Association of low CYP3A activity with p53 mutation and CYP2D6 activity with Rb mutation in human bladder cancer

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Introduction
Bladder cancer is a heterogeneous group of tumors in terms of its morphology (low grade versus high grade), tumor number and size, and at presentation (single versus multiple), stage (superficial versus invasive) and prognosis (recurrence, progression and survival rate) (1,2). These different tumor behaviors suggest a heterogeneous disorder in which there may be different pathways of carcinogenesis and different mechanisms and carcinogens causing genetic mutations (3).

Several oncogenes and tumor suppressor genes have been found in mutated forms in human tumors. Among the tumor suppressor genes, the p53 gene has been found to be frequently mutated in a wide variety of tumors, including bladder. The normal p53 protein has been implicated in controlling cell cycle regulation, cell differentiation and the surveillance of genomic integrity (4-7). A second tumor suppressor gene frequently mutated in bladder cancer is the retinoblastoma (Rb) gene. It encodes a nuclear phosphoprotein which is believed to function as a cell cycle regulator (8-10). It has been shown in both animal and human studies that specific chemical carcinogens can induce both high mutation rates and specific mutation spectra of certain oncogenes or tumor suppressor genes. For example, all rat mammary tumors induced by N-methyl-N-nitrosourea show a G to A mutation in the H-Ras oncogene at the second nucleotide of codon 12 (11). Similarly a high mutation rate, especially at codon 249, of the p53 gene has been identified in hepatocellular carcinoma and is strongly associated with dietary aflatoxin exposure (12).

Even unknown pro-carcinogens associated with tobacco smoke generate specific mutation spectra or genetic fingerprints, in cancer cells. For example, the mutation spectrum of p53 in lung cancer, which is mostly smoking related, is significantly different from the mutation spectrum of p53 in colon cancer, which is not strongly smoking related (5,13-17). This suggests that different proximate carcinogens target specific gene sequences.

One way to identify the individual pathways of carcinogenesis is to analyze the association of intermediate biomarkers related to different steps in cancer progression. The environmental pro-carcinogen hypothesis predicts that a specific procarcinogen or its metabolite will be selectively metabolized, either by detoxification or activation, by specific drug/xenobiotic metabolizing enzymes. The specific proximate carcinogen(s) generated will subsequently interact with specific gene sequences to selectively induce mutations. Previously, we and others have shown that low CYP3A activity (18), assessed by dapsone N-hydroxylation, high CYP2D6 (19) activity, assessed by debrisoquine 4-hydroxylation and the presence of the null genotype of GSTM1 (20,21) were statistically significant susceptibility risk factors for the development of aggressive bladder cancer. In addition, smoking and occupational exposure histories are well-documented risk factors for bladder cancer.

Both p53 and Rb gene mutations are associated with bladder cancer (3,22-24). In bladder cancer, the most commonly identified genetic changes have been p53 gene mutations which can be observed in 40-62% of patients (22,25-28) followed by Rb gene mutations which can be identified in approximately...
Assay of drug metabolizing enzyme activities were screened by immunohistochemistry and p53 RFLP, type C) is associated with cancer. In the present study, altered patterns of p53/Rb mutations in bladder patients with lung cancer (P < 0.05, odds ratio 2.15) (32). However, no information is available concerning the relationship between measures of drug metabolizing enzyme activities and p53/Rb mutations in bladder cancer. In the present study, altered patterns of p53 and Rb expression were screened by immunohistochemistry and compared to specific in vivo drug metabolizing enzyme activities in bladder cancer patients to determine if the mutations were selectively associated with a particular enzyme.

Materials and methods

Subjects for this study were part of a larger case-controlled study of patients receiving barbiturates or who were within 1 week of having had a general anesthesia. All individuals with hepatic disease, congestive heart failure and those receiving barbiturates or who were within 1 week of having had a general anesthesia were excluded from the study. The only drug use allowed at the time of the study were diuretics, digitoxin and aspirin. All other medications were discontinued for at least 3 days.

Assay of drug metabolizing enzyme activities

Total body activities of four drug metabolizing enzyme activities were measured as previously described (18,19). Briefly, each subject underwent a cocktail protocol in which debrisoquine (10 mg orally, for the study of debrisoquine hydroxylation), racemic mephenytoin (100 mg orally, for the study of S-mephenytoin hydroxylation) and debrisoquine (100 mg orally, for the study of debrisoquine hydroxylation and acetylation) were administered concurrently. The concentrations of specific drug metabolites were analyzed by an H-plasma or urine sample to calculate the ratio of parent drug to metabolite. Namely, CYP3A activity was measured by the dapsone recovery ratio (DPRR), CYP2D6 activity by the debrisoquine recovery ratio (DBRR). CYP2C19 activity by the mephenytoin R/S ratio (RDR) and N-acetyltransferase activity by the monoclonal dapsone ratio (MDR) as shown in Table I.

GSTM1 genotyping

A DNA extraction kit (Saratagen Cloning Systems, La Jolla, CA) was used to extract genomic DNA from snap frozen tissue samples from the bladder cancer cases. The GSTM1 null genotype genetic polymorphism was characterized by differential PCR using a slightly modified protocol (20,21,33). Briefly, fragments of both the GSTM1 and β-globin genes were co-amplified by PCR. Twenty microliters of the PCR product was separated electrophoretically on a 8% polyacrylamide gel and visualized by ethidium bromide staining. A 268 b.p. band amplified from the β-globin gene and a 215 b.p. band amplified from GSTM1 gene were identified. The absence of the 215 b.p. band in combination with the presence of a 268 b.p. band was classified as GSTM1 null genotype.

Pathological grading and preparation

All cases in this study were categorized following the guidelines set by the Union Internationale Contre Le Cancer (UICC) for classifying tumors according to the TNM classification (34). All tumors were examined by the same consultant pathologist. In instances where tumors showed two or more histologic grades, the carcinoma was graded according to its highest grade. We defined grades 1 and 2 as non-aggressive and grade 3 only as aggressive bladder cancer Grades 1 and 2 of the UICC classification represent grades 1 and 2 of the Ash classification (35) of the World Health Organization (WHO) classification (36). Grades 3 and 4 of the UICC represents grades 3 and 4 of Ash's classification (35) and grade 3 and undifferentiated carcinoma of the WHO classification (36). In addition, grades 1 and 2 of the UICC represent grade G1 and G2 of the TNM classification and grades 3 and 4 of the UICC represent TNM grades G3 and G4 (34). The cases examined for p53 and Rb mutations included 37 non-aggressive bladder cases and 56 aggressive bladder cancer cases.

Sections (3 mm in thickness) of formaldehyde fixed, paraffin-embedded tumor tissues were cut and mounted on slides at the Bristol Royal Infirmary and transported to the University of Pittsburgh for immunohistochemical staining. Sections of transitional cell carcinoma of the bladder previously identified as positive for p53 gene mutations and sections of retinoblastoma previously identified as positive for Rb gene mutations were used as positive control slides. As a negative control, 250 ml of phosphate buffered saline (PBS) (136 mM sodium chloride, 10 mM phosphate buffer salts) replaced the primary antibodies. This was added to a second positive control slide in each set of slides stained.

Detection of overexpression of p53 protein by immunohistochemistry

Slides were placed in a slide rack and de-paraffinized by heating at 56°C for 30 min immediately followed by two incubations (2X3 min) with xylene. Rehydration of tissue sections was achieved by sequential 5 min incubations in 100%, 95% and 80% ethyl alcohol and rinsed with water. Endogenous peroxidase activity was quenched with a 30 min incubation in 3% hydrogen peroxide/100% methanol solution. To expose the antigenicity of the tissue, slides were boiled in 6 M urea for 10 min in a microwave oven (900 watt) followed by a 15 min cooling and 15 min incubation in PBS at room temperature (RT). Non-specific protein binding was blocked by coating 250 ml of 2% gelatin horse serum (NHS) in PBS for 30 min in a humid chamber at RT. NHS was shaken off and 250 ml of 1/350 dilution of polyclonal rabbit anti-mouse secondary antibody in 2% NHS (prepared with PBS) for 30 min at RT in a humid chamber. This incubation was again followed by washing in PBS for 5 min. Sureperoxidin-peroxidase conjugate (ABC kit, Vector Laboratories, Burlingame, CA), freshly made and allowed to sit for 30 min, was added to the slides for 30 min in a hummid chamber followed by another 5 min PBS washing. Finally, diaminobenzidine (DAB) substrate solution (Pierce, Rockford, IL) was applied for 1–3 min, monitored under microscope for optimal contrast of positive staining (~3 min), to determine the expression of p53 protein by a brown color. The reaction was stopped by washing away the DAB in water. The slide was then counterstained lightly with a 1/2 dilution of Mayer's hematoxylin (Sigma, St Louis, MO) for 15 sec. Dehydration of slides was achieved by sequential 2 min incubations in 95%, 100% and 100% ethyl alcohol and rinsed with water. Endogenous peroxidase activity was quenched with a 30 min incubation in 2.4% hydrogen peroxide/100% methanol solution. To expose the antigenicity of the tissue, slides were boiled in 6 M urea for 10 min in a microwave oven (900 watt) followed by a 15 min cooling and 15 min incubation in PBS at room temperature (RT). Non-specific protein binding was blocked by coating 250 ml of 2% gelatin horse serum (NHS) in PBS for 30 min in a humid chamber at RT. NHS was shaken off and 250 ml of 1/350 dilution of polyclonal rabbit anti-mouse secondary antibody in 2% NHS (prepared with PBS) for 30 min at RT in a humid chamber. This incubation was again followed by washing in PBS for 5 min. Sureperoxidin-peroxidase conjugate (ABC kit, Vector Laboratories, Burlingame, CA), freshly made and allowed to sit for 30 min, was added to the slides for 30 min in a hummid chamber followed by another 5 min PBS washing. Finally, diaminobenzidine (DAB) substrate solution (Pierce, Rockford, IL) was applied for 1–3 min, monitored under microscope for optimal contrast of positive staining (~3 min), to determine the expression of p53 protein by a brown color. The reaction was stopped by washing away the DAB in water. The slide was then counterstained lightly with a 1/2 dilution of Mayer’s hematoxylin (Sigma, St Louis, MO) for 15 sec. Dehydration of tissue sections was achieved by sequential 2 min incubations in 95%, 100% ethyl alcohol and two exchanges of xylene. Permount was used to mount slides with coverslips. The slides were interpreted by the same pathologist, Dr. Michael Beech. A similar protocol for staining Rb protein by immunohistochemistry was used. The primary antibody was a monoclonal mouse antibody PMG3–245 (PharMingen, San Diego, CA) which recognizes the 110 kDa Rb gene product with an epitope between amino acid 300–380. The slides were classified as positive or negative staining by the same pathologist, Dr. Michael Beech. Negative Rb staining indicates a loss of Rb protein due to Rb mutations. Of the 93 bladder cancer slides stained for p53 mutations, 90 were stained for Rb protein detection as duplicate slides were unavailable.

Statistical analysis

Data were recorded in a dBase III file. The database was then transformed and analyzed in a SAS statistic computer program (SAS Institute). The Chi-square test was used to show the difference of frequency distribution of categorical variables, for example p53 or Rb mutation status. Because the continuous variables of drug metabolizing enzyme activities violated the normal distribution assumption for the t-test, the simple predictor of logistic regression analysis was used for univariate analysis.

Results

Clinical histories, including follow-up information, were available for 93 patients with a diagnosis of grades 1, II or III
CYP3A and p53 mutation, CYP2D6 and Rb mutation

Fig. 1. The left panel of the figure shows an aggressive bladder cancer with diffuse positive p53 nuclear staining. The right panel illustrates an aggressive bladder cancer with negative p53 staining.

Fig. 2. The right panel of the figure demonstrates an aggressive bladder cancer which had negative Rb staining only in the cancer cells. The infiltrating lymphocytes, endothelial cells of the blood vessel still expressed Rb protein and stained positive. The left panel shows a non-aggressive bladder cancer in which all the cancer cells stained positive for Rb protein.

Table II. p53 and Rb immunohistochemistry results in bladder cancer

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<tr>
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<th>Aggressive bladder cancer p53</th>
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<tr>
<td></td>
<td>wild-type (- stain)</td>
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<td>wild-type (- stain)</td>
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<tr>
<td>Rb</td>
<td>wild-type (+ stain)</td>
<td>24</td>
<td>9</td>
<td>17</td>
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Of the 93 non-aggressive and aggressive bladder cancer cases, six samples did not have both p53 and Rb staining results.
transitional cell cancer of the bladder as described previously (37). The mean age of the aggressive bladder cancer group was slightly but significantly greater than the control group (72 versus 65 years old, \( P < 0.001 \)). However, the mean age of the non-aggressive or all cancers group was not different from control. The proportion of alcohol drinkers in the aggressive cancer group was greater than that in the control group (70% versus 54%) which conferred a two-fold increase in risk of developing aggressive cancer \( (P < 0.05) \). The frequency of smoking was twice as high in non-aggressive bladder cancer patients than in controls (44% versus 22%, \( P < 0.02 \)). The frequency of a history of occupational exposure was higher in both non-aggressive and aggressive bladder cancer patients than in controls (32%, 33% versus 15%, \( P < 0.01 \) respectively).

Five morphologically normal bladder specimens examined showed absence of p53 staining and presence of Rb staining in urothelial and stromal cells consistent with wild-type expression of these two genes. Mutant p53 gene products have a longer half-life than the wild-type protein, thus rendering it detectable by immunohistochemical analysis. The left panel of Figure 1 shows an example of aggressive bladder cancer with diffuse (>20% tumor cells) p53 positive nuclear staining. In contrast, the right panel illustrates an aggressive bladder tumor with negative p53 staining (Figure 1). The right panel of Figure 2 demonstrates an aggressive bladder tumor which had negative Rb staining only in the tumor cells. The infiltrating lymphocytes and endothelial cells of the blood vessel still expressed Rb protein and stained positive. The left panel shows a non-aggressive bladder tumor in which all the tumor cells stained positive for Rb protein.

The overall altered expression pattern of this series of patients was 47.3% (44/93) for p53 and 22.2% (18/90) for Rb (Figure 3). Among the 93 cases examined for over-expression of p53 protein, 29.7% (11/37) of the non-aggressive and 58.9% (33/56) of the aggressive bladder tumors were classified as p53 positive staining \( (P < 0.01, \text{Chi-square test}) \). Among 90 cases evaluated for expression of mutated Rb protein in tumor cells (negative staining), 5.7% (2/35) of the non-aggressive and 32.7% (18/55) of the aggressive bladder cancer cases were classified as Rb mutation \( (P < 0.01, \text{Chi-square test}) \). These results showed significant associations of both p53 and Rb mutations with aggressive bladder cancer. Two (5.7%) non-aggressive bladder cancer cases and 13 (25%) aggressive bladder cancer cases showed mutations for both p53 and Rb (Table II). The incidence of p53 or Rb mutations was not significantly associated with smoking history or pack-years of smoking (Table III).

Using p53 and Rb mutations as the end point of analysis, it was found that low DPRR was significantly associated with p53 mutations (Table IV) and high CYP2D6 activity (DBRR) was significantly associated with Rb mutations (Table V). In contrast, the RSR, MDR and GSTM1 null genotype were not significantly associated with either p53 or Rb mutations.
Individuals with both low DPRR and high DBRR did not have significantly higher rates of altered expression of either p53 ($P = 0.58$, Chi-square test) or Rb ($P = 0.73$, Chi-square test). When we compared the drug metabolizing enzyme activities between non-aggressive bladder cancer and aggressive bladder cancer, only the DPRR was significantly associated with aggressive bladder cancer (Table VI) (38). The DBRR was not significantly associated with aggressive bladder cancer.

Discussion

The novel observation reported in this study is the finding of selective associations between measures of activity of individual drug metabolizing enzymes and tumor suppressor gene mutations in transitional carcinoma of the bladder. Thus, low CYP3A activity was selectively associated with p53 mutations and high CYP2D6 activity was selectively associated with Rb mutations while MDR, RSR, GSTM1 null genotype were not associated with either tumor marker. These findings support and extend the observations of our previous studies (18,19). These observations are consistent with the hypothesis that CYP3A detoxifies an unknown bladder pro-carcinogen(s) in the environment that preferentially induces p53 mutations. In contrast, CYP2D6 may activate some unknown bladder pro-carcinogen(s) in the environment that preferentially attack DNA sequences found in the Rb gene. These different patterns of associations suggest different mechanisms of bladder carcinogenesis. The GSTM1 null genotype was found to be a susceptibility risk factor for aggressive bladder cancers (20) but was not preferentially associated with either p53 or Rb mutations. This may also reflect the role of GSTM1 as a general protective mechanism with less substrate specificity. N-acetyltransferase and CYP2C19 activities were not significantly associated with p53/Rb mutations or aggressive bladder cancer. These serve as useful negative controls for evaluating the relationship of drug metabolizing enzyme activities and p53/Rb mutations.

The significant association between p53 over-expression and low CYP3A activity should be interpreted with caution. Although it is reasonable to suggest the significant association is related to sequential events, we cannot rule out the possibility that the association between p53 mutation and CYP3A activity may be due to another related variable. For example, both markers were also associated with the morphological appearance of aggressive bladder cancer. The probability was that CYP3A activity was more closely related to p53 mutation ($P = 0.016$, Table IV) than its association with aggressive bladder cancer ($P = 0.049$, Table VI). However, regression analysis alone is unable to discriminate between alternative explanations for a given relationship. The interpretation of the significant association between CYP2D6 activity and Rb mutation is unlikely to be confounded by the tumor type, because CYP2D6 activity was not significantly different between non-aggressive and aggressive bladder cancer (Table VI). However, the sample size is small and may be insufficient to exclude such an association.

A second concern is the reliability of immunohistochemistry to detect p53 mutations compared to direct gene sequencing. False negatives are possible in that frameshifts, nonsense mutations or deletions which abolish protein production will not be detected by immunohistochemistry. Furthermore, not all missense mutations will result in protein stabilization (39). In addition, false positives are also possible with immunohistochemistry. The magnitude of this potential for misclassification appears to be dependent on tumor type. The correlation between p53 mutations detected by immunohistochemistry and gene sequencing is controversial in breast cancer (40–44). In lung cancer, detection of p53 mutations by PCR and direct sequencing were corroborated by immunohistochemistry (45) but the level of expression of p53 mutants was dependent on the type of mutation (46). A good correlation was found between immunohistochemistry and PCR–SSCP analysis for p53 mutations in bladder tumors (39,47) and immunohistochemistry and loss of heterozygosity studies (48). Thus, while it appears that p53 mutations are present in a large proportion of aggressive bladder cancer cases and can be quickly screened by immunohistochemistry, the pattern of p53 mutations, that is the mutational spectra, in combination with complete molecular epidemiological analyses may help to identify possible carcinogens.

In current models of carcinogenesis, neoplastic transformation involves an accumulation of adverse genetic and epigenetic events. This study confirms previous observations that genetically derived differences in the activity of several drug metabolizing enzymes play a role in bladder cancer susceptibility (38). Mutations in tumor suppressor genes and oncoproteins are often detected in bladder cancer and are probably influenced by these predisposing factors. This study represents the first observation of these associations between p53/Rb mutations and drug metabolizing enzyme activities in bladder cancer. The results require confirmation in future investigations including the characterization of mutational spectra by sequencing.

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References
