Genetic Instability in Bladder Cancer Assessed by the Comet Assay

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Background: Latent genetic instability has been associated with an increased risk for several cancers. We used the comet assay (single-cell gel electrophoresis) to assess whether genetic instability, as reflected by susceptibility to DNA damage, was associated with the risk of bladder cancer in a case-control study. Methods: We used the comet assay to measure baseline and benzo[a]pyrene diol epoxide (BPDE)- and γ-radiation-induced DNA damage in individual peripheral blood lymphocytes from 114 incident case patients with bladder cancer and 145 matched healthy control subjects. All subjects provided personal information, including smoking history. DNA damage was visualized with the comet assay and quantified by the Olive tail moment parameter, a relative measure. Multivariable analysis was used to assess relative risks for bladder cancer associated with DNA damage. All statistical tests were two-sided. Results: Baseline levels of DNA damage were statistically significantly higher in case patients (tail moment = 1.40) than in control subjects (tail moment = 1.21) (difference = 0.19, 95% confidence interval [CI] = 0.04 to 0.32; \( P = .015 \)), as were γ-radiation-induced (tail moment = 4.76 versus 4.22; difference = 0.54, 95% CI = 0.11 to 0.96; \( P = .013 \)) and BPDE-induced (tail moment = 4.06 versus 3.45; difference = 0.61, 95% CI = 0.23 to 0.99; \( P = .002 \)) DNA damage. When data were dichotomized at the median value for DNA damage in control subjects and adjusted for age, sex, ethnicity, and smoking status, an increased estimated relative risk of bladder cancer was statistically significantly associated with DNA damage at baseline (odds ratio [OR] = 1.84, 95% CI = 1.07 to 3.15) and after γ-radiation (OR = 1.81, 95% CI = 1.04 to 3.14) but not after BPDE treatment (OR = 1.69, 95% CI = 0.98 to 2.93). Conclusion: Latent genetic instability as measured by the comet assay is associated with an increased estimated relative risk of bladder cancer. [J Natl Cancer Inst 2003;95: 540–7]

DNA repair involves complex defense systems that maintain the integrity of the genome. Disruption of these processes can lead to cell death or neoplasia. Rare, recessive chromosome instability syndromes are characterized by various defects in DNA repair and cell-cycle control, and these syndromes are associated with a substantially increased cancer risk. The DNA of patients with chromosome instability syndromes is often more susceptible to mutagen damage than that of the general population. Latent genetic instability has been associated with an increased risk for cancer. In an extensive review of mutagen sensitivity and cancer, Berwick and Vineis (1) noted consistent and positive associations between suboptimal DNA repair capacity and cancer risk. Numerous attributes, including allelic variations in genes involved in DNA repair pathways, can alter an individual's ability to repair DNA damage, resulting in increased sensitivity to exogenous and endogenous agents. We have previously shown (2–4) that mutagen sensitivity varies widely and that this variation translates into interindividual variations in susceptibility to a carcinogenic challenge.

A variety of assays can assess genetic instability and DNA repair capacity in population-based studies, including those that assess metaphase chromosomal aberrations, micronuclei, sister chromatid exchanges, and host cell reactivation. However, these methods are typically laborious and time-consuming (e.g., host cell reactivation) or require highly trained technicians to accurately read and interpret slides (e.g., micronuclei and chromosomal aberrations). Over the past decade, the comet assay, or single-cell gel electrophoresis, has been used to study processes ranging from DNA repair to genotoxicity, a measure of the adverse effects of a toxin on DNA (5). Automated imaging technology facilitates use of this assay to determine DNA damage, which has been shown to be associated with an individual's susceptibility to cancer. The comet assay is relatively high-throughput, requires small numbers of cells, detects primary DNA damage in individual cells (5), provides results within a few hours of sampling, and is cost-effective (6). It is also a simple, sensitive, and reliable laboratory method that is suited for assessing in vitro genetic instability in molecular epidemiologic studies.

The challenge mutagen induces a specific type of DNA damage and provides information on possible deficiencies in DNA repair pathways. γ-Radiation induces single- and double-strand breaks that are repaired by base excision and/or double-strand break repair pathway(s). Benzo[a]pyrene diol epoxide (BPDE), a metabolite of the tobacco smoke procarcinogen benzo[a]pyrene, induces DNA adducts that are repaired by the nucleotide excision repair pathway. Two DNA repair genes, XPD (belonging to the nucleotide excision repair pathway) and XRCC1 (belonging to the base excision repair pathway), have been shown to be associated with an increased cancer risk in several case-control studies. Previous reports (7–14) indicate that variant genotypes in XPD (exons 10 and 23) and in XRCC1 (exon 6 wild-type and exon 10) are putative adverse genotypes that appear to be associated with increased levels of DNA damage.

In an ongoing hospital-based case-control study, we used the comet assay to quantify the baseline and BPDE- and γ-radiation-induced DNA damage in the peripheral blood lymphocytes of...
114 patients with incident bladder cancer and 145 healthy control subjects and assessed whether individuals with latent genetic instability, as reflected in higher levels of DNA damage, have an increased estimated relative risk of bladder cancer.

**PATIENTS AND METHODS**

**Study Population**

Patients with incident urinary bladder cancer were accrued from August 10, 1999, through August 17, 2001, from the patient population at The University of Texas M. D. Anderson Cancer Center in Houston, Texas. All case patients had been diagnosed with bladder cancer within 1 year of accrual, their bladder cancer was histologically confirmed, and they had not been previously treated with chemotherapy or radiotherapy. There were no age, sex, ethnic, or cancer-stage restrictions. Case patients were identified by M. D. Anderson staff interviewers from daily review of computerized appointment schedules for clinics in which patients with bladder cancer were treated (Departments of Urology and Genitourinary Medical Oncology). Each new patient was initially screened by using a brief eligibility questionnaire that assessed prior cancer therapy and willingness to participate in the epidemiologic study. If the patient was willing to participate, the interviewer escorted the study participant to a private room or examination room to conduct the interview. We accrued a total of 114 case patients with bladder cancer and 145 healthy matched controls for this study.

The control subjects without a history of cancer (except nonmelanoma skin cancer) were recruited from a large pool of potential volunteers from the Kelsey-Seybold clinics, Houston’s largest multispecialty physician group. As of May 1, 2001, there were more than 79,000 potential control subjects in the database, with 1000–1200 subjects added monthly. A control subject was selected to match to a case patient by age (±5 years), sex, and ethnicity. Control subjects were then contacted by telephone to confirm their willingness to participate, and an appointment was scheduled at a Kelsey-Seybold clinic convenient to the volunteer.

**Epidemiologic Data**

After written informed consent was provided, all study participants completed a 45-minute personal interview that was administered by trained M. D. Anderson staff interviewers. The interview elicited information regarding demographics, smoking history, alcohol consumption, family history of cancer, medical history, and occupational history. Additionally, a 60-minute food-frequency questionnaire was administered to assess diet during the year before diagnosis for case patients and diet during the year before the interview for control subjects. Both questionnaires consisted of a fixed script and included introductory and transitional statements. All interviewers were trained in the appropriate use of probes. A Spanish language version of both questionnaires was available. At the conclusion of the interview, a 40-mL blood sample was drawn into coded heparinized tubes. A 40-mL aliquot of the blood culture was gently spread onto each end of a precoated slide and covered with a fresh glass coverslip. The agarose on the slides was allowed to solidify on a metal plate at 4°C for approximately 10 minutes. A final layer of 0.5% low-glucosamine solution was added to the plates before the slide was placed in a 60°C oven for 45 minutes to complete gelification of the agarose. After gelification, the slides were quickly placed on ice to slow DNA repair. Cells were embedded in agarose, which was attached to slides approximately 15 minutes before the comet assay was sufficient to induce nuclear DNA damage. After γ-irradiation, blood cultures were placed on ice to slow DNA repair. Cells were embedded in agarose, which was attached to slides approximately 15 minutes after γ-irradiation. Thus, the comet cells reflected the net result of DNA damage and repair because the fast component of DNA repair was unavoidable with this approach.

**Comet Assay**

To minimize extraneous DNA damage from ambient ultraviolet radiation, all steps were performed with reduced illumination. We slightly modified the comet assay under alkaline conditions as originally described by Singh et al. (16). Briefly, fully frosted slides were precoated on each end with 50 μL of 1% agarose in phosphate-buffered saline (PBS, pH 7.4), covered with a 22-mm × 22-mm glass coverslip, and allowed to stand at room temperature for 20–30 minutes. For both baseline and mutagen-exposed cells, 50 μL of the blood culture was gently mixed with 150 μL of 0.5% low-melting-point agarose (Life Technologies) in PBS and maintained at 42°C on a dry-bath incubator (Fisher Scientific, Houston, TX). A 50-μL aliquot of the mixture was immediately spread onto each end of a precoated slide and covered with a fresh glass coverslip. The agarose on the slides was allowed to solidify on a metal plate at 4°C for approximately 10 minutes. A final layer of 0.5% low-
melting-point agarose in PBS was placed on top of the cell suspension/low-melting-point agarose layer and covered with a new glass coverslip, and the slide was placed on a metal plate at 4 °C for an additional 10 minutes. Cells were lysed by submersing the slides in freshly prepared 1× lysis buffer (2.5 M NaCl, 100 mM EDTA, 1% Na-lauroylsarcosine sodium salt, 10 mM Tris, adjusted to pH 10 with NaOH; 10% dimethyl sulfoxide and 1% Triton X-100 were added before use) for approximately 1 hour at 4 °C. To allow for DNA denaturation and unwinding and the exposure of the alkali-labile sites, slides were kept for 30 minutes in a horizontal electrophoresis box without power that was filled with freshly prepared alkali buffer (300 mM NaOH and 1 mM EDTA at pH >13) at 4 °C. To separate the damaged DNA from the intact nuclei, a constant electric current of 295–300 mA was then applied for 23 minutes at 4 °C. After electrophoresis, the slides were neutralized with three 5-minute washes in 0.4 M Tris–HCl (pH 7.4). Finally, the slides were fixed in 100% methanol for 5–10 minutes and stored in the dark at room temperature until analysis.

Quantification of DNA Damage

Immediately before imaging analysis, slides were hydrated in fresh 0.4 M Tris–HCl (pH 7.4) for approximately 30 minutes and then stained for 1 minute with a fresh solution of 0.4 M Tris–HCl containing 300 μL of ethidium bromide at 10 mg/mL. Fifty consecutive cells (25 cells from each end of the slide) were manually selected and were automatically quantified with Kometa version 4.0.2 (Kinetic Imaging Ltd., Bromborough, Wirral, U.K.) imaging software attached to a fluorescent microscope (Nikon, Melville, NY), which also determined the Olive tail moment parameter [(tail mean – head mean) × (% tail DNA/100)] used to quantify DNA damage (17). There are no expressible units for DNA damage measured by the comet assay. The head of the comet is the nucleus of the cell, and the tail of the comet is the damaged DNA that has been liberated from the nucleus by electrophoresis. The tail mean is the tail DNA intensity subtracted from background intensity, the head mean is the head DNA intensity subtracted from background intensity, and the percentage of tail DNA is the fraction of DNA that has migrated from the head. The difference between the tail mean and the head mean represents the difference in the distance between the center of gravity of the DNA distribution in the comet head and the center of gravity of the DNA distribution in the comet tail. For each subject, the averages of the Olive tail moment were calculated for the baseline comets, γ-radiation-induced comets, and BPDE-induced comets.

Polymerase Chain Reaction–Restriction Fragment Length Polymerism Assays for XPD and XRCC1

Previously described polymerase chain reaction–restriction fragment length polymorphism assays were used to amplify the polymorphic regions for XPD exons 10 and 23 (13) and XRCC1 exons 6 and 10 (14). For the XPD exon 10 polymorphism (Asp312Asn), the wild-type (Asp/Asp) genotype produced two DNA bands (507 and 244 base pairs [bp]), the variant genotype (Asn/Asn) produced three bands (474, 244, and 33 bp), and the heterozygous genotype (Asp/Asn) produced all four bands (507, 474, 244, and 33 bp). For the XPD exon 23 polymorphism (Lys751Gln), the homozygous wild-type genotype (Lys/Lys) produced two DNA bands (290 and 146 bp), the variant genotype (Gln/Gln) produced three DNA bands (227, 146, and 63 bp), and the heterozygous genotype (Lys/Gln) produced all four bands (290, 227, 146, and 63 bp). For the XRCC1 exon 6 polymorphism (Arg194Trp), the wild-type genotype (Arg/Arg) produced one band (490 bp), the heterozygous genotype (Arg/Trp) produced three bands (490, 294, and 196 bp), and the variant genotype (Trp/Trp) produced two bands (294 and 196 bp). For the XRCC1 exon 10 polymorphism (Arg399Gln), the homozygote genotype (Arg/Arg) produced two bands (269 and 133 bp), the heterozygous genotype (Arg/Gln) produced three bands (402, 269, and 133 bp), and the variant genotype (Gln/Gln) produced one band (402 bp). Positive and negative controls were used in each experiment, and 10% of the total samples were randomly selected and reanalyzed with 100% concordance.

Statistical Analysis

All statistical analyses were performed with the Intercooled Stata 7.0 statistical software package (Stata Corporation, College Station, TX). The χ² test was used to test for differences between the case patients and the control subjects in the distribution of sex, ethnicity, and smoking status, and the Student’s t test was used to test for differences between the case patients and control subjects for age, pack-years (among ever smokers), and Olive tail moments. Odds ratios (ORs) were calculated as estimates of relative risk by dichotomizing the tail moment at the median (50th percentile) value in control subjects. Unconditional multivariable logistic regression was performed to control for possible confounding by age, sex, ethnicity, and smoking status, where appropriate. To detect patterns of association between the degree of risk and higher levels of DNA damage for baseline and mutagen-induced comets, the tail moment was categorized by the tertile values in the control subjects, and the two new variables were analyzed simultaneously in one model. All statistical tests were two-sided.

RESULTS

One hundred fourteen case patients with bladder cancer and 145 healthy control subjects were available for this analysis (Table 1). Essentially no differences were observed in terms of age, sex, and ethnicity between case patients and control subjects. Approximately 70% of the case patients and 68% of the control subjects were male. Approximately 11% of the case patients and 5% of the control subjects were non-Caucasian. Current smokers were statistically significantly over-represented (13.2%) among case patients compared with control subjects (3.5%). Case patients were also self-reported heavier smokers (40.1 ± 30.3 pack-years; mean ± standard deviation [SD]) than control subjects (27.7 ± 27.9 pack-years) (P = .01). Case patients (65.8 ± 10.1 years; mean ± SD) were slightly older than control subjects (63.6 ± 9.4 years), but the difference was not statistically significant (P = .067).

Although there was no statistically significant difference between case patients and control subjects in the XPD genotype distribution, the putative adverse genotypes (variant genotypes for both exons) were more frequently observed among case patients than among control subjects (Table 1). Specifically, the XPD exon 10 variant genotype (Asn/Asn) was found in 14.8% of case patients and in 8.8% of control subjects; the XPD exon 23 variant genotype (Gln/Gln) was found in 15.8% of case patients and in 10.1% of control subjects. There were no statistically significant differences in the XRCC1 genotype distribution between case patients and control subjects; however, putative
adverse genotypes (variant genotype for exon 10 and wild-type genotype for exon 6) were also observed more frequently among case patients than among control subjects. The XRCC1 exon 6 wild-type genotype (Arg/Arg) was found in 91.2% of case patients and in 85.4% of control subjects. No case patient or control subject had the homozygote variant genotype for exon 6. The XRCC1 exon 10 variant genotype (Gln/Gln) was found in 14.6% of case patients and in 12.3% of control subjects.

Because the quality of some slides was poor, slides for only 112 of 114 case patients and 141 of 145 control subjects were available for the baseline comet analysis, slides for 108 case patients and 138 control subjects were available to assess BPDE-induced comets. When the DNA damage data, as measured by the tail moment parameter, were dichotomized at the median (50th percentile) value in the control subjects, an increased estimated relative risk for bladder cancer was statistically significantly associated with baseline DNA damage (OR = 1.84, 95% CI = 1.07 to 3.15) and γ-radiation-induced DNA damage (OR = 1.81, 95% CI = 1.04 to 3.14) after adjusting for age, sex, and ethnicity (Table 3). The increased estimated relative risk for bladder cancer associated with elevated BPDE-induced DNA damage was of only borderline statistical significance (OR = 1.69, 95% CI = 0.98 to 2.93). However, an OR of 2.22 (95% CI = 1.11 to 4.43) was evident when the DNA damage induced by both BPDE and γ-radiation exceeded the median value for each mutagen.
An intermediate category, therefore, was created that was composed of individuals classified as above the median value for either BPDE or γ-radiation. One intermediate category was created instead of two because of the small number of participants. Only 13 case patients and 24 control subjects were classified as having the median value or more for γ-radiation sensitivity and less than the median value for BPDE sensitivity, and only eight case patients and 25 control subjects were classified as having less than the median value for γ-radiation sensitivity and the median value or more for BPDE sensitivity. The OR associated with the intermediate category was 0.75 (95% CI = 0.35 to 1.63). When DNA damage data in control subjects were separated into tertiles, the estimated relative risk of bladder cancer was positively associated with increasing levels of DNA damage across the tertiles for baseline and γ-radiation-induced DNA damage but was not associated with BPDE-induced DNA damage, although the highest estimated relative risk for bladder cancer was noted in the third tertile for BPDE damage (Table 4).

We also analyzed the data by smoking status for case patients and control subjects (Table 5) by comparing never smokers to former smokers, current smokers, and ever smokers separately.

Table 4. Relative risk estimates of bladder cancer for baseline and induced DNA damage quantified by the Olive tail moment parameter

<table>
<thead>
<tr>
<th>Treatment and level of DNA damage</th>
<th>No. of case patients</th>
<th>No. of control subjects</th>
<th>Univariate OR (95% CI)*</th>
<th>Multivariable OR (95% CI)†</th>
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<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; Median‡</td>
<td>72</td>
<td>70</td>
<td>1.83 (1.09 to 3.04)</td>
<td>1.84 (1.07 to 3.15)</td>
</tr>
<tr>
<td>&lt; Median</td>
<td>40</td>
<td>71</td>
<td>1.0 (referent)</td>
<td>1.0 (referent)</td>
</tr>
<tr>
<td>γ-Radiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; Median‡</td>
<td>71</td>
<td>70</td>
<td>1.86 (1.11 to 3.13)</td>
<td>1.81 (1.04 to 3.14)</td>
</tr>
<tr>
<td>&lt; Median</td>
<td>37</td>
<td>68</td>
<td>1.0 (referent)</td>
<td>1.0 (referent)</td>
</tr>
<tr>
<td>BPDE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; Median‡</td>
<td>67</td>
<td>71</td>
<td>1.59 (0.95 to 2.68)</td>
<td>1.69 (0.98 to 2.93)</td>
</tr>
<tr>
<td>&lt; Median</td>
<td>39</td>
<td>66</td>
<td>1.0 (referent)</td>
<td>1.0 (referent)</td>
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<tr>
<td>Both BPDE and γ-radiation</td>
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<td></td>
</tr>
<tr>
<td>&gt; Median‡</td>
<td>55</td>
<td>42</td>
<td>2.18 (1.14 to 4.16)</td>
<td>2.22 (1.11 to 4.43)</td>
</tr>
<tr>
<td>&lt; Median</td>
<td>21</td>
<td>49</td>
<td>0.71 (0.33 to 1.56)</td>
<td>0.75 (0.35 to 1.63)</td>
</tr>
<tr>
<td>Intermediate‡</td>
<td>24</td>
<td>40</td>
<td>1.0 (referent)</td>
<td>1.0 (referent)</td>
</tr>
</tbody>
</table>

*OR = odds ratio; CI = confidence interval; BPDE = benzo[a]pyrene diol epoxide.
†Adjusted by age, sex, ethnicity, and smoking status.
‡Dichotomized by the median (50th percentile) value of control subjects.
§Subjects classified as either >median for γ-radiation sensitivity or <median for γ-radiation sensitivity and ≥median for BPDE sensitivity.

For case patients and control subjects, no differences were observed between never smokers and former smokers in the level of DNA damage at baseline and after γ-radiation or BPDE treatment. In control subjects, a statistically significant difference in DNA damage was observed between never smokers and current smokers; however, it should be noted that only five control subjects were current smokers. Conversely, there were no differences in DNA damage between the never smokers and current smokers among the case patients. Because of the small number of current smokers, we combined current smokers and former smokers into ever smokers. No differences in the level of DNA damage were observed between never smokers and ever smokers for both groups at baseline or after γ-radiation and BPDE treatment.

Finally, we explored the association between BPDE-induced DNA damage and the XPD genotypes (reflecting nucleotide excision repair) and between γ-radiation-induced DNA damage and the XRCC1 genotypes (reflecting base excision repair). Fig. 3, A, shows the mean γ-radiation-induced DNA damage for control subjects according to the XRCC1 genotypes. A higher level of γ-radiation-induced DNA damage appeared to be associated with the combined putative adverse genotypes of XRCC1 (exon 6 wild-type and exon 10 variant genotypes [N = 55]; tail moment = 4.19, 95% CI = 3.79 to 4.59) than with favorable XRCC1 genotypes (exon 6 heterozygote and exon 10 wild-type [N = 14]; tail moment = 3.54, 95% CI = 2.74 to 4.36) (P = .147). Among case patients, similar levels of γ-radiation-induced DNA damage were observed in those with the XRCC1 exon 6 adverse genotypes and for those with the favorable genotypes. Similar patterns of γ-radiation-induced DNA damage were observed for the exon 10 genotypes among case patients (data not shown).

Fig. 3, B, shows the mean BPDE-induced DNA damage for control subjects according to the XPD genotype. For the XPD exon 10 polymorphism, a non-statistically significantly lower level of BPDE-induced DNA damage was observed for wild-type genotypes of XPD exon 10 (N = 65; tail moment = 3.23, 95% CI = 2.95 to 3.52) than for variant genotypes (N = 75; tail moment = 3.46, 95% CI = 3.18 to 3.65) (P = .236). For the XPD exon 23 polymorphism, a non-statistically significantly...
lower level of BPDE-induced DNA damage was noted for wild-type genotypes (N = 62; tail moment = 3.24, 95% CI = 2.95 to 3.53) than for variant genotypes (N = 79; tail moment = 3.46, 95% CI = 3.18 to 3.65) (P = .247). A similar pattern was noted for the BPDE-induced DNA damage associated with the variant genotypes of XPD exons 10 and 23 combined (N = 64; tail moment = 3.45, 95% CI = 3.14 to 3.66) and with wild-type genotypes of XPD exons 10 and 23 combined (N = 50; tail moment = 3.13, 95% CI = 2.85 to 3.52) (P = .135). Similar levels of BPDE-induced DNA damage were observed among case patients with the wild-type XPD genotypes (exons 10 and 23) and those with the variant XPD genotypes (exons 10 and 23) (data not shown).

**DISCUSSION**

In this article, we used the comet assay to assess baseline and mutagen-induced DNA damage to determine whether genetic instability is associated with the estimated relative risk of bladder cancer. The main finding of this study was that the peripheral blood lymphocytes from case patients with bladder cancer showed higher levels of DNA damage than those from control subjects both at baseline (without a mutagen challenge) and after BPDE and γ-radiation exposure in vitro.

When the data were dichotomized at the median value for the control subjects, there was a statistically significantly increased estimated relative risk of cancer associated with higher levels of baseline (spontaneous or uninduced) DNA damage (OR = 1.84, 95% CI = 1.07 to 3.15). Thus, categorizing individuals into discrete groups may be useful for identifying subsets of individuals who are at higher risk for cancer. Other studies using the comet assay have also shown greater baseline DNA damage in patients with cancer, including those with oral squamous cell carcinoma (18), cancer of the uterine cervix (19), and breast cancer (20,21). McKelvey-Martin et al. (22) noted statistically significantly greater DNA damage in cells from bladder washings of patients with bladder cancer than in cells from bladder washings of control subjects. We also observed this association between genetic instability and the estimated relative risk of bladder cancer when data on DNA damage were separated into tertiles. Specifically, individuals in the highest tertile of DNA damage at baseline were at a 1.3-fold greater estimated relative risk (OR = 1.31, 95% CI = 0.65 to 2.62). Therefore, our data support the hypothesis that individuals with genetic instability are at an increased estimated relative risk for bladder cancer. Indeed, categorizing the level of DNA damage by this approach may be particularly useful for identifying individuals with susceptible genetic profiles. Results of the comet assay could be combined with data on cigarette smoking, family history of cancer, occupation, and dietary intake to construct comprehensive risk profiles.

Latent genetic instability can be identified by a mutagen challenge. Therefore, a mutagen challenge with both BPDE and γ-radiation, which induce different types of DNA damage, can assess overall host susceptibility to bladder cancer because it yields data on the net result of DNA damage and repair for two distinct repair pathways. BPDE and γ-radiation have both been used previously to induce DNA damage that can be detected with the comet assay (23,24). We observed an increased estimated relative risk of bladder cancer associated with γ-radiation- and BPDE-induced DNA damage and a 2.2-fold increased estimated relative risk associated with elevated levels of both (Table 3). The OR for the intermediate category containing individuals classified as above the median value for either mutation challenge was not statistically significant (OR = 0.75, 95% CI = 0.35 to 1.63). This result may reflect the small sample size or may represent biologic effects in that the individuals segregated readily to both high levels of BPDE- and γ-radiation-induced DNA damage or low levels of BPDE- and γ-radiation-induced DNA damage. When the data from control subjects were separated into tertiles, we observed an increased estimated relative risk with higher levels of γ-radiation- and BPDE-induced DNA damage (Table 4). Previous studies using the comet assay have demonstrated similar case–control differences in induced DNA damage with a variety of test mutagens for other cancers. Colleu-Durel et al. (25) noted that lymphocyte DNA from patients with various cancers had higher levels of damage than that of control subjects with or without irradiation. Rajaei-Bebahani et al. (26) reported that the number of comet cells in bleomycin-treated peripheral blood lymphocytes, measured before DNA repair could occur, was statistically significantly higher in lung cancer patients than in tumor-free hospital control subjects. Kleinsasser et al. (27) used the comet assay to demonstrate that N'-nitrosodiethylamine induced statistically signifi-
24-hour incubation should reflect poor nucleotide excision repair. A similar argument could be made for \( \gamma \)-radiation-induced DNA damage; the observed differences should be the net results of DNA damage and repair. Approximately 50% of \( \gamma \)-radiation-induced DNA damage occurs within 15 minutes (29), and the rapid repair of DNA, but not the slow component, occurs within 5 minutes. Additionally, cells from patients with ataxia-telangiectasia have a reduced fast-repair component in both \( G_1 \) and \( G_2 \)-phase cells that operates on DNA double-strand breaks and chromosomal breaks. However, these differences are not observed in the slow component of DNA repair (29). In our approach, \( \gamma \)-irradiation was performed at room temperature. Cells were then placed on ice blocks to slow DNA repair, embedded in agarose, and attached to the slides approximately 15 minutes after \( \gamma \)-radiation. Consequently, \( \gamma \)-radiation-induced comet cells should reflect the net result of DNA damage and repair because the fast component of DNA repair was not inhibited. Our data therefore indicate that the latent genetic instability, identified by using a mutagen challenge followed by the comet assay, is associated with an increased estimated relative risk of bladder cancer.

The data were also analyzed by smoking status to ensure that smoking behavior did not contribute to the differences observed between the case patients and the control subjects (Table 5). There were no differences in DNA damage when former smokers and ever smokers were compared with never smokers. Thus, smoking status apparently does not explain the substantial differences in DNA damage between case patients and control subjects. Further analysis of the DNA damage by case–control status revealed a normal distribution, which supports the idea that the DNA damage in case patients was apparently not influenced by a subset of case patients with higher values, which would have contributed to an artificially increased estimated relative risk (data not shown).

We also report preliminary data showing a non-statistically significant association between DNA damage as measured by the comet assay and the polymorphic DNA repair genes XRCC1 (exons 6 and 10) and XPD (exons 10 and 23). XRCC1 is an important gene in the base excision repair pathway and is involved in the removal of \( \gamma \)-radiation-induced DNA damage. The deleterious XRCC1 genotypes (exon 6 wild-type genotype and exon 10 variant genotypes) were associated with greater \( \gamma \)-radiation-induced DNA damage than genotypes thought to be associated with more proficient DNA repair (exon 6 variants and exon 10 wild-type). Additional support for our findings comes from previous studies showing that the XRCC1 exon 10 variant genotypes are associated with greater mean sister chromatid exchange frequencies (7) and higher levels of aflatoxin B1–DNA adducts and erythrocyte glycoporphin A somatic mutations (variant NN) (8) than the wild-type genotype. XRCC1 exon 10 variant genotypes have also been associated with increased risk for head and neck cancer (9) and gastric cancer, and XRCC1 exon 6 genotypes modulate the risks for bladder cancer (11), oral and pharyngeal cancers (9), and gastric cardia cancer (10).

XRPD is an essential gene in the nucleotide excision repair pathway and is involved in the removal of BPDE-induced DNA adducts. In our study, a higher level of BPDE-induced DNA damage among control subjects was associated with the variant genotypes of XPD (exons 10 and 23) than with the XPD wild-type genotypes (exons 10 and 23). These results are consistent with previous findings of more efficient DNA repair with XPD.

cantly more DNA migration in lymphocytes from patients with nasopharyngeal cancer (tail moment = 9.8 ± 3.1) than in lymphocytes from nontumor patients (tail moment = 8.2 ± 2.3) \((P = .03)\).

A useful feature of the comet assay is that it can be modified to measure various types of DNA damage and to measure the repair of that damage. We propose that the mutagen-induced comet cells in our study reflect the net result of DNA damage and repair. In the BPDE experiments, the peripheral blood lymphocytes were treated once with BPDE and incubated for an additional 24 hours before the comet assay was performed. During this 24-hour period, cells were continuously removing and repairing BPDE-induced DNA damage. Breaks would be only transiently present as cells repaired lesions by nucleotide excision (28), so that a high level of breaks in our study after a
wild-type genotypes than with XPD variant genotypes at both of these exons \([12,13]\). The small sample size in the present study limited extensive analyses of the XPD and XRCC1 genotypes. Nonetheless, we did note that the putative adverse genotypes of XPD and XRCC1 were observed more frequently among case patients than among control subjects, but these differences did not reach statistical significance. The small sample size could also explain why we did not observe a statistically significant association between the DNA repair genotypes and DNA damage in case patients. Because cancer is a multigenic disease and each gene involved has only a small independent effect on the disease phenotype \([13]\), a much larger sample size is required to determine whether each polymorphism was associated with risk for bladder cancer.

In conclusion, genetic instability (both spontaneous and mutagen-induced) appears to be associated with the estimated relative risk of bladder cancer. Measuring mutagen sensitivity with the comet assay is a promising approach for determining genetic instability and thus the susceptibility of an individual to cancer. Patients with bladder cancer and control subjects appear to have subtle differences in the degree of genetic instability, as shown by the mean differences in spontaneous and mutagen-induced DNA damage that were detected by tail moments of comet cells. However, by separating the data into categories, we may identify individuals at higher estimated relative risk for developing bladder cancer. Although this study may not be large enough (sufficiently powered) to detect the complex associations between DNA repair genotypes and DNA damage, this study is an initial step to demonstrate that the comet assay can be used as a tool to assess genetic instability for cancer risk. Large population-based cohort studies are required to further validate these findings and determine whether the comet assay will have clinical utility and applications in large-scale molecular epidemiologic studies.

REFERENCES


NOTES

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