. Primer küçük hücreli olmayan akciğer kanserli 21 hastanın ve 20 sağlıklı kontrolün, 24 saatlik idrar örneklerinde gaz kromatografi–kütle spektrometre (GC-MS) metodu ile 8OHdG, ve serum örneklerinde nefelometrik metot ile CRP düzeyleri ölçüldü. Hastaların CRP ve 8OHdG düzeyleri, kontrollerinkinden belirgin derecede yüksekti (p<0.0001 ve p<0.005, sırası ile). Hasta grubunda 8OHdG ve CRP arasında belirgin pozitif bir korelasyon (r=0.636, p=0.002) vardı. Sigara içen hastalarda ve ilerlemiş kanser hastalarında bu korelasyon daha belirgindi (r=0.830, p=0.0001 ve r=0.744, p=0.002, sırası ile). Sonuçlarımız, inflamatuar hücrelerden salınan reaktif oksijen türlerinin akciğer kanserinin ilerlemesinde DNA hasarına katkıda bulunabileceğini öne sürmektedir.

Anahtar Kelimeler: *C-reaktif protein, 80HdG, akciğer kanseri.*

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INTRODUCTION

There are many stages where both environmental and endogenous oxidants and nitrosants potentially influence lung carcinogenesis. The oxidants from metabolic activity, inflammation, radiation, or toxins such as cigarette smoke, can damage macromolecules, generating lesions that can contribute to cancer (1,2). Evidence suggests that chronic inflammation in tissues contribute to about one-third of all the known cancers (3,4).

An infection or cellular damage may initiate inflammation process, which then stimulate the synthesis and activation by macrophages of interleukin (IL)-6, nuclear factor (NF)-κB, and tumor necrosis factor (TNF)-α. These molecules induce some proteins such as p-selectin that contribute sending more leukocytes to the infected area, which produce ROS/RNS (5,6). Activated neutrophils release excess of $O_2^{-\cdot}$ and cause beginning of the production of more toxic reactive species. Neutrophils also release the myeloperoxidase (MPO), together with H_2O_2, which is produced by dismutation of $O_2^{-\cdot}$. This enzyme catalyzes the reaction of H_2O_2 with Cl^-, resulting in the formation of HOCl. Also reaction of H_2O_2 with HOCl produces $^1O_2$, and reaction of $O_2^{-\cdot}$ with nitric oxide ($NO^{\cdot}$) produces $ONOO^-$. Formation of $^{\cdot}OH$ results from reaction of $O_2^{-\cdot}$, H_2O_2, and iron or reaction of $O_2^{-\cdot}$ and $NO^{\cdot}$. (7-8). These reactive species kill the pathogens by halogenation or by protein and lipid peroxidation.

Excessive and continuous ROS/RNS produced by inflammatory cells leads to oxidative modification of lipids, proteins, and DNA bases and stimulates oncogenes. ROS/RNS interact with DNA resulting in genomic alterations such as point mutations, deletions, or rearrangements (9). Reaction of ROS/RNS with purine and pyrimidine bases of DNA will generate several oxidative products, such as 8OHdG, 8OHGua (8-hydroxyguanine), thymine glycol, 5-hydroxymethyluracil, 5-hydroxycytosine and others (reviewed in 10).

Reactive species can also attack lipids and induce formation of lipid peroxidation products. Some of these products, such as HNE or MDA, can directly react with DNA (11). These aldehydes can also react with some amino acids in DNA repair proteins and destroy protein functions (12). Therefore, they can reduce DNA repairing capacity. These effects caused by intracellular aldehydes play an important role in mutagenesis and carcinogenesis (13).

In this study, we wished to determine the oxidative status by the levels of MDA, and 80HdG in patients with non-small cell lung cancer in comparison with healthy controls and to examine whether these parameters are associated with inflammatory response by the levels of CRP.

MATERIALS and METHODS

Study Population

Baseline characteristics of the total patients and controls were given in Table I. Twenty one patients with primary non-small cell lung cancer; all men (age range 45 to 78) were included in the study. All patients had been newly diagnosed by bronchoscopic punch biopsy (or) and bronchial lavage cytology, and had no any clinical or radiological anti-cancer treatment at the time of the study. Patients had histologically proven adenocarcinoma (n:8), epidermoid carcinoma (n:13). The patients were classified according to the TNM system (14). None of the patients had any treatment.

Patients were followed up by the Chest Diseases Department of Social Insurance Educational Hospital in Ankara. Informed consent was obtained from all subjects before the study. This study was approved by Ethics Committee of Social Insurance Educational Hospital of Ankara.

Twenty age- and sex-matched healthy controls; all men (with age range 46 to 77) were also included in the study. Controls were selected from healthy subjects attending the Clinics of Chest Disease in the hospital. All controls were apparently healthy and without a history of hypertension, diabetes, renal disease and any acute disease.

Urine samples (24 h) were collected from the patient and control groups and were kept frozen at –80°C.

Measurements of Parameters

Determination of 8-Hydroxy-2′-deoxyguanosine (8OHdG)

The level of 8OHdG was determined in urine by the gas chromatography-mass spectrometry (GC-MS) according to the method of Mei et al. with some modifications in sample preparation procedure (15). Urine samples were thawed and centrifuged at 2000g for 10 min. pH of the urine was adjusted to approximately by the addition of dilute HCl. 10 mL methanol, 10 mL water and 10 mL of 5 mM KH2PO4, pH 7.5 (buffer A) were used for preconditioning of the solid phase extraction columns (C18 OH, 500 mg) (Varian, Harbor City, CA, USA). A 5 mL volume of urine spiked with a 2.5 mL certain concentration 8OHdG standard was applied to the column. The column was washed with 3 mL of buffer A, and 8OHdG was eluted with 3 mL of 15% methanol in buffer A. After mixing of the eluate with 6 mL acetonitrile, it was evaporated to become dry at 40°C under a stream of nitrogen and the residue was dissolved in 1 mL methanol. The final methanol solution of solid-phase extraction was transferred into a derivatization glassware, and the organic phase was evaporated to dryness at 48°C under a stream of nitrogen. The residue was trimethylsilylated with a 50 μL mixture of BSTFA and acetonitrile (2:1, v/v) (1 mL acetonitrile containing 100 mg pyrene as an internal standard) by heating for 1 h at 100°C. After the incubation, samples were directly injected onto a GC-MS system.

HP 6890 gas chromatograph and HP 5972A mass spectrometer (Hewlett Packard) were used in the analysis of 8OHdG. The samples were applied onto a ZB-5 capillary column (30 m=0.25 mm i.d., 0.25 mm film thickness) with helium as carrier gas (flow rate 1 mL/min). Injection port temperature was 260°C and initial oven temperature was 210°C for 5 min rising to 270°C at 10°C/min for 6 min. The ions at m/z 383.0, m/z 368.0 and m/z 643 for 8OHdG were monitored under selective ion recording conditions (ionization energy 70 eV) with a 30-ms dwell time on each ion.

C-Reactive Protein (CRP)

CRP was measured by the N-latex assay in an automated analyzer (a Dade-Behring BN 100 nephelometer, Marburg, Denmark).

Statistical Analysis

Data were expressed as the mean ± SD, and statistical analyses were performed by student's t-test and Mann-Whitney U test (for groups of small numbers). Pearson correlation coefficients were calculated for the relationship among parameters. Statistical analyses were performed with a SPSS 10.0 Package (SPSS Inc., USA).

RESULTS

Baseline characteristics of the patients and controls are given in Table 1. The age of the patients and controls was similar. BMI of patients was lower than those of controls (p=0.05). There were several metastases in twelve patients.

Plasma levels of 8OHdG and CRP were significantly higher in the patients than those in controls, as shown in Table 2 ($p<0.005$ and $p<0.0001$, respectively). When the patients were grouped according to the stage of disease (I+II and III+IV), there were no significant differences in the measured parameters between two groups.

Table 1: Baseline characteristics of the patients and controls

Variables	Patients (n=21)	Controls (n=20)
Age (years)	62.1 ± 11.0*	62.7 ± 8.4
BMI (kg/m2)	23.5 ± 3.5*	25.8 ± 3.9
Never smoker (%)	9.5	5.0
Smoker Included (%)	71.4	35.0
Ex-smoker (%)	19.0	60.0
Using Alcohol (%)	9.5	20
Family History of Cancer (Y/N)	6/15	1/19
Metastase (Y/N)	12/9	---
Stage of Disease (I/II/III/IV)	5/2/2/12	---

* Non-significant when compared with controls

Table 2: The levels of 8-OHdG and CRP in the patients and controls

	Mean Levels ± SD (95%Confidence Interval)	
	NSCLC (n=21)	Controls (n=20)
CRP (mg/L)	70.36 ± 40.32a (52.01-88.72)	6.93 ± 4.88 (4.64-9.21)
8-OHdG (nmol/l)	1.80 ± 0.87b (1.40-2.19)	1.12 ± 0.46 (0.90-1.33)

[a] P<0.0001, [c] P<0.02 compared with controls

By bivariate correlation analysis of the measured parameters, 8OHdG and CRP was positively correlated in whole patients (R=0.636, p=0.002) (Figures 1). According to the stage of the disease, there was a strong correlation (R=0.744, p=0.002) in the advanced stage (stages III and IV) (Figure 2), whereas there was no correlation in the early stage (stages I and II). According to the sub-type of NSCLC, there were positive correlations between parameters in both epidermoid carcinoma and adenocarcinoma groups (R=0.535, p=0.06 and R=0.789, p=0.02, respectively) (Figures 3 and 4). We also found significantly high correlation between parameters in the current smoker NSCLC patients (Figure 5).

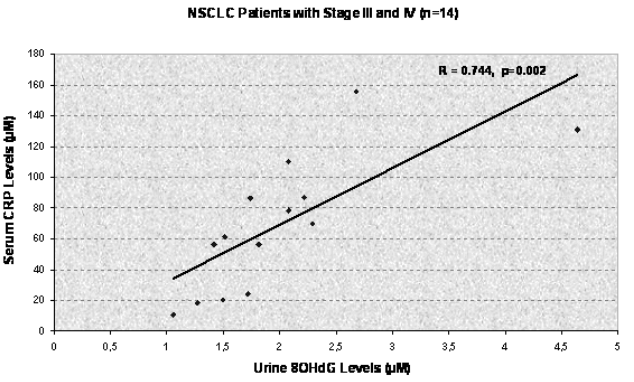

Figure 2. Correlations between CRP and 8OHdG levels in NSCLC patients with Stage III and IV.

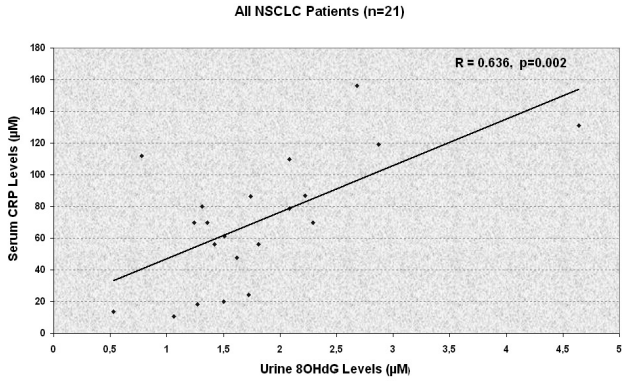

Figure 1. Correlations between CRP and 8OHdG levels in all NSCLC patients.

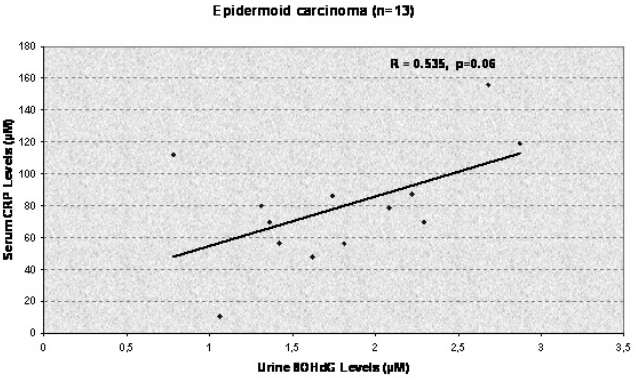

Figure 4. Correlations between CRP and 8OHdG levels in epidermoid carcinoma patients.

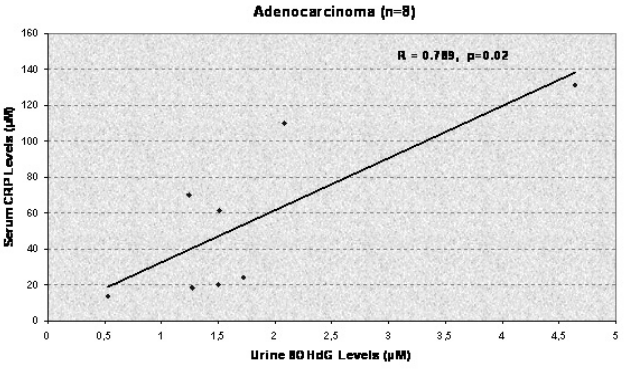

Figure 3. Correlations between CRP and 8OHdG levels in adenocarcinoma patients.

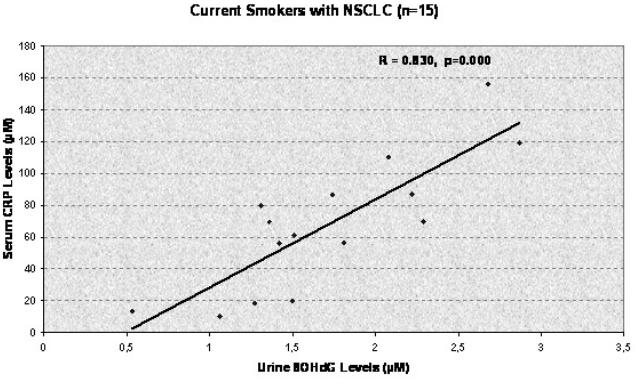

Figure 5. Correlations between CRP and 8OHdG levels in current smokers with NSCLC.

DISCUSSION

There is growing evidence that demonstrate the direct relationship between chronic inflammation and cancer. As part of the immune system, phagocytic cells, such as neutrophils, macrophages, and monocytes play very important role during an inflammatory response. Phagocytic cells produce toxic substances upon activation to kill bacteria, cells infected with bacteria, and apoptotic/necrotic cells. These toxic substances include proteolytic enzymes, nitric oxide, and reactive oxygen species. Reactive oxygen/nitrogen species (ROS/RNS) kill the pathogens by halogenation or by protein and lipid peroxidation. Excessive and continuous ROS/RNS produced by inflammatory cells leads to oxidative modification of lipids, proteins, and DNA bases and stimulates oncogenes. (4,5,6,16)

In several studies, markers of oxidative stress and inflammatory response were separately investigated in lung cancer (17-22). C-reactive protein is a well-known acute phase reactant, synthesized in response to general inflammatory episodes in humans. It is expressed principally in hepatocytes, and to be less in adipocytes and coronary artery smooth muscle cells (23). Trichopoulos et al. demonstrated higher CRP levels in lung cancer patients than those in healthy controls, and suggested that plasma CRP is a potential marker of increased cancer risk (24). Similarly, other studies showed higher CRP levels in the patients with lung cancer (19,25,26). Consistent with other studies, we found higher CRP levels in NSCLC patients than in healthy controls.

ROS/RNS produced by inflammatory cells also stimulate some oncogenes such as jun and fos. It was reported that overexpression of jun to be directly associated with lung cancer (27,28). It was established that DNA damage produced by activated phagocytes is a result of attack by •OH radical. In general, •OH radical is the major source of DNA damage that attacks phosphate, deoxyribose, and base sites, resulting in strand breakage. The hydroxylation of guanine in the 8-position is the frequent mutagenic lesion induced by oxidative species. (29,30) 8OHdG is one major product of guanine nucleotide oxidation in DNA. Proteins and lipids are also significant targets of ROS/RNS. Modification of protein molecules may indirectly result in mutagenesis through oxidative modification of DNA polymerase or inhibition of DNA repair enzymes (31-34). In addition, interactions of ROS and lipids result in formation of genotoxic lipid peroxidation by-products that react with the DNA. (31,32). These products can also react with some amino acids in DNA repair proteins and destroy protein functions (12). Therefore, they can reduce DNA repairing capacity (13). Urinary 8OHdG levels were intensively studied in several types of cancer, including lung cancer and other diseases (35). In the current study, 8OHdG levels in urine were significantly higher in NSCLC patients than in healthy controls. Consistent with all the other studies, this study also showed correlation between inflammation and free radicals; we found strong correlations between CRP and 8OHdG in all NSCLC and sub-groups of disease. There was also high correlation between these parameters in advanced NSCLC patients, not in early stage of the disease.

Smoking is a well-known risk factor in the initiation and progression of lung cancer. Cigarette smoke contains highly reactive oxygen/nitrogen species in addition to a number of known carcinogens (2,36). The reactive oxidant species (ROS) generated by smoking induce inflammation in the lung and its airways as well as cause mutations in airway epithelial cell DNA. The risk of developing lung cancer does not disappear after giving up smoking. (23,37,38). Although some researchers found high 8OHdG levels in smokers (39,40), others did not observe any significant differences between smokers and non-smokers. (41,42) In addition, a link has been shown between smoking and several chronic inflammatory diseases, such as chronic obstructive pulmonary diseases (43), rheumatoid arthritis (44). In our study, we couldn't observe the effects of smoking on 8OHdG and CRP levels; this might be because we had a small number of non-smoker patients. However, we observed very high correlation between CRP and 8OHdG in patients who smoked.

In conclusion, our results may suggest that reactive oxygen species secreted by inflammatory cells may contribute to DNA damage in progressing of lung

carcinogenesis. Studies on reversing plasma antioxidant levels by supplementation could not usually be successful in cancer patients. Anti-inflammatory therapy may be beneficial to improve the plasma antioxidant levels and decrease releasing ROS by neutrophils in lung cancer patients, especially in early stage of disease. Further studies are needed to put forth much more detailed information on this subject.

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