Introduction

Bladder cancer (BC) is a heterogeneous disease with a variable natural history. It is currently the fourth most common malignancy among men in the western world, following prostate, lung and colon cancers. In 2012, it will be the second most common genitourinary cancer in the USA, with 73,150 estimated new cases and 14,880 estimated deaths. Currently, it is estimated that there are >1 million United States and European men and women who have a history of bladder urothelial carcinoma, which is 2.5–4 times more common in men (2).

Individuals with non-muscle-invasive BC (Tis-Ta-T1) have a high risk for developing another bladder tumor, reflected by a recurrence rate of up to 70% at 3 years of initial treatment. They also may progress to muscle-invasive disease (up to 50% in T1 high-grade lesions) (3). In contrast, tumors that are muscle invasive at diagnosis (T2-T3-T4) have a high malignant potential associated with significant progression and cancer death rates. Of this population, nearly 50% demonstrate distant metastasis within 2 years, and 60% die within 5 years despite aggressive treatment. If left untreated, only 14% of patients survive beyond 2 years (4,5).

The etiology of BC is a typical example for the concept of multistep carcinogenesis, reflecting that multiple lesions in the DNA are required for tumor development. Interaction of genetic susceptibility and environmental exposures is crucial. Among the latter, the most relevant risk factors associated with the occurrence of BC are tobacco smoking, occupational exposure to aromatic amines, ionizing radiation (IR) and arsenic-contaminated drinking water (6). Exposure to these carcinogens causes different types of DNA damage, including double-strand breaks (DSB). The latter is a serious lesion, potentially lethal to the cell if not fully repaired. Alternatively, broken DNA strands can lead to genomic instability, increasing the occurrence of cancer (7). Therefore, identification of susceptibility to DSB DNA damage may provide valuable information about individual cancer risk (8).

The histone H2AX plays a crucial role in the signaling of DNA damage events, enhancing the recruitment of different factors required for DSB repair. Upon induction of the latter, H2AX becomes rapidly phosphorylated at serine 139 on the carboxyl terminus to form γ-H2AX, one of the earliest events in cells following exposure to DNA damaging agents (9,10). This reaction depends on complex interactions between phosphoinositide 3-kinases, including ataxia telangiectasia-mutated, ataxia telangiectasia and Rad3-related and DNA-dependent protein kinase. γ-H2AX is easily detected with a high sensitivity by using specific antibodies, allowing reliable quantification of DSB foci in a cell by requiring a small sample of biological material. Therefore, it has become a widely used marker for DSB damage in translational studies, including areas such as carcinogenesis, radiation research, drug development and clinical assessment of DNA-targeted therapies (9,11).

Peripheral blood lymphocytes (PBLs) are the easiest cells to obtain for assessment of γ-H2AX formation in vivo. Analysis of PBLs after exposure to a given agent allows determination of the extent of DNA damage caused to normal cells in that individual (7,9). By these means, we have developed a high-throughput, image-based pheno-
typic assay to measure IR-induced DSBs in PBLs.

Studies evaluating the relationship of γ-H2AX activity with BC are few in the literature. In fact, its association with risk of the disease has not been evaluated to date. Since susceptibility to DNA damage and the consequent genomic instability are related to cancer risk, we hypothesized that an increased amount of DSBs may be a risk factor for BC. In a large ongoing BC hospital-based case–control study, we investigated whether levels of γ-H2AX in PBLs obtained after stimulation by IR are capable to predict BC risk. In addition, we examined the association of γ-H2AX level with epidemiological variables that may affect BC risk.

Materials and methods

Study population

Patients were enrolled from a large ongoing case–control study, which started in 1999, recruiting patients based at The University of Texas MD Anderson Cancer Center. The subject recruitment methodology and eligibility criteria have been described previously (12). Briefly, all case subjects were patients with primarily diagnosed and histologically confirmed BC who had not previously received any chemotherapy or radiation therapy. The control subjects were healthy individuals with no prior history of cancer (except non-melanoma skin cancer). All controls were recruited from the Kelsey-Seybold Clinic, the largest multispecialty physician group in the Houston metropolitan area. Most of them were enrolled during annual health checkups. Controls were matched to cases by age (±5 years), sex and race. However, only white Caucasian people were included in the analysis due to the small proportion of minority populations. This design has been shown to be a valid and efficient method for large epidemiologic cancer studies (13).

Data collection

Informed consent was obtained from all participants by trained MD Anderson staff interviewers before the collection of epidemiological data (demographics, smoking history, family history of cancer and medical history). After the interview, a blood sample was taken from each subject by clinical or specialized
staff and then delivered by the interviewers to the laboratory for molecular analyses. Institutional review boards of MD Anderson Cancer Center, Baylor College of Medicine and Kelsey-Seybold Clinic approved the study. Response rates were ~92% for cases and 76.7% for controls. For this study, which required fresh blood samples, subjects for cases and controls included only those recruited since 2009.

γ-H2AX assay

All of the experiments were performed by a skilled research staff (Y.G.) to minimize interoperator variation and blinded to case–control status of all samples. Each procedure was followed strictly in the same manner using fresh whole blood samples. A total of 0.4 ml of whole blood from each study subject was divided equally into two 0.2 ml aliquots and plated in two 30 mm dishes with 1.8 ml of RPMI 1640 (JRM Biosciences, Lenexa, KS), each containing 15% fetal calf serum and 1.25% phytohemagglutinin (Wellcome Research Laboratories, Research Triangle Park, NC). Samples were then cultured at 37°C for 72 h. One culture was used for detecting baseline DSBs (untreated) and the other for detecting IR-induced DSBs (treated). To avoid stress-induced DNA damage caused by lymphocytes isolation, all whole blood samples were processed for IR and γ-H2AX detection. For the γ-radiation treatment, cultured whole blood cells were exposed to 25 Gy γ-radiation from a cesium-137 source (cesium irradiator Mark 1, Model 30; J.L. Shepherd and Associates, Glendale, CA) at room temperature. Following γ-radiation, whole blood cultures were immediately returned to a 37°C incubator for 1 h, based on the fact that the highest induced γ-H2AX levels are detected 1 h after IR exposure (14). H2AX phosphorylation detection was performed by using a modification of a previously described protocol (14). Briefly, the leukocytes were fixed in 4% formaldehyde (Sigma) for 10 min after complete lysis of erythrocytes, followed by addition of Triton X-100 (0.12% in phosphate-buffered saline (PBS)) to the leukocytes. Then, cells were washed and fixed again in cold 50% methanol in PBS for 10 min. The leukocytes were spotted onto slides and blocked in PBS with 4% bovine serum albumin for 30 min, incubated with mouse monoclonal γ-H2AX antibody (BioLegend, San Diego, CA) for 30 min, washed in PBS and incubated with FITC-conjugated horse anti-mouse secondary antibody (Vector Laboratories) for 30 min. After extensive washing, slides were mounted with propidium iodide counterstain (Abbott Molecular, Abbott Park, IL) and covered with cover slips. All experiments were conducted at room temperature except when specific temperature is mentioned. The above immunocytochemistry assay was conducted simultaneously for the paired untreated blood sample, and the latter was considered as baseline.

An iCys™ laser scanning cytometer (Compucyte, Cambridge, MA) was used to measure the fluorescence signals. Slides were scanned automatically using the 488 nm argon laser and cell count data were obtained with a ×40 objective at a 4 μm pixelation (Figure 1). The clustered cells and fragments of fluorescence were excluded in order to eliminate uncountable cells and non-specific fluorescent signals. Counting was performed until 5000 dispersed and contoured cells were registered. The mean fluorescent intensity per cell was calculated for each slide. The ratio of γ-H2AX level of IR-treated cells to that of non-treated cells (baseline) derived from the same blood sample reflected the final DSB sensitivity to mutagen and was used as core variable to assess BC risk.

Statistical analysis

Patient characteristics according to γ-H2AX status were assessed by means of the Pearson’s chi-square test, comparing cases with controls. Differences in continuous variables were evaluated by the Student’s t-test. The γ-H2AX ratio was analyzed as a continuous variable. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated as estimates of relative risk by using unconditional multivariable logistic regression, adjusting for possible confounding by age, sex and smoking status. To assess for the presence of a trend in BC risk according to degree of DNA damage, we analyzed the data of γ-H2AX ratio using tertiles of the controls as cut-off points. All statistical analyses, including box plot charts, were performed and generated with the STATA 10.1 software package (Stata Corp, College Station, TX). All P values were two sided, and associations were considered statistically significant at P < 0.05.

Results

The study included 174 newly diagnosed, previously untreated BC patients and 174 healthy control subjects, matched by age and gender. The epidemiological data for the whole cohort are summarized in Table I. As expected, there were significantly more ever smokers among cases (58.6 versus 47.7%; P = 5.26 × 10−10).

We assessed the γ-H2AX ratios according to host characteristics as shown in dot plots (Figure 2). Ratios were significantly higher for cases (overall 1.43 ± 0.14 versus 1.35 ± 0.12; P = 8.45 × 10−3). This was irrespective of age (1.44 ± 0.14 versus 1.35 ± 0.12 for individuals ≤66 years; P = 0.00003 and 1.42 ± 0.15 versus 1.35 ± 0.12 for individuals >66 years; P = 0.0006), sex (1.43 ± 0.15 versus 1.36 ± 0.10 for males; P = 6.90 × 10−6 and 1.43 ± 0.15 versus 1.32 ± 0.18 for females; P = 0.007) and smoking status (1.47 ± 0.12 versus 1.35 ± 0.14 for never smokers; P = 1.72 × 10−4 and 1.43 ± 0.15 versus 1.35 ± 0.10 for ever smokers; P = 0.0001). Interestingly, γ-H2AX ratio was significantly higher for never smokers when compared with ever smokers within cases (1.47 versus 1.43; P = 0.03), however, there was no difference within controls in terms of smoking status (1.35 versus 1.35; P = 0.96). Meanwhile, age and sex did not modify γ-H2AX ratio, neither in cases nor in controls (Figure 2).

The risk estimates of BC for induced DNA damage by tertiile distributions in controls are listed in Table II. After adjusting for age, sex and smoking status, there was a significant trend for higher risk at higher tertiles, being more significant at the highest tertile for the whole cohort (OR = 3.78; 95% CI = 2.26–6.33; P = 4.18 × 10−7) and for each category, including age (OR = 3.47; 95% CI = 1.70–7.06 for ≤66 years and OR = 4.16; 95% CI = 1.97–8.79 for >66 years), sex (OR = 3.46; 95% CI = 1.94–6.16 for male and OR = 5.34; 95% CI = 1.68–16.95 for female) and smoking status (OR = 10.48; 95% CI = 3.62–30.36 for never smokers and OR = 3.54; 95% CI = 1.74–7.21 for ever smokers).

We further assessed the multiplicative joint effects of mutagen sensitivity and smoking status on BC risk (Supplementary Table 1, available at Carcinogenesis Online). Cases and controls were categorized into four groups according to γ-H2AX ratio (low or high as dichotomized by the median value in controls) as well as by smoking status. Subjects with no risk factors (i.e. ‘low γ-H2AX ratio and...
never smoker’) were used as the reference group. Compared with this group, ‘high ratio and never smoker,’ ‘low ratio and ever smoker’ and ‘high ratio and ever smoker’ groups showed a gradual increase of BC risk (OR = 6.22, 9.06 and 21.08, respectively; $P$ for trend = $4.32 \times 10^{-11}$). However, no significant interaction between γ-H2AX ratio and smoking status was detected ($P = 0.109$).

![Fig. 2. Dot plot charts of γ-H2AX ratios according to host characteristics comparing cases and controls among (A) overall subjects; (B) subjects age ≤66 years; (C) age >66 years; (D) males; (E) females; (F) never smokers and (G) ever smokers. Horizontal line with crosses indicates mean value with upper and lower dotted lines marking the upper and lower values of mean ± SD. The mean ± SD values for cases and controls and the $P$ values for each comparison are shown.](https://academic.oup.com/carcin/article-abstract/34/11/2543/2464027)
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It requires only a small sample of biological material and the response time for endpoint after DSB induction is very rapid. Moreover, detection can be performed in intact cells, allowing direct visualization of DSBs by immunofluorescence through secondary antibodies. The latter has been enhanced by the introduction of high-throughput systems for counting of γ-H2AX foci (9). It is important to point out, that similar to most of the mentioned assays, there is probably an overlap between detection of DNA damage and repair (14). However, since detection of IR-induced γ-H2AX foci was performed at 1 h, it more likely reflected the degree of mutagen-induced DSB formation, including only a minor proportion of early-phase DNA repair. For all the previous reasons, the γ-H2AX assay is currently a powerful tool to assess DNA DSBs in translational cancer research.

PBLs have been used as a surrogate tissue in several studies evaluating DNA damage and can be considered as the current standard (8, 14, 16, 20, 21). In addition to its simple collection from widely available blood samples, they provide favorable settings for measurement of basal DNA damage before exposure to the mutagenic agent due to their quiescent nature, avoiding interference by DNA damage occurring during S-phase (9). Meanwhile, assessment of DNA damage after mutagen exposure by the ratio of γ-H2AX level of IR-treated lymphocytes to that of non-treated lymphocytes allowed us to reflect variations following treatment more accurately, reducing the potential confounding effect of variability of baseline activity.

A number of different environmental carcinogens have been implicated in the development of BC. The most relevant of them is tobacco smoke. It is associated with 50% of cases among men and with 52% among women according to a recent large analysis, conferring a relative risk of 4.06 (95% CI = 3.66–4.50) and 2.22 (95% CI = 2.03–2.44) to current and former smokers, respectively (22). In our study, the significant association found for high γ-H2AX levels with BC risk was irrespective of smoking status. However, risk was more pronounced in never smokers than in ever smokers (OR = 12.41 and 3.27, respectively). This suggests that genetically susceptible individuals might present with BC at lower environmental carcinogen exposure than those with a significant exposure, e.g. heavy smokers. Therefore, susceptibility to DNA damage may have a more significant role in the development of BC in never smokers. However, overall results of our study are consistent with previous studies, showing that susceptibility to DNA damage in BC exists regardless of smoking status (16).

Implementation of the γ-H2AX assay in prevention and screening of BC will have to be further defined in future studies, focusing on high-risk cohorts and incorporating well-known environmental and genetic risk factors in predictive models. Active screening for BC is currently not recommended by any major organization due to insufficient evidence supporting it (23). The most important arguments against its implementation are the low prevalence of the disease in the general population as well as the low sensitivity and specificity of available screening methodologies. However, small studies testing urinary markers have shown benefits in terms of survival for screened men in addition to be cost-effective when implemented in high-risk populations, e.g. heavy smokers (24, 25). The significant 21-fold increase of risk when combining mutagen sensitivity with smoking status favors the eventual inclusion of this assay to future screening trials focused on high-risk cohorts. This may be a reasonable approach to further assess its value in BC primary prevention.

The limitations of our study include the small number of cases and the absence of an independent external validation. Furthermore, reverse causation of retrospective studies is an inherent limitation to any retrospective case–control study, since results may be affected by disease status. In our case, postdiagnostic samples may not be ideal for assessing the predictive value of phenotypic assays due to the possible effect on outcome by disease status or clinical factors such as therapy. Nevertheless, response to mutagens has been shown to have a high heritability and consistency suggesting a strong genetic component (26–28). Moreover, variations in γ-H2AX baseline activity due to differences in the interval between blood sample collection and the cell-based experiment may have been the case. To avoid this, the interval
time between blood draw and whole blood culture was minimized. However, we were not able to predict a priori or to completely eliminate this problem. Finally, the necessity of using fresh blood samples for proper comparison precluded us from using another method, such as flow cytometry, to assess γ-H2AX activity. Future prospective trials are needed to confirm the strong association of high γ-H2AX levels and BC risk and for overcoming all of these limitations.

Conclusions

Individuals with BC presented a higher susceptibility to induction of DSBs than controls as measured by the γ-H2AX assay in PBLs. This might help to identify individuals at high risk for this cancer, adding new perspectives to established epidemiological and genetic risk factors. Further research of the role of γ-H2AX in biological processes of BC is warranted.

Supplementary material

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

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References


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