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DNA Damage and Repair Capacity in Lung Cancer Patients: Prediction of Multiple Primary Tumors

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Abstract

Purpose—Patients who survive one non-small cell lung cancer (NSCLC) are at higher risk of a second malignancy. Capacity to repair damaged DNA may modulate individual susceptibility to develop lung cancer. Therefore, we evaluated constitutive and induced DNA damage, and repair capacity, in patients with multiple NSCLC (cases) and compared the results to those obtained in patients with single NSCLC (controls).

Methods—One-hundred and eight cases and 99 controls matched by age, gender and time since diagnosis were studied. DNA damage was assessed on peripheral blood lymphocytes by the comet assay before and after exposing cells to a tobacco-derived carcinogen, using the tail moment (TM), and the tail intensity (TI) as measures to assess baseline damage, induced damage and repair capacity.

Results—Constitutive DNA damage, BPDE-induced damage, and repair after BPDE-induced damage were all significantly higher in cases than in controls. These results were confirmed in regression analyses adjusted for potential confounders.

Conclusion—DNA damage as measured by the comet assay is associated with the development of multiple primary tumors in individuals with NSCLC.

Introduction

Lung cancer is the second most frequent cancer in both males and females in the U.S. after breast and prostate cancer; and the most common cause of death from cancer in men and women aged from 40 and 60, respectively¹. A major etiologic factor is exposure to tobacco

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smoke. However, only a fraction of heavy smokers develop lung cancer. It is probable that people differ in their susceptibility to tobacco smoke-inflicted damage and that their interindividual differences, attributed to heritable traits, modulate the risk of lung cancer².

Regardless of the etiology, the high incidence and poor prognosis of lung cancer make this disease a major health problem worldwide. Patients who survive one primary cancer are at considerably higher risk of a second malignancy. Current evidence suggests that the annual incidence of second primary lung cancers is in the region of 1–2%^{3,4}. A second primary lung cancer is potentially curable by surgical resection if discovered early and if the initial primary is also cured. Distinguishing patients that are at higher risk to develop multiple lung tumors is of great clinical relevance. Moreover, risk factors that are identified for a second primary lung cancer are highly likely to be risk factors for lung cancer in general. Thus studies of factors that differ significantly between patients with second primary lung cancer and patients with a solitary lung cancer are convenient vehicles for identifying new risk factors for the disease⁵. This study design has been utilized, for example, in studies of contralateral breast cancer^{6,7}.

There is considerable evidence that individual variation in the ability to limit DNA damage from endogenous and exogenous carcinogens contributes to cancer risk^{8–10}. A number of epidemiological studies in which a variety of global measures of DNA repair capacity were used found that individuals with lung cancer displayed a higher rate of spontaneous and carcinogen-induced chromosomal aberrations than the healthy controls^{11–14}. Similarly, patients with lung cancer displayed a lower DNA repair capacity than controls^{13,15}, which in the presence of a deficient cell cycle checkpoint increased the risk for developing lung cancer even further¹⁴. Paz-Elizur *et al* evaluated a more specific aspect of repair. The enzymatic 8-oxoguanine DNA N-glycosylase (OGG) activity was significantly decreased in lung cancer patients and the risk of developing non-small cell lung cancer for smokers with low OGG1 activity was observed to be 34 to 124-fold higher than for smokers with a normal OGG1 activity¹⁶.

In the present study we sought to identify the risk of developing multiple primary lung tumors in relation to levels of constitutive and induced DNA damage among patients with a single non-small cell lung cancer (NSCLC). For this purpose, constitutive, induced, and unrepaired damage were quantified by measuring single strand DNA breaks and abasic sites of untreated and treated peripheral blood lymphocytes using the alkaline comet assay¹⁷. This assay provides a measure of the net effect of each cell's anti-oxidant and DNA repair capacity to prevent and repair oxidative damage, the major endogenous source of genomic instability. To evaluate the cells' sensitivity to the potent tobacco smoke constituent benzo[a]pyrene (BaP) which is also found in fuel combustion, car exhaust, occupational settings, and contaminated air, cells were exposed to the main metabolic product and protein reactive benzo[a]pyrene diol epoxide (BPDE)¹⁸. *In vitro* assays indicate that the BPDE-DNA adducts are formed in less than an hour, while the removal of the induced damage can take several hours^{19,20}, and depending on the position on the DNA, some adducts remain unrepaired²¹. With the comet assay, the BPDE-modified DNA strands are revealed as fragmented DNA due to alkaline lability, presence of apurinic sites, and cleavage in the DNA at guanine, adenine or cytosine sites²². BPDE-DNA adducts are repaired mainly by

the nucleotide excision repair pathway, although some unstable adducts may produce depurination that can be repaired by the base excision repair pathway^{23,24}. In this study we hypothesized that endogenous, BPDE induced, and unrepaired damaged DNA as measured by the comet assay would be higher in patients who develop multiple primaries than in those who develop single primary tumors.

Materials and Methods

Study Subjects

All participants were recruited at Memorial Sloan-Kettering Cancer Center and gave written informed consent to participate in this case-control study approved by the institutional review board. One-hundred and eight individuals diagnosed with a second primary cancer (cases) and 99 individuals diagnosed with a first primary lung cancer (controls) participated, matched by age (± 5 years), sex, and time since diagnosis. The distinction between a second primary tumor and a metastasis from the first primary was established as previously described by Martini and Melamed²⁵. Briefly, patients met the criteria for having multiple primary lesions if both lung tumors had a different histology, or if the histology was the same but the second tumor originated from a carcinoma in situ, or was located in a different lung or lobe with no evidence of lymphatics common to both and no extrapulmonary metastases at the time of diagnosis. All participants donated blood and completed a questionnaire that provided detailed information on demographic data, exposure to tobacco smoke, occupational exposures, family history of cancer, dietary and exercise habits, medications, reproductive history, and past treatments for non-cancer conditions. In addition, clinicopathologic information was collected from the medical records.

Blood samples

Blood samples were collected in 2 to 3 green top heparinized Vacutainer tubes. These were light-protected, and immediately transported at room temperature to the Molecular Epidemiology Laboratory at Memorial Sloan-Kettering Cancer Center. Lymphocytes were isolated with standard procedures (Ficol) and cryopreserved in liquid nitrogen at a concentration of $1 \times 10^6/\text{mL}$. Ninety-six hours prior to the comet assay, lymphocyte cultures were established in three T-25 flasks using RPMI-1640 media supplemented with 20% fetal calf serum, antibiotics and 1.5% phytohemagglutinin (PHA) in a final volume of 10 mL.

Comet Assay or single-cell gel electrophoresis

This assay was utilized to measure constitutive genetic instability, or baseline damage. Briefly, by this method, one can determine the constitutive or unrepaired DNA damage represented by shorter DNA strands that migrate faster in the electrophoretic field and resemble the tail of a comet. This assay has been used to monitor genotoxic effects of certain compounds, as well as to measure genetic instability associated with certain conditions including cancer.

We performed the comet assay under alkaline conditions as described by Singh et al¹⁷ with minor modifications. Briefly, ten microliters of a cell suspension containing approximately 11000 cells were mixed with 100 μL of 0.5% low-melting-point agarose in PBS, kept at

37°C in a dry-bath incubator, spread on CometSlides™ HT slides (Trevigen, Gaithersburg, MD). The agarose solidified at 4°C for 10 minutes. Cells were lysed for 1h at 4°C in a freshly prepared lysis buffer (pH 10) containing 2.5M NaCl, 100mM EDTA, 100mM Trizma base, 10% dimethyl sulfoxide (DMSO) and 1% Triton X-100. Slides were then rinsed three times in 0.4M Tris-HCl (pH 7.5) for 5 minutes to remove detergents and salts and placed on a horizontal electrophoretic unit without power for 30 minutes in freshly prepared alkaline buffer (300mM NaOH and 1mM EDTA, pH>13) at 4°C. Electrophoresis was carried out in a TECA2222 electrophoresis unit (Ellard Instrumentation Ltd, Monrow, WA) during 30 minutes at 25 volts, adjusting the current to 295–300 mAmp and with a constant recirculating flow of 100mL/min. All these steps were carried out in the dark. Finally, the slides were rinsed in neutralization buffer (0.4M Tris-HCl, pH 7.5) for 5 minutes three times, fixed in cold 100% ethanol for 15 minutes and air dried. To control the comet assay performance, each run included an internal laboratory control comprised of a pool of lymphocytes. In addition, room temperature and humidity levels were recorded.

Response to induced damage and DNA repair capacity

A modification of the basic alkaline comet assay was introduced in order to test the cells' response to and their capacity to repair after *in vitro* induced damage. In addition to the baseline comet assay described above, we grew two culture flasks with cells that were exposed to 0.25µM benzo[a]pyrene diol epoxide (BPDE). After one hour of incubation, all cells were washed with fresh media. Cells from one flask were processed for the comet assay in order to measure the sensitivity to BPDE while cells from the second flask were allowed to recover from the damage for 24 hours and then processed in order to measure the DNA repair capacity. The BPDE (CAS registry number 58917-67-2) was purchased from the National Cancer Institute Chemical Carcinogen Repository (Midwest Research Institute, Kansas, MO, USA), dissolved in DMSO (Sigma Chemical Co) at a concentration of 2mM, and stored at –20°C in the dark to prevent photo-oxidation.

Scoring of DNA damage

Immediately before imaging analysis, slides were stained with 10µl of a 1µg/mL ethidium bromide solution for 5 minutes. Observations were made at 200× magnification using a fluorescent microscope (Olympus BX51) connected to a CoHu 4915-2010 CCD camera. One hundred consecutive cells (50 from each duplicate slide) were randomly selected with care to avoid borders, starting with the center of the slide, and quantified with the Komet 5.5 software (Kinetic Imaging, UK). The extent of the damage was quantified by the tail moment (TM) and the tail intensity (TI). The most commonly reported parameter is the tail moment (TM), defined as the product of the percentage of DNA in the comet tail and the distance between the means of the tail and head fluorescence distributions, where 'mean' is the profile center of gravity, divided by 100²⁶. The TM is expressed in arbitrary units. The tail intensity is most useful as it bears a linear relationship to the density of abasic sites and DNA breaks. In addition, it is relatively unaffected by threshold settings, allows discrimination of damage over the widest possible range (0 to 100%), and gives an impression of the comet's appearance. The tail intensity (TI) is defined as percentage of DNA (fluorescent) in the tail. Final results were expressed as the mean, median, and standard deviation of TMs and TIs of 100 cells from 2 duplicate slides. For quality control

assurance, independent intra- and inter-observer readings were compared between two readers, for a total of 25 samples. For the analysis of sensitivity to BPDE, the relative induced damage (RID) was calculated as:

$$RID = \frac{(\text{DNA damage at } t_{\text{im}0} \text{ after exposure} - \text{DNA damage before exposure})}{(\text{DNA damage before exposure})}$$

and for the analysis of repair kinetics, the percentage of residual DNA damage (%RD) after exposure to BPDE was calculated as follows²⁷:

$$RD\% = \frac{100 \times (\text{DNA damage at } t_{\text{im}1} \text{ after exposure} - \text{DNA damage before exposure})}{(\text{DNA damage at } t_{\text{im}0} \text{ after exposure} - \text{DNA damage before exposure})}$$

where DNA damage at time t_0 corresponds to the damage found immediately after replacing BPDE with fresh media and DNA damage at time t_1 corresponds to the DNA damage found after allowing the cells to recover from the BPDE induced damage.

Statistical analysis

Differences in distribution between the patients with multiple non-small lung cancer (cases) and those with single primary non small lung cancer (controls) with regard to age at consent and other lung cancer risk factors were determined using chi-square and t-test statistics. Analysis of variance was conducted to estimate differences in the logarithms of the measures of DNA damage between subgroups (e.g. sex, age). The t-test was used to compare the differences between cases and controls in terms of log DNA damage measured by the tail moment (TM) and the tail intensity (TI). Linear regression analyses were conducted to adjust these comparisons for potential confounders using the SAS software v. 9.1 (SAS Institute, Cary NC). All statistical tests were 2-sided.

Results

A total of 108 patients with multiple primary lung cancer (cases) were recruited, along with 99 patients with a single primary lung cancer (controls). All patients were recruited at Memorial Sloan-Kettering Cancer Center between March 2003 and November 2006. The majority of patients were White Non-Hispanic (89%) with the remaining as Asian, Hispanic, Black, or other. Sixty-three of the 108 second primary tumors were diagnosed within two years of the initial primary. Cases and controls were similar with respect to age at first diagnosis, age at consent, gender, histology, and smoking history (Table 1).

Intra and inter-observer agreement was tested, and based on 25 scored slides and median tail moment (TM) values, Kendall's correlation coefficient was 0.84. Storage time and room conditions at the time of the experiments such as humidity and temperature did not affect the internal controls. Constitutive or baseline levels of DNA damage with respect to these factors are shown in Table 2. Endogenous DNA damage appears to be significantly increased among males.

Significant differences were observed in the levels of constitutive, induced and unrepaired DNA damage measured by the TM and the TI between cases and controls (Table 3). After adjustment for sex, age, smoking history and treatment in a linear regression analysis of the logarithms of the comet assay measures, significant differences were confirmed in the levels of constitutive, induced and unrepaired DNA damage for TM and TI (see final column of Table 3). The histograms in Figure 1 show clearly that the preponderance of patients with high levels of DNA damage were cases for the three measures under investigation.

Discussion

Findings from this study reveal a consistent association of increased DNA damage in patients with multiple primary non-small cell lung cancer (NSCLC) when compared to the level of DNA damage in individuals with single primary tumors after controlling for age, sex, smoking history and treatment. The main aim of the present work was to identify markers that may help predict which lung cancer patients have the greatest risk to develop multiple tumors. For this, we compared a group of patients with lung cancer with single primary NSCLC (controls) with a group of patients with multiple independent NSCLC (cases).

Our genetic measures were obtained with the comet assay, also known as single-cell gel electrophoresis, one of the current standard methods for assessing DNA damage. The assay integrates the effects of exposure to exogenous and endogenous genotoxins due to both the amount of damaging agent, and the individual's metabolic and DNA repair capacities. The analysis of constitutive DNA damage in circulating lymphocytes indicates that cases have a reduced capacity to limit endogenous damage than controls (Table 3 and Figure 1a). As the most important etiologic risk factor for lung cancer is tobacco smoke, we also considered it appropriate to challenge the patients' cells to BPDE in order to measure the sensitivity to the compound and the capacity to repair the induced damage. For this measure, we also found an association between DNA damage and the development of multiple tumors in NSCLC patients (Figure 1b,c). Linear regression indicates that these differences are statistically significant even when controlling for age, gender, smoking habits and treatment status (Table 3).

The present findings are consistent with those from previous studies in that constitutive DNA damage and capacity to repair damaged DNA may modulate an individual's susceptibility to develop NSCLC. Specifically, others have found that, when compared to unaffected individuals, patients with lung cancer were characterized by higher rate of spontaneous chromosomal aberrations¹¹, elevated endogenous single stranded breaks¹², higher sensitivity to gamma-radiation, bleomycin, and BPDE¹²⁻¹⁴, and lower DNA repair capacity¹⁵.

In our study we did not detect significant differences in the comet assay measures by age, histologic subtypes, or family history. However, men showed increased endogenous and induced DNA damage as well as higher levels of unrepaired DNA than females, regardless of case-control status. Gender differences in the sensitivity to tobacco-carcinogens and in the susceptibility to lung cancer have been reported^{15, 28-31}. Several authors have hypothesized

that women are at higher risk to develop lung cancer than men of the same age and smoking history. However evidence from several large prospective studies found no such differences^{32–34}. Women with lung cancer survive their disease longer than men^{35–38}. This gender difference in survival may be due to the overall levels of damaged DNA. Future studies should examine in more depth gender differences in the repair capacity of patients with lung cancer.

Our study has some limitations. Use of post-diagnostic samples for assessing the predictive value of phenotype assays such as the comet assay is not ideal because the assay outcome may be affected by disease status or treatment. In fact, eighteen of our study participants received chemotherapy, radiation therapy, or a combination of both. Treatment occurred within 5 years prior to recruitment. Levels of DNA damage were higher in individuals treated with chemotherapy, followed by those with radiation therapy (data not shown). To further characterize this type of measure as a potential biomarker of lung cancer development, the study should be replicated with pre-diagnostic samples collected in a prospective study. Nevertheless, in this pilot study after adjustment for confounding factors including treatment status the difference between cases and controls remained significant. These measures of DNA damage may prove to be helpful markers of risk for the development of multiple tumors, and for the development of *de novo* NSCLC. A further potential limitation is the possibility that some of the subsequent primaries may be clonal products of the initial tumor, i.e. metastases. This issue is an active topic of research, and the literature to date suggests that lung tumors designated as second primaries by the Martini-Melamed criteria are predominantly, but not exclusively, independent occurrences of the disease^{39–41}. Clearly, it is probable that some of our cases may be misdiagnosed metastases. However, this phenomenon would only have the effect of attenuating any observed differences between cases and controls.

In summary, these results suggest that elevated levels of damaged DNA are associated with the development of multiple NSCLC tumors in patients with NSCLC. If replicated in a large prospective study, relatively simple functional assays such as this could contribute to the identification of individuals with increased cancer susceptibility and may prove useful to follow-up patients diagnosed with their initial NSCLC.

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Figure 1.

(A) Constitutive, (B) BPDE-induced, and (C) unrepaired DNA damage in patients with NSCLC. *Left:* bar graphs show the distribution of DNA damage among patients with single (green) and multiple (blue) primary tumors. *Right:* representative nucleoids and median TI values obtained with the comet assay (200 $\times$). Note the extent and intensity of the tails in cases 16 and 21 (arrows).

Table 1

Characteristics of study subjects

	Cases (N=108)	Controls (N=99)	p-value
Mean Age (years $\pm$ S.D.)			
At first diagnosis	63.4 $\pm$ 8.3	63.8 $\pm$ 8.5	0.72
At second diagnosis	66.5 $\pm$ 8.1	--	
At consent	68.4 $\pm$ 7.9	67.7 $\pm$ 8.2	0.53
Gender (N)			
Male	40	37	
Female	68	62	0.96
Histology ⁽¹⁾ (N)			
Adenocarcinoma & BAC	66	62	
Non-small cell carcinoma	20	18	
Squamous cell carcinoma	14	16	
Other	8	3	0.52
Smoking History ⁽²⁾ (N)			
Never	8	6	
Former	89	78	
Current	5	6	0.77*

¹ Adenocarcinoma & BAC: adenocarcinoma, adenocarcinoma associated with bronchoalveolar carcinoma (BAC) and BAC;

² Never smokers: less than 100 cigarettes in lifetime;

* current *versus* former/never combined p-value=0.64

Table 2DNA damage according to patient characteristics¹

	Constitutive damage Mean TM $\pm$ SD	p-value ²	Constitutive damage Mean TI $\pm$ SD	p-value ²
Age (years)				
45–54 (N=13)	0.51 $\pm$ 0.45		1.69 $\pm$ 0.71	
55–64 (N=53)	0.38 $\pm$ 0.36		1.54 $\pm$ 0.59	
65–74 (N=91)	0.49 $\pm$ 0.42		1.66 $\pm$ 0.68	
75–84 (N=49)	0.41 $\pm$ 0.35		1.52 $\pm$ 0.60	
85–94 (N=1)	0.26	0.47	1.67	0.64
Gender				
Males (N=77)	0.52 $\pm$ 0.48		1.73 $\pm$ 0.69	
Females (N=130)	0.40 $\pm$ 0.32	0.03	1.52 $\pm$ 0.60	0.02
Histology				
Adenocarcinoma & BAC (N=128)	0.43 $\pm$ 0.35		1.58 $\pm$ 0.62	
Non-small cell carcinoma (N=38)	0.46 $\pm$ 0.47		1.58 $\pm$ 0.66	
Squamous cell carcinoma (N=30)	0.43 $\pm$ 0.35		1.65 $\pm$ 0.63	
Other (N=11)	0.59 $\pm$ 0.60	0.65	1.75 $\pm$ 0.84	0.82
Family history of cancer³				
Yes (N=139)	0.43 $\pm$ 0.41		1.57 $\pm$ 0.64	
No (N=10)	0.47 $\pm$ 0.26	0.78	1.65 $\pm$ 0.69	0.72
Smoking History				
Never (N=14)	0.29 $\pm$ 0.16		1.37 $\pm$ 0.41	
Current & Former (N=182)	0.46 $\pm$ 0.41	0.11	1.63 $\pm$ 0.65	0.15

¹ All results are presented as logarithms of the defined measures defined in the text;² analysis of variance;³ affected first-degree relatives

Table 3

DNA damage according to case-control status

	Cases (N=108)	Controls (N=99)	Mean Effect difference	p-value ¹	p-value ^{2,3}
	Mean ± SD	Mean ± SD	95% CI		
Constitutive DNA damage					
TM	0.55 ± 0.47	0.33 ± 0.23	0.22 (0.12, 0.32)	<0.01	<0.01
TI	1.73 ± 0.73	1.46 ± 0.49	0.27 (0.10, 0.43)	<0.01	<0.01
DNA damage post BPDE					
TM	1.30 ± 0.84	0.98 ± 0.64	0.32 (0.12, 0.53)	<0.01	<0.01
TI	2.70 ± 0.78	2.45 ± 0.72	0.25 (0.05, 0.46)	0.02	0.01
Repair after BPDE-induced damage					
TM	0.77 ± 0.54	0.56 ± 0.36	0.21 (0.08, 0.33)	<0.01	<0.01
TI	2.12 ± 0.66	1.93 ± 0.56	0.20 (0.03, 0.36)	0.02	0.03
Relative induced damage					
TM	1.40 ± 0.75	1.42 ± 0.80	−0.02 (−0.23, 0.19)	0.87	0.95
TI	1.16 ± 0.72	1.22 ± 0.68	−0.06 (−0.25, 0.13)	0.54	0.35
Residual damage (%)					
TM	2.82 ± 1.83	2.76 ± 1.88	0.05 (−0.45, 0.56)	0.84	0.17
TI	2.86 ± 1.76	2.94 ± 1.86	−0.08 (−0.58, 0.41)	0.74	0.46

¹ T-test;² Linear regression of logarithms of the comet assay measures adjusted for age at consent, gender, treatment history, and smoking history³ Induced damage and residual damage further adjusted for constitutive DNA damage

Abbreviations: TM, tail moment; TI, tail intensity; S.D., Standard deviation