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

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p53 and Rb gene mutations are intermediate biomarkers useful for the prediction of neoplastic progression in bladder cancers. Previously, we have shown that low CYP3A activity, measured by dapsone *N*-hydroxylation, and high CYP2D6 activity, assessed by debrisoquine 4-hydroxylation, were significant susceptibility risk factors in developing aggressive bladder cancer. However, no information is available about the relationship between drug/xenobiotic metabolizing enzyme activities and *p53*/*Rb* mutations that may suggest mechanisms of bladder carcinogenesis. We evaluated *in vivo* CYP3A activity by the dapsone recovery ratio (DPRR), CYP2D6 activity by the debrisoquine recovery ratio (DBRR), CYP2C19 activity by the mephenytoin R/S ratio (RSR), *N*-acetyltransferase activity by the monoacetyl dapsone to dapsone ratio and glutathione-S-transferase M1 (*GSTM1*) genotype by PCR. In immunohistochemical studies of bladder tumor tissue, over expression of *p53* protein was detected with antibody pAb1801 and loss of *Rb* protein expression was evaluated with antibody PMG3–245 in patients with transitional cell carcinoma of the bladder. Low CYP3A activity was significantly associated with over expression of or mutated p53 protein ($P < 0.05$). High CYP2D6 activity (within the extensive metabolizer group) was significantly associated with loss of expression of or mutated *Rb* protein ($P < 0.05$). Positive *p53* staining also predicted aggressive bladder cancer histopathology ($P < 0.05$, odds ratio 2.9), and the lowest tertile of DPRR predicted *p53* positivity ($P < 0.01$, odds ratio 3.9 comparing means of lower tertile versus upper tertile of DPRR). These selective associations are consistent with the hypothesis that an environmental pro-carcinogen fails to be detoxified by CYP3A which may preferentially induce *p53* mutations, whereas, an alternative pro-carcinogen that may be activated by CYP2D6, may selectively induce *Rb* mutations.

Introduction

Bladder cancer is a heterogeneous group of tumors in terms of its morphology (low grade versus high grade), tumor number 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*-nitrosurea 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 pro-carcinogen 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

*Abbreviations: DPRR, dapsone recovery ratio; DBRR, debrisoquine recovery ratio; *GSTM1*, glutathione-S-transferase M1; RSR, mephenytoin R/S ratio; MDR, monoacetyl dapsone ratio; UICC, Union Internationale Contre le Cancer; WHO, World Health Organization; PBS, phosphate buffered saline; RT, room temperature; NHS, normal horse serum; DAB, diaminobenzidine.

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Table I. Equations of drug metabolizing enzyme activities

Parameter	Equation
Dapsone recovery ratio	DPRR = dapsone hydroxylamine/dapsone + dapsone hydroxylamine
Debrisoquine recovery ratio	DBRR = 4-hydroxydebrisoquine/ 4-hydroxydebrisoquine + debrisoquine
Monodapsone acetylation ratio	MDR = monoacetyldapsone/dapsone
Mephenytoin R/S ratio	RSR = R-mephenytoin/S-mephenytoin

30% (9,10,29,30). Due to this high frequency, both are used as prognostic predictors of bladder cancers (9,10,31). The precedent for associating selective routes of metabolism with gene mutations was the observation that the *CYP1A1* genetic polymorphism (*MspI* RFLP, type C) is associated with *p53* mutations in Japanese 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

Patient recruitment

Subjects for this study were part of a larger case-controlled study of patients with transitional cell cancer of the bladder ($n = 93$) or controls ($n = 85$) recruited from the hematuria clinic at the Bristol Royal Infirmary, Bristol, UK between November 1988 and April 1991. Only incident cases were recruited. All individuals with hepatic disease, congestive heart failure and those receiving barbiturates or who were within 1 week of having had a general anesthetic 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 dapsone (100 mg orally, for the study of dapsone hydroxylation and acetylation) were administered concurrently. The concentrations of specific drug metabolites were analyzed from an 8-h plasma or urine sample to calculate the ratios 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 (RSR) and *N*-acetyltransferase activity by the monoacetyl dapsone ratio (MDR) as shown in Table I.

GSTM1 genotyping

A DNA extraction kit (Stratagene 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 G_1 and G_2 of the TNM classification and grades 3 of the UICC represents TNM grades G_3 and G_4 (34). The cases examined for *p53* and *Rb* mutations included 37 non-aggressive bladder cases and 56 aggressive bladder cancer cases.

Sections (5 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 analyzes. Sections of transitional 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) (138 mM sodium chloride, 10 mM phosphate buffer salts) replaced the primary antibody. 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 (2×3 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 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% normal horse serum (NHS) in PBS onto each slide for 20 min in a humid chamber at RT. NHS was shaken off and 250 ml of 1/350 dilution of polyclonal mouse antibody pAb1801 (*p53* Ab-2; Oncogene Science, Manhasset, NY) in PBS was applied and incubated in a humid chamber at 4°C overnight. This antibody recognizes a human specific epitope near the amino terminus of the p53 protein and detects both normal and mutated p53 proteins. Slides were subsequently washed with PBS for 10 min. The slides were exposed to 250 ml of 1/200 dilution of biotinylated 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. Streptavidin–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 humid 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 (1–3 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 Becich, and graded as negative, focal (<5% cells stained positive), patch (5–20% cells stained positive) and diffuse (>20% cells stained positive). The presence of *p53* mutations was defined as a specimen in which more than 20% of the cells stained positive (26).

Detection of Rb protein by immunohistochemistry

A similar protocol for staining Rb protein 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 Becich. 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 single 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 I, II or III

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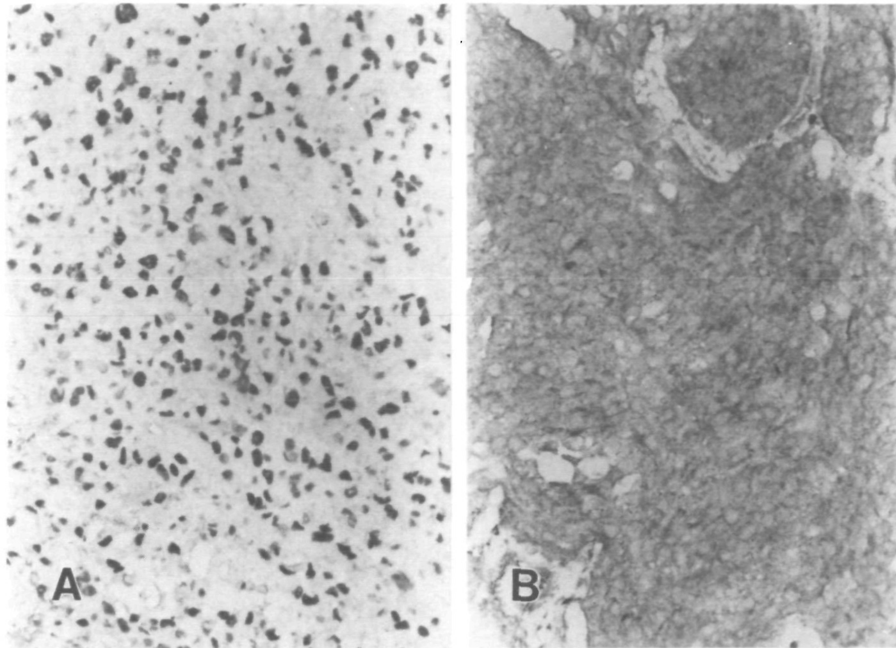

Fig. 1. The **left** panel of the figure shows an aggressive bladder cancer with diffuse positive *p53* nuclear staining. The **right** panel illustrates an aggressive bladder cancer with negative *p53* staining.

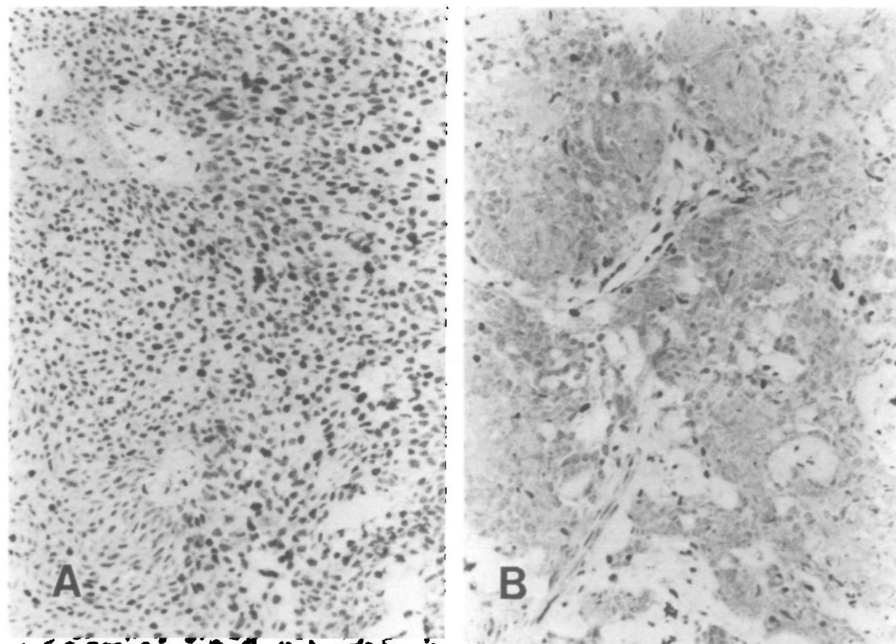

Fig. 2. The **right** panel of the figure demonstrates an aggressive bladder cancer which had negative *Rb* staining only in the cancer cells. The infiltrating lymphocytes, endothelial cells of the blood vessel still expressed *Rb* protein and stained positive. The **left** panel shows a non-aggressive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

Table II. *p53* and *Rb* immunohistochemistry results in bladder cancer

		Non-aggressive bladder cancer *p53*		Aggressive bladder cancer *p53*	
		wild-type (- stain)	mutant (+ stain)	wild-type (- stain)	mutant (+ stain)
Rb	wild-type (+ stain)	24	9	17	18
Rb	mutant (- stain)	0	2	4	13

Of the 93 non-aggressive and aggressive bladder cancer cases, six samples did not have both p53 and Rb staining results.

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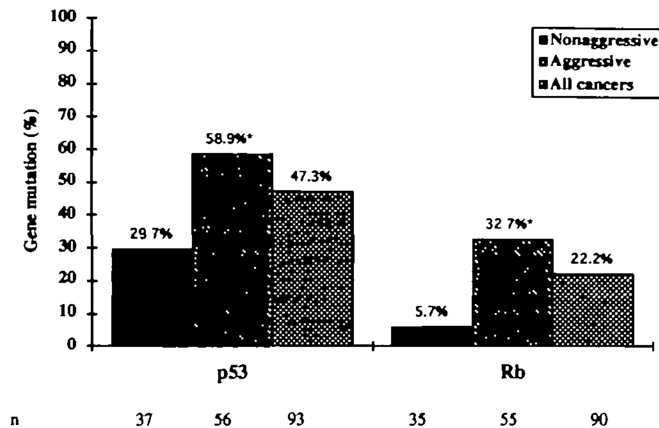

Fig. 3. *p53* and *Rb* mutation rates in non-aggressive, aggressive and all bladder cancer patients. *$P = 0.001$ for comparison of non-aggressive versus aggressive bladder cancer, Chi-square test.

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%, $P < 0.03$ and $P < 0.01$ 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 ($P < 0.01$, 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 ($P < 0.01$, 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.

Table III. *p53* and *Rb* mutation status and smoking history

Immunohistochemistry Results	Smoker n (%)	Nonsmoker n (%)	Pack-year[a] mean ± SD
p53 wild-type	42 (85.7%)	7 (14.3%)	37.90 ± 24.2
p53 mutant	36 (81.8%)	8 (18%)	37.03 ± 25.1
Rb wild-type	59 (84.3%)	11 (15.7%)	37.73 ± 23.9
Rb mutant	16 (80%)	4 (20%)	35 64 ± 28.3

[a]One pack-year is defined as one pack (20) of cigarettes/day for 1 year.

Table IV. Drug metabolizing enzymes as single predictor of *p53* protein expression

	p53 wild-type mean ± SD	n	*p53* mutant mean ± SD	n	P*
DBRR	0.51 ± 0.23	48	0.51 ± 0.24	44	n.s.
DPRR	0.51 ± 0.17	49	0.41 ± 0.21	43	0.016*
RSR	3.70 ± 4.31	49	3.15 ± 3.00	43	n.s.
MDR	0.39 ± 0.27	49	0.41 ± 0.29	42	n.s.
% *GSTM1* null genotype	56.3	48	66.7	39	n.s.

*Single predictor of logistic regression analysis.
n.s., not significant.
DBRR, missing value for case no.139; DPRR, missing value for case no. 153; RSR, missing value for case no. 185; MDR, missing value for case no. 18,38; GSTM1, missing value for cases no. 33,44,47,65,184.

Table V. Drug metabolizing enzymes as a single predictor of *Rb* protein expression in aggressive bladder cancer

	Rb wild-type mean ± SD	n	*Rb* mutant mean ± SD	n	P
DBRR	0.48 ± 0.24	69	0.61 ± 0.16	20	0.025*
DPRR	0.47 ± 0.19	69	0.46 ± 0.22	20	n.s.
RSR	3.65 ± 3.94	69	2.82 ± 3.37	20	n.s.
MDR	0.38 ± 0.28	69	0.45 ± 0.28	18	n.s.
% *GSTM1* null genotype	64.2	67	50.0	18	n.s.

*Single predictor of logistic regression analysis.
n.s., not significant.
DBRR, missing value for case no. 139; DPRR, missing value for case no. 153; RSR, missing value for case no. 185; MDR, missing value for case no. 18,38,177; GSTM1, missing value for cases no. 33,44,47,65,184.

Table VI. DBRR and DPRR as single predictors of non-aggressive versus aggressive bladder cancer

	Non-aggressive		Aggressive		*P
DBRR (mean)	0.52	n = 42	0.52	n = 64	0.987
DPRR (mean)	0.52	n = 43	0.44	n = 63	0.049

*Single predictor of logistic regression analysis.

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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 can not 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 oncogenes 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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