

## P53 alterations in bladder tumors from arsenic and tobacco exposed patients

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Previous studies demonstrated that tobacco and arsenic exposure are risk factors for bladder cancer. A case–case study was conducted to compare *p53* mutations in 147 bladder tumors from South American patients by tobacco and arsenic exposure. Information on residential history and lifestyle factors was collected. The prevalence of *p53* mutations and protein expression was examined in relation to tumor stage, grade, patient age, gender, tobacco and arsenic exposure. Smokers were grouped as ever/never smokers and by pack years of exposure (0, 1–20, >20). Patients were also grouped into four arsenic exposure categories based on the average of the five highest years arsenic concentration in their drinking water: group 1, non-detectable to <10 µg/l (*n* = 50); group 2, 10–99 µg/l (*n* = 31); group 3, 100–299 µg/l (*n* = 35); group 4, >300 µg/l (*n* = 30). The proportion of tumor samples with *p53* mutations and P53 immunopositivity increased strongly with both stage and grade, but not with arsenic exposure or smoking. The prevalence of tumors containing mutational transitions increased markedly with tumor stage (from 14 to 52%,  $P_{\text{trend}} = 0.005$ ) and grade (from 11 to 48%,  $P_{\text{trend}} = 0.004$ ) and was higher in smokers than in non-smokers (34 versus 18%, respectively,  $P = 0.10$ ). An increasing trend was observed with pack years of smoking ( $P = 0.09$ ). The majority of mutations in tumors from both smokers and non-smokers were G → A transitions, however, in smokers a preference for G → A transitions at CpG sites was observed ( $P = 0.07$ , two-tailed) and a positive trend was observed with pack years of exposure ( $P = 0.04$ ). A hotspot was found at codon 273 in 12% of the tumors from smokers but was not observed in never smokers ( $P = 0.05$ ) and a positive trend was observed with pack years of tobacco exposure ( $P = 0.001$ ). Neither stage nor grade demon-

strated a preference for CpG site mutation, suggesting that these changes may be early exposure-related events in carcinogenesis and are not related to tumor progression. Arsenic exposure was not associated with an increased prevalence of *p53* mutation or P53 immunopositivity and there was no evidence of interaction between arsenic and smoking with these outcome variables.

### Introduction

Inactivation of the *p53* tumor suppressor gene is found in most human cancers, including bladder cancer (1). Such mutations are frequently found in cancer because mutation of the *p53* gene provides the cell with a selective advantage for growth and proliferation. Normal P53 protein functions in cell cycle regulation, in maintenance of genomic stability and in controlled cell death (apoptosis). Mutation of the *p53* gene is believed to be a key mechanism of gene inactivation. Most inactivating mutations in *p53* consist of point mutations in evolutionarily conserved domains, leading to changes in the amino acid composition of the P53 protein. It is thought that examination of the presence and pattern of molecular changes in the *p53* gene in tumor tissue could provide clues to early carcinogenic events and the mechanism of tumor formation (2).

In cancer epidemiology studies, the case–case study design can be used to compare the prevalence of *p53* mutations in cases exposed and unexposed to an etiological agent, after adjusting for potential confounding factors such as sex, age and stage and grade of tumors that may be related to both exposure and mutational patterns in tumors. *p53* gene mutation is a useful biomarker in such studies because it is frequently mutated in nearly all types of human cancer and because exogenous and endogenous mutagenic events cause distinctly different mutational patterns (3). In addition to *p53* mutation, aberrant P53 protein expression and or degradation can also be detected in tissues through immunohistochemical (IHC) methods. The prevalence of tumors observed to be positive or negative for IHC stain can be compared in exposed and unexposed cases to provide further understanding of etiology. Although a mutated *p53* gene frequently results in a protein that is observable through IHC methods, the two assays measure different outcomes and results of mutational sequencing and immunohistochemical analysis of the same tumors can be inconsistent. This is because mutations that affect IHC staining can occur outside the exons sequenced or within extragenic promoter sites leading to reduced gene expression or because they result in stop codons that interrupt protein translation (1). The P53 protein can be transcriptionally down-regulated leading to ‘false negative’ IHC. ‘False positive’ IHC results are also possible if the *p53* gene is overexpressed or if the P53 protein is bound to viral or cellular proteins (1).

The *p53* gene is mutated in ~40% of bladder tumors (4,5). About 18% of all the mutations (i.e. including insertions and

**Abbreviations:** 4-ABP, 4-aminobiphenyl; BPDE, (+)-anti-7β,8α-dihydroxy-9α,10α-epoxy 7,8,9,10-tetrahydrobenzo[*a*]pyrene; 95% CI, 95% confidence intervals; H&E, hematoxylin and eosin; IHC, immunohistochemical; OR, odds ratios; TCC, transitional cell carcinoma.

deletions) in bladder tumors result in a change from G:C to A:T at CpG dinucleotide sites. These sites are susceptible to mutation via deamination of a 5'-methylcytosine to thymidine and also through DNA adduct formation (4). It is thought that CpG islands may be preferential targets for many chemical carcinogens (6,7).

The pattern of *p53* mutations has been studied in bladder cancer, but limited attention has been given to environmental exposures. Some studies have found predominantly G → A transitions, while others reported mostly transversions (1,8). Although bladder cancer incidence is between two and four times higher in smokers than in non-smokers (9,10), an association of *p53* mutation and/or P53 IHC positivity with cigarette smoking has not been conclusively demonstrated.

In this study, bladder tumors from patients in Argentina and Chile that reported ever or never smoking tobacco and patients exposed to various levels of arsenic in drinking water were compared (11). Small studies comparing arsenic-exposed and unexposed bladder and skin tumors have suggested the possibility of arsenic-specific mutational patterns in the *p53* gene (12–15). P53 alterations were also examined in relation to patient age, gender and tumor stage and grade.

## Materials and methods

### Case selection and interviews

Cases were obtained from two bladder cancer case–control studies conducted in Argentina and Chile, principally for the purpose of investigating the association with drinking water arsenic levels. These studies were reviewed and approved by the Committee for the Protection of Human Subjects at the University of California Berkeley and by local Institutional Review Boards. In Argentina the study included all newly diagnosed cases of transitional cell carcinoma (TCC) of the bladder diagnosed among residents of Union County (population 96 000) between 1996 and 2000 and Marcos Juarez County (population 97 000) from 1998 to 2000. These counties were selected because of previously reported high bladder cancer mortality rates (16) and a known wide range of arsenic levels in drinking water. Bladder cancer cases were verified as TCC by pathologic review. Smoking, in this paper, has been categorized into those who reported ever smoking cigarettes, a pipe or cigars and those who reported never having smoked tobacco. Tumor biopsies were also collected from a hospital-based bladder cancer case–control study conducted in northern Chile which had the same design and was conducted simultaneously with a lung cancer case–control study in the same population (17). The Chilean case–control study included many individuals exposed to very high concentrations (>500 µg/l) of arsenic in their drinking water. Briefly, nurses identified bladder cancer cases in the public hospitals of regions I–III of northern Chile between November 1994 and July 1996. Cases were confirmed by biopsy and diagnosis was either during the current hospital admission or no more than 1 year before the current admission. In both studies, each patient was interviewed about their lifetime residential histories, current and past drinking water and tobacco consumption and occupational histories.

### Exposure assessment

Smokers were first categorized as ever or never smokers and then by pack years of tobacco exposure. For cases from Argentina, data were also available to categorize smokers as current or ex-smokers, although information on whether the Chilean subjects were smokers at the time of interview was not available. Smoking by pack years was grouped in two ways, first as those below and those at or above the median number of pack years (1–44 and ≥45) and then as 1–20 or >20 pack years, since it appeared that most of the smoking-associated mutations were observed at smoking levels >20 pack years.

Arsenic exposures for the Argentine and Chilean cases were calculated as previously described (17,18). Briefly, water samples from the study area in Argentina were frozen at –20°C in 50 ml aliquots before being brought to the USA on dry ice. Arsenic was measured by hydride generation using a previously published method (19). Both bottled and aljibe (rain water collection tank) water had only very low levels of arsenic contamination.

For the Chilean subjects, almost 100% of urban households were served by city water systems and the large majority of the remaining population in this desert region received water from town or village sources distributed by small

canals. Since 1950, measurement of arsenic in water has been carried out extensively. We obtained historic data on arsenic concentrations in drinking water from 1950 to 1994 (20).

Cases were grouped into four categories based on identifying the five years with the highest concentration of arsenic in each participant's drinking water during the period 5–40 years prior to their diagnosis with bladder cancer. Group 1 consisted of tumors from cases that had an average maximum 5-year arsenic concentration of: group 1, 0–<10 µg/l (*n* = 50); group 2, 10–99 µg/l (*n* = 31); group 3, 100–299 µg/l (*n* = 35); group 4, ≥300 µg/l (*n* = 30). Nearly all of those in the highest exposure category were from region II of Chile.

### Tumor samples and tissue selection

To confirm diagnoses, both pathology reports and tumor sections were reviewed by study pathologists as previously described (18). One hematoxylin and eosin (H&E) stained slide was used to confirm the grade of each tumor. For Argentine cases, the tumor stage described in the pathology report was used, since only one paraffin block was obtained for each case. For Chilean cases, all existing tumor blocks were collected for each case and H&E sections from all blocks were reviewed to confirm tumor grade and stage. Staging and grading of tumors was in accordance with World Health Organisation guidelines (21,22).

Paraffin sections (5 µm) were placed on glass slides for microdissection. Using an adjacent H&E stained slide for orientation, one or two 5 µm deparaffinized, methyl green (0.1%) stained sections were microdissected, as described previously (23). An area of the tumor containing the smallest proportion of normal cells was selected. Areas containing necrotic tissue and cautery artifacts were excluded. When multiple tumor pieces were available, the largest area of tumor that was representative of the primary lesion was selected. The minimal size needed was at least 0.3 mm<sup>2</sup> or ~500 cells. The volume of tissue extraction buffer was adjusted to contain ~500–1000 cells/15 µl. DNA isolation and PCR amplification were conducted as described previously (23,24).

### Immunohistochemistry

Formalin fixed and paraffin embedded tumors were cut into 5 µm sections, placed on charged slides and baked in preparation for P53 IHC staining. Antigen retrieval on fixed stained slides was used as described previously (25). Briefly, P53 immunostaining was performed using pAb1801, a purified IgG mouse monoclonal antibody to human P53 that reacts with a denaturation-stable epitope between amino acids 32 and 79 (Cambridge Research Biochemicals, Wilmington, DE). Deparaffinized slides were incubated with a 1:4000 dilution of primary antibody in phosphate-buffered saline and 1% bovine serum albumin at 4°C for 18 h. Antibody staining was observed using biotinylated anti-mouse antibody (Vector Laboratories, Burlingame, CA) and streptavidin-conjugated horseradish peroxidase (Zymed Laboratories, San Francisco, CA) followed by diaminobenzidine. Sections were counterstained using methyl green (1%) and then dehydrated and coverslipped with mounting medium. Known negative and positive control slides of cell lines (MDA 231 and MDA 453, respectively) were stained with each batch of slides for quality control of staining. Cells that displayed a finely granular staining pattern confined to the nucleus were interpreted as 'positive'. The total number of positive and negative tumor cells was estimated by counting at least 1000 cells at 400× magnification. If <10% of all tumor cells were positive, the sample was classified as negative. If at least 10% of all tumor cells were positive, the sample was classified as positive.

### Sequencing

Fluorescent DNA sequencing of exons 5–8 of the *p53* gene was carried out using BigDye™ dye terminator sequencing chemistry (PE Biosystems). Primers used have been previously described (26). Before sequencing, PCR products were purified using the PCR product pre-sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ) to remove excess primers and nucleotides. First, exons 5–8 were sequenced in the forward direction. If the presence of a mutation was indicated, confirmatory sequencing in the reverse direction was run from an independent PCR reaction.

### Statistical analyses

Contingency table analysis to examine variation in mutation and staining frequency for categorical variables (age, gender, stage, grade, smoking and arsenic) was carried out using  $\chi^2$  tests for differences between groups. The proportion of cases with mutations or positive IHC was calculated by dividing the number of abnormal cases by the total number of tumors in the group. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated with logistic regression incorporating potential confounding factors using Stata 6.0 (Stata Corp., College Station, TX). All statistical tests were two-sided.

## Results

The distributions of tumors from Argentina and Chile by age, gender, stage, grade and smoking status (ever/never) are shown in Table I. There was a higher proportion of male cases and a lower proportion of female cases in the Argentinean group than in the Chilean group.

P53 alteration in bladder cancer tissue was assessed using both sequence ( $n = 126$ ) and IHC analyses ( $n = 147$ ) (Table II). In 21 of the samples analyzed, at least one exon of the *p53* gene was not amplifiable and these samples were excluded from the mutational analysis study. Of the 126 tumors analyzed by sequencing, 45 (36%) contained at least one mutation. Mutations were distributed across all four exons, 30% in exon 5, 8% in exon 6, 11% in exon 7 and 51% in exon 8 (data not shown). Sixty (41%) of the 147 tumors studied by IHC were positive for P53 staining.

As seen in Table II, the prevalence of tumors with *p53* mutations and those with positive staining increased significantly with both stage ( $P_{\text{trend}} = 0.01$  and  $0.01$ , respectively) and grade ( $P_{\text{trend}} = 0.003$  and  $0.008$ , respectively). No significant associations with *p53* mutations nor P53 positive IHC staining were observed for age, gender, tobacco and arsenic exposure categories.

The prevalence of each mutation class is shown in Table III. Single base substitutions (transitions and transversions) were the most common type of mutation observed. A transition is the replacement of one purine by another purine or of one pyrimidine by another pyrimidine. A transversion is the replacement of a purine by a pyrimidine or vice versa. Transitions and transversions are thought to arise through different mechanisms and different chemical exposures have been associated with one predominant type of mutation (1,4). The prevalence of tumors containing transitions but not transversions increased with both tumor stage ( $P_{\text{trend}} = 0.005$ ) and grade ( $P_{\text{trend}} = 0.004$ ) (Table III). The prevalence of tumors with transitions from patients who ever smoked was slightly higher

than in never smokers, 34 versus 18%, respectively (OR = 1.63, 95% CI 0.83–7.94,  $P = 0.10$ , two-tailed). A trend was observed with increasing pack years of smoking, the highest percentage being found in those with >20 pack years of exposure ( $P_{\text{trend}} = 0.09$ ). Only tumors from smokers with >20 pack years of exposure contained double mutations ( $n = 5$ ; data not shown). Trends were not observed between the prevalence of transitions and patient age or arsenic exposure category. There was a suggestion of a trend with transversion prevalence and the maximum 5-year peak concentration of arsenic in drinking water category ( $P_{\text{trend}} = 0.11$ ) and perhaps tumor grade ( $P_{\text{trend}} = 0.22$ ).

The prevalence of G → A transitions is presented in Table IV. In the group as a whole, 56% (25 of 45) of all *p53* mutations observed were G → A transitions, of which 72% (18 of 25) were located at CpG sites. Mutations at CpG sites are thought to be caused either by endogenous factors, such as deamination of methylated cytosine, or by exposure to adduct-forming chemicals, such as (+)-anti-7β,8α-dihydroxy-9α,10α-epoxy 7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE), a carcinogenic component of tobacco smoke. A higher prevalence of tumors with G → A transitions at CpG sites was observed in ever smokers than in never smokers (17 versus 4%,  $P = 0.07$ , two-tailed). A trend with pack years of tobacco exposure was also observed ( $P_{\text{trend}} = 0.04$ ). A mutational hotspot at codon 273 was observed in 12 (12%) tumors from ever smokers but none of the tumors from never smokers ( $P = 0.05$ , two-tailed). As pack years of tobacco exposure increased, so did the prevalence of G → A transitions located at CpG sites ( $P = 0.04$ ) and codon 273 ( $P = 0.001$ ). This type of mutation was specific to tobacco exposure and, unlike other types of mutations, did not increase with stage or grade. Neither G → A transitions located within exons 5–8 nor G → A transitions at CpG sites nor at codon 273 were associated with arsenic exposure. Arsenic-exposed ever smokers and never smokers in each exposure category had similar prevalences of G → A transitions. Comparisons of results from regression analyses were conducted with and without interaction (smoking history and arsenic exposure) using a likelihood ratio test. Ever smoking and arsenic exposure combined did not cause an increase in the prevalence of *p53* mutations or IHC positivity than either factor alone (data not shown).

**Table I.** Age, gender, stage, grade and smoking status for bladder cancers from Argentina and Chile

|                     | Argentina <i>n</i> (%) | Chile <i>n</i> (%) | Total <i>n</i> (%) |
|---------------------|------------------------|--------------------|--------------------|
| Total               | 105 (71)               | 42 (29)            | 147 (100)          |
| Age                 |                        |                    |                    |
| ≤60 years           | 27 (26)                | 9 (21)             | 36 (24)            |
| 61–75 years         | 50 (48)                | 18 (42)            | 68 (46)            |
| ≥76 years           | 28 (27)                | 16 (37)            | 43 (29)            |
| Gender <sup>a</sup> |                        |                    |                    |
| Male                | 90 (86)                | 25 (60)            | 115 (78)           |
| Female              | 15 (14)                | 17 (40)            | 32 (22)            |
| Stage               |                        |                    |                    |
| Ta                  | 31 (30)                | 12 (29)            | 43 (29)            |
| T1                  | 40 (38)                | 20 (48)            | 60 (41)            |
| T2–T4               | 34 (32)                | 10 (24)            | 44 (30)            |
| Grade               |                        |                    |                    |
| 1                   | 31 (30)                | 6 (14)             | 37 (25)            |
| 2                   | 51 (49)                | 25 (60)            | 76 (52)            |
| 3                   | 21 (22)                | 11 (26)            | 34 (23)            |
| Smoking             |                        |                    |                    |
| Never               | 23 (22)                | 14 (33)            | 37 (25)            |
| Ever                | 81 (78)                | 28 (67)            | 110 (75)           |

<sup>a</sup> $P < 0.01$ ,  $\chi^2$  test.

## Discussion

In this study, sequencing and IHC were used to characterize the prevalence, location and class (transition or transversion) of *p53* mutations and immunopositivity in bladder tumors. Tobacco exposure was associated with a distinct class and pattern of mutation. Tobacco exposure was associated primarily with G → A transitions at CpG sites and a ‘hotspot’ was found at codon 273, a CpG site previously found to contain BPDE adducts and mutations in tobacco-associated lung tumors (27–29).

The chemical carcinogen 4-aminobiphenyl (4-ABP) is now considered to be a major etiological agent of human bladder cancer based on similarities observed in the *p53* alteration database at the International Agency for Research on Cancer (IARC) (8) and *in vitro* toxicological evidence demonstrating that chemical exposure forms adducts preferentially at non-CpG sites such as codons 280 and 285 (30–32). It should be noted, however, that because the IARC TP53 database

**Table II.** *p53* mutations and P53 immunohistochemistry for bladder cancers from Argentina and Chile

|                           | Cases with <i>p53</i> mutations/total cases |      |       |            | Cases positive for P53 staining/total cases |      |       |            |
|---------------------------|---|------|-------|------------|---|------|-------|------------|
|                           | <i>n</i>                                    | (%)  | OR    | 95% CI     | <i>n</i>                                    | (%)  | OR    | 95% CI     |
| Total                     | 45/126                                      | (36) |       |            | 60/147                                      | (41) |       |            |
| Smoking <sup>a</sup>      |   |      |       |            |   |      |       |            |
| Never                     | 9/28  | (32) | 1.00  |            | 15/37                                       | (41) | 1.00  |            |
| Ever                      | 36/98                                       | (37) | 1.27  | 0.50–2.99  | 41/100                                      | (41) | 1.01  | 0.45–2.23  |
| <i>P</i>                  |   |      | 0.66  |            |   |      | 0.99  |            |
| Pack years <sup>a</sup>   |   |      |       |            |   |      |       |            |
| 0                         | 9/28  | (32) | 1.00  |            | 15/37                                       | (41) | 1.00  |            |
| 1–20                      | 5/18  | (28) | 1.13  | 0.29–4.40  | 9/21  | (43) | 1.61  | 0.52–4.95  |
| >20                       | 31/80                                       | (39) | 1.31  | 0.49–3.50  | 36/87                                       | (41) | 0.99  | 0.43–2.24  |
| <i>P</i> <sub>trend</sub> |   |      | 0.59  |            |   |      | 0.78  |            |
| Arsenic <sup>ab</sup>     |   |      |       |            |   |      |       |            |
| 1                         | 13/43                                       | (30) | 1.00  |            | 17/49                                       | (35) | 1.00  | –          |
| 2                         | 10/29                                       | (34) | 1.46  | 0.50–4.21  | 13/32                                       | (41) | 1.48  | 0.57–3.86  |
| 3                         | 12/27                                       | (44) | 2.26  | 0.78–6.54  | 16/34                                       | (47) | 2.05  | 0.80–5.23  |
| 4                         | 10/26                                       | (38) | 1.36  | 0.47–3.96  | 14/31                                       | (45) | 1.47  | 0.57–3.83  |
| <i>P</i> <sub>trend</sub> |   |      | 0.43  |            |   |      | 0.33  |            |
| Stage                     |   |      |       |            |   |      |       |            |
| Ta                        | 8/35  | (23) | 1.00  |            | 11/43                                       | (26) | 1.00  |            |
| T1                        | 17/51                                       | (33) | 1.69  | 0.63–4.50  | 26/60                                       | (43) | 2.22  | 0.95–5.23  |
| T2–T4                     | 20/40                                       | (50) | 3.38  | 1.24–9.20  | 23/44                                       | (52) | 3.19  | 1.23–7.88  |
| <i>P</i> <sub>trend</sub> |   |      | 0.01  |            |   |      | 0.01  |            |
| Grade                     |   |      |       |            |   |      |       |            |
| 1                         | 4/28  | (14) | 1.00  |            | 9/37  | (24) | 1.00  |            |
| 2                         | 24/65                                       | (37) | 3.51  | 1.08–11.34 | 32/76                                       | (42) | 2.26  | 0.94–5.46  |
| 3                         | 17/33                                       | (52) | 6.38  | 1.81–22.46 | 19/34                                       | (56) | 3.94  | 1.43–10.83 |
| <i>P</i> <sub>trend</sub> |   |      | 0.003 |            |   |      | 0.008 |            |
| Age                       |   |      |       |            |   |      |       |            |
| ≤60 years                 | 11/30                                       | (37) | 1.00  |            | 12/36                                       | (33) | 1.00  |            |
| 61–75 years               | 24/58                                       | (41) | 1.22  | 0.5–3.0    | 32/68                                       | (47) | 1.78  | 0.77–4.1   |
| ≥76 years                 | 10/38                                       | (26) | 0.62  | 0.2–1.7    | 16/43                                       | (37) | 1.19  | 0.5–3.0    |
| <i>P</i> <sub>trend</sub> |   |      | 0.56  |            |   |      | 0.88  |            |
| Gender                    |   |      |       |            |   |      |       |            |
| Females                   | 9/23  | (39) | 1.00  |            | 14/32                                       | (43) | 1.00  |            |
| Males                     | 36/100                                      | (36) | 0.84  | 0.33–2.12  | 46/115                                      | (40) | 0.86  | 0.39–1.89  |
| <i>P</i>                  |   |      | 0.71  |            |   |      | 0.70  |            |

<sup>a</sup>Analysis results adjusted for tumor stage and grade.

<sup>b</sup>Average 5-year peak concentration (with proxy concentrations from nearest well of similar depth when necessary): group 1, 1–<10 µg/l; group 2, 10–99 µg/l; group 3, 100–299 µg/l; group 4, >300 µg/l.

contains data from bladder tumors from patients with many unique exposures, the mutational spectrum may be biased as it is probably not representative of bladder cancer in general. We compared our findings reported here with those summarized in the IARC *p53* database for human bladder cancer (8). When the IARC tumor group was examined as a whole, regardless of exposure, the majority of bladder cancer mutations reported in the database were G → A transitions (32.9%), and 20.2% of all G → A transitions occurred at CpG islands (8), results that are similar to those found in the current study. Unlike the current study, the most frequently reported bladder cancer mutations reported were located at codon 248 (8%) and codons 266, 280 and 285 (4.5% each). Only 3% of mutations were located at codon 273, the major bladder cancer hotspot observed in this study (10%). However, when the analysis of the IARC database was limited to include only bladder tumors from smokers, 42.4% of mutations reported were G → A transitions, 6/33 (18.1%) mutations were located at CpG sites and 3/33 (10%) mutations were located at codon 273, similar to the 12% found in this study among smokers. Similarly, when the IARC database was limited to include only *p53* mutations from cases

exposed to aromatic amines, the major hotspot observed was again located at codon 273. We were unable to limit this analysis to include only *p53* mutations from non-smokers exposed to aromatic amines, therefore we were unable to determine whether these codon 273 G → A transitions were due to tobacco smoking or aromatic amine exposure. Only when several large population-based studies are conducted with good exposure data on tobacco use and occupational exposures will the true mutational spectrum of bladder cancer be known. In this study, non-smoking bladder cancer cases did not have codon 280/285 mutations. In smokers two mutations were found at codon 280 and three at codon 285, however, the major mutational hotspot was observed at codon 273, and only in smokers.

Also noteworthy is the finding that these results are in sharp contrast to the results from mutation studies conducted on chromosomal genes and shuttle vectors. In those studies primarily G → T transversion mutations were observed after treatment with the bladder carcinogen *N*-hydroxy-4-acetylaminobiphenyl (33–36). Although both *in vitro* and *in vivo* 4-ABP derivatives have been shown to react mainly with the C8

**Table III.** Types of mutations in bladder cancers from Argentina and Chile

|                           | Total tumors | Transitions <sup>a</sup> |      |       |            | Transversions <sup>b</sup> |      |      |            |
|---------------------------|--------------|--------------------------|------|-------|------------|----------------------------|------|------|------------|
|                           |              | <i>n</i>                 | (%)  | OR    | 95% CI     | <i>n</i>                   | (%)  | OR   | 95% CI     |
| Total                     | 126          | 38                       | (30) |       |            | 10                         | (8)  |      |            |
| Smoking <sup>c</sup>      |              |                          |      |       |            |                            |      |      |            |
| Never                     | 28           | 5                        | (18) | 1.00  |            | 4                          | (14) | 1.00 |            |
| Ever                      | 98           | 33                       | (34) | 1.63  | 0.83–7.94  | 6                          | (6)  | 0.42 | 0.11–1.71  |
| <i>P</i>                  |              |                          |      | 0.10  |            |                            |      | 0.23 |            |
| Pack years <sup>c</sup>   |              |                          |      |       |            |                            |      |      |            |
| 0                         | 28           | 5                        | (18) | 1.00  |            | 3                          | (14) | 1.00 |            |
| 1–20                      | 18           | 4                        | (22) | 2.03  | 0.43–9.66  | 2                          | (6)  | 0.45 | 0.04–4.72  |
| >20                       | 80           | 29                       | (36) | 2.67  | 0.85–8.38  | 4                          | (6)  | 0.42 | 0.10–1.79  |
| <i>P</i> <sub>trend</sub> |              |                          |      | 0.09  |            |                            |      | 0.25 |            |
| Arsenic <sup>c,d</sup>    |              |                          |      |       |            |                            |      |      |            |
| 1                         | 43           | 11                       | (26) | 1.00  |            | 2                          | (5)  | 1.00 | –          |
| 2                         | 29           | 9                        | (31) | 1.62  | 0.53–4.93  | 1                          | (3)  | 0.77 | 0.07–9.04  |
| 3                         | 27           | 11                       | (41) | 2.52  | 0.83–7.64  | 3                          | (11) | 2.90 | 0.44–19.1  |
| 4                         | 26           | 7                        | (27) | 1.00  | 0.31–3.20  | 4                          | (15) | 3.44 | 0.58–20.6  |
| <i>P</i> <sub>trend</sub> |              |                          |      | 0.73  |            |                            |      | 0.11 |            |
| Stage                     |              |                          |      |       |            |                            |      |      |            |
| Ta                        | 35           | 5                        | (14) | 1.00  |            | 3                          | (9)  | 1.00 |            |
| T1                        | 51           | 17                       | (33) | 2.50  | 0.83–7.68  | 3                          | (6)  | 0.67 | 0.13–3.51  |
| T2–T4                     | 40           | 21                       | (53) | 4.91  | 1.58–15.25 | 4                          | (10) | 1.19 | 0.25–5.73  |
| <i>P</i> <sub>trend</sub> |              |                          |      | 0.005 |            |                            |      | 0.80 |            |
| Grade                     |              |                          |      |       |            |                            |      |      |            |
| 1                         | 28           | 3                        | (11) | 1.00  |            | 1                          | (4)  | 1.00 |            |
| 2                         | 65           | 24                       | (37) | 3.70  | 1.00–13.70 | 5                          | (8)  | 2.25 | 0.25–20.20 |
| 3                         | 33           | 16                       | (49) | 6.94  | 1.75–27.60 | 4                          | (12) | 3.73 | 0.39–35.44 |
| <i>P</i> <sub>trend</sub> |              |                          |      | 0.004 |            |                            |      | 0.22 |            |
| Age                       |              |                          |      |       |            |                            |      |      |            |
| ≤60 years                 | 30           | 10                       | (33) | 1.00  | –          | 1                          | (3)  | 1.00 | –          |
| 61–75 years               | 58           | 20                       | (34) | 1.05  | 0.41–2.67  | 7                          | (12) | 3.98 | 0.47–34.0  |
| ≥76 years                 | 38           | 8                        | (21) | 0.53  | 0.18–1.58  | 2                          | (5)  | 1.61 | 0.14–18.7  |
| <i>P</i> <sub>trend</sub> |              |                          |      | 0.24  |            |                            |      | 0.87 |            |

<sup>a</sup>Purine to purine or pyrimidine to pyrimidine base change.

<sup>b</sup>Purine to pyrimidine or pyrimidine to purine base change.

<sup>c</sup>Analysis adjusted for tumor stage and grade.

<sup>d</sup>Average 5-year peak concentration (with proxy concentrations from nearest well of similar depth when necessary): group 1, 1–<10 µg/l; group 2, 10–99 µg/l; group 3, 100–299 µg/l; group 4, >300 µg/l.

position of guanine, forming a major DNA adduct, dG<sup>8-ABP</sup>, the exact reason for finding primarily G → A transitions, as opposed to G → T transitions, is unclear, however consistent with other studies of p53 mutations and bladder cancer.

The present study corroborates previous reports that p53 gene mutation and P53 immunopositivity increase with tumor stage and grade (40,41). The prevalence of tumors with G → A transitions increased with stage and grade (*P* = 0.04 and 0.02, respectively), but unlike smoking, a positive trend in the prevalence of G → A transitions at CpG sites or at codon 273 was not observed. This finding could suggest that while overall G → A mutation prevalence within exons 5–8 of the p53 gene are associated with tumor progression, those found at CpG sites and more specifically at codon 273 may be earlier tobacco-related mutations in bladder tumorigenesis. Early event p53 mutation has been implicated as an important factor in determination of tumor prognosis in bladder cancer (42–44).

In the current study, the percentage of tumors containing a G → A mutation at CpG sites increased from 4% in never smokers to 17% in ever smokers and a hotspot at codon 273 was identified in smokers. Ten of the 12 mutations located at codon 273 (82%) resulted in Arg → His amino acid substitutions. Unlike tumors containing other p53 mutations, codon

273 mutation did not result in positive IHC staining. This discordance may, at least partially, explain why p53 transition prevalence was higher in tumor tissue from ever smokers than never smokers and IHC positivity was not. This type of mutation has been shown to stabilize and increase expression of the MDM2 oncoprotein in human tumor cell lines that may contribute to tumorigenesis (45).

The p53 mutation pattern associated with smoking in the current study was different to those reported in other studies of p53 mutation in tobacco-associated bladder cancers. One study of Japanese bladder tumors (*n* = 61) found primarily A → G transitions (46). Another study of American (*n* = 62) and Danish (*n* = 18) tumors found primarily G → C transversions in smokers (47). In a study from Finland (*n* = 28) an increase in G → A transitions at CpG sites was reported in smokers, although they were scattered between codons 270 and 288 (48). In a study by La Rue *et al.* (49) a significant positive association between the presence of p53 point mutations and the number of years of smoking was found (*P* = 0.04), but none of the observed mutations were located at codon 273, the predominant site of mutation in this study. One reason for the differences observed may be the selection criteria used in each study. Unlike previous studies, the current study utilized

**Table IV.** G → A transitions at CpG sites and codon 273 mutation

|   | Total cases | G → A transitions |      | G → A transitions at CpG sites |      | Codon 273 mutations |       |
|---|-------------|-------------------|------|--------------------------------|------|---------------------|-------|
|   |             | n                 | (%)  | n                              | (%)  | n                   | (%)   |
| Total                                       | 126         | 25                | (25) | 18                             | (14) | 12                  | (10)  |
| Smoking <sup>a</sup>                        |             |                   |      |                                |      |                     |       |
| Never                                       | 28          | 4                 | (14) | 1                              | (4)  | 0                   | (0)   |
| Ever  | 98          | 21                | (21) | 17                             | (17) | 12                  | (12)  |
| P   |             |                   | 0.40 |                                | 0.07 |                     | 0.05  |
| Pack years <sup>a</sup>                     |             |                   |      |                                |      |                     |       |
| 0   | 28          | 4                 | (14) | 1                              | (4)  | 0                   | (0)   |
| <20   | 18          | 2                 | (11) | 2                              | (11) | 1                   | (6)   |
| >20   | 80          | 19                | (24) | 15                             | (19) | 11                  | (14)  |
| P   |             |                   | 0.40 |                                | 0.04 |                     | 0.001 |
| Arsenic <sup>a,b</sup> (never smokers only) |             |                   |      |                                |      |                     |       |
| 1   | 10          | 0                 | (0)  | 0                              | (0)  | 0                   | (0)   |
| 2   | 5           | 1                 | (20) | 1                              | (20) | 0                   | (0)   |
| 3   | 3           | 1                 | (33) | 0                              | (0)  | 0                   | (0)   |
| 4   | 10          | 2                 | (20) | 0                              | (0)  | 0                   | (0)   |
| P <sub>trend</sub>                          |             |                   | 0.20 |                                | 0.72 |                     | N/A   |
| Arsenic <sup>a,b</sup> (smokers only)       |             |                   |      |                                |      |                     |       |
| 1   | 33          | 7                 | (21) | 6                              | (18) | 5                   | (15)  |
| 2   | 24          | 4                 | (17) | 3                              | (13) | 2                   | (8)   |
| 3   | 24          | 7                 | (29) | 6                              | (25) | 2                   | (8)   |
| 4   | 16          | 3                 | (19) | 2                              | (13) | 3                   | (19)  |
| P <sub>trend</sub>                          |             |                   | 0.82 |                                | 0.99 |                     | 0.97  |
| Stage                                       |             |                   |      |                                |      |                     |       |
| Ta  | 35          | 4                 | (11) | 4                              | (3)  | 3                   | (9)   |
| T1  | 51          | 10                | (20) | 7                              | (14) | 5                   | (10)  |
| T2–T4                                       | 40          | 11                | (28) | 7                              | (18) | 4                   | (10)  |
| P <sub>trend</sub>                          |             |                   | 0.08 |                                | 0.45 |                     | 0.84  |
| Grade                                       |             |                   |      |                                |      |                     |       |
| 1   | 28          | 3                 | (11) | 3                              | (11) | 4                   | (14)  |
| 2   | 6           | 13                | (20) | 9                              | (14) | 5                   | (8)   |
| 3   | 33          | 9                 | (27) | 6                              | (18) | 3                   | (9)   |
| P <sub>trend</sub>                          |             |                   | 0.11 |                                | 0.40 |                     | 0.52  |

<sup>a</sup>Analysis adjusted for tumor stage and grade.

<sup>b</sup>5-year peak value (with proxy well levels): group 1, 1–<10 µg/l; group 2, 10–99 µg/l; group 3, 100–299 µg/l; group 4, >300 µg/l.

well-defined, broad selection criteria for inclusion in the study, thus reducing selection bias (50,51). Although the previous studies involved smaller numbers of tumors than the present study, the finding of an apparent hotspot in codon 273 in our study must be interpreted with caution in view of the multiple comparisons involved in investigating mutations in tumor DNA.

In this study, arsenic exposure via drinking water was not associated with an increase in *p53* mutation. This was clear from the fact that the mutation prevalence in the highest exposure group showed little difference from the lowest, practically unexposed group. Based on these findings, the mechanism of arsenic-induced carcinogenesis is more likely to be indirect, such as through inhibition of DNA repair, increasing oxidative damage or genetic instability (52,53). In a previous study, we used comparative genomic hybridization to analyze tumors for chromosome gains and losses associated with arsenic exposure (18). We found that even after controlling for stage and grade, tumors from patients exposed to arsenic contained more genetic changes than tumors from less exposed patients, suggesting that arsenic-exposed tumors were less genetically stable. The results from the present study suggest

that the genetic instability associated with arsenic exposure occurs through a P53-independent mechanism. One very small study of arsenic-exposed bladder tumors from an arsenic endemic region of Taiwan ( $n = 13$ ) observed several mutations at codon 175, a CpG site previously associated with inflammatory agents in transitional cell carcinomas of the bladder (12). Lack of an unexposed comparison group in this study means that the present study is the first to investigate the possible association of *p53* mutations and P53 IHC positivity in arsenic-related bladder cancers.

This study demonstrates how mutational spectrum analysis of the *p53* gene, combined with IHC methods to examine the P53 protein, may be used to investigate whether there are distinct chemical-specific patterns of *p53* gene, P53 protein or pathway alteration. An increase in G → A mutations at CpG sites was observed in smokers with >20 pack years of exposure and a mutational hotspot at codon 273 was associated with smoking. Arsenic exposure was not associated with a prevalence of *p53* mutation or P53 IHC positivity and there was no evidence of interaction between arsenic exposure and smoking for these outcome variables.

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