A Hot Spot for *p53* Mutation in Transitional Cell Carcinoma of the Bladder: Clues to the Etiology of Bladder Cancer[1]

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Abstract

Twenty-eight transitional cell carcinomas of the bladder, grade 2 or 3, were analyzed for the presence of *p53* mutations. Thirteen tumors were found to contain 14 mutations. These were all base substitution mutations, of which nine were GC→AT transitions (three at CpG sites). The remaining five mutations were transversions (three GC→CG, one GC→TA, and one AT→TA). Four of the mutations were found at codon 280. A comparison with other studies of bladder tumors reveals that a region encompassing codons 280 and 285 represents a hot spot for *p53* mutation in bladder cancer. The 280/285 hot spot lies within two purine-rich sequences that may provide some clues to the identity of potential bladder carcinogens. A comparison of mutations from bladder tumors of smokers and nonsmokers reveals no significant differences.

Introduction

Bladder cancer is the fifth most common neoplasm in the United Kingdom and the United States. The commonest form of bladder cancer is TCC.[3] A number of genetic abnormalities have been found to be associated with TCC. Loss of markers on chromosome 9 (1) and chromosome 14 (2) occurs in approximately 50 and 25% of bladder tumors, respectively. In addition, *p53* mutations have been identified with an incidence of around 50% seen in moderately (grade 2) and poorly differentiated (grade 3) TCCs (3–6).

A number of occupational exposures have been associated with bladder cancer, especially those involving aromatic amines such as those in the dye and rubber industries (7). People exposed to high levels of arsenic in Taiwan have elevated levels of bladder cancer (8). Animal studies have demonstrated that chemical carcinogens such as nitrosamines are bladder carcinogens (9). In addition, patients with chronic schistosomal infections such as bilharzia (10) or paraplegics with chronic urinary infections (11) have an increased risk of bladder cancer. However, the strongest epidemiological link concerns exposure to tobacco smoke. People who smoke regularly have a 2–3-fold higher risk of developing bladder cancer (12). There is evidence for the presence of smoking-related DNA adducts in the bladder epithelial cells of smokers (13), in an analogous manner to observations made in lung tissue (14). Therefore, there is strong evidence of a role for occupational and environmental carcinogens in the etiology of bladder cancer.

In this study, we wished to examine the molecular epidemiology of TCC of the bladder with respect to *p53* mutation. The scientific literature describes approximately 270 *p53* mutations in bladder tumors from the United States, Japan, the Netherlands, Egypt, and the United Kingdom. The intention of this study was to increase the database of *p53* mutations in bladder tumors from United Kingdom patients and to analyze the total mutational spectrum, particularly with respect to cigarette smoke exposure, to see if any clues might be obtained concerning the etiology of bladder carcinogenesis.

Materials and Methods

Detection of *p53* Mutations in Fresh Tumor Material. Grade 2 or 3 TCC samples were obtained by intraurethelial resection from 28 patients over a 1-year period and frozen for subsequent DNA extraction using a Nucleon I DNA extraction kit (Scotlab). In one case, the DNA was extracted from urine sediment spun out of a 40-ml urine sample and washed four times with sucrose solution [0.25 M sucrose, 25 mM KCl, 1.8 mM $CaCl_2$, and 50 mM Tris-HCl (pH 7.5)]. DNA sequences from exons 4–9 of the *p53* gene were amplified using the PCR method. The oligonucleotide amplimers and amplification conditions were as described in Brash *et al.* (15), except that 30 cycles of amplification were performed. The downstream amplimer of each exon pair was biotinylated. The DNA amplification products were extracted from agarose gels by spinning the excised agarose blocks in Spin-X tubes (Costar). The DNA was then bound to streptavidin-coated magnetic Dynabeads (Dynal, Wirral, United Kingdom) and converted into single-strand DNA template. These templates were then directly sequenced using a T7 DNA polymerase sequencing kit (Pharmacia) with [^{33}P]dATP. Sequence alterations were confirmed by repeating the PCR and sequencing reactions. The possibility of alterations being polymorphisms or germ-line mutations was discounted through sequencing of normal DNA samples. Standard precautions and controls were performed to exclude the possibility of cross-contamination.

Statistical Analysis. The *p53* mutational spectra from smokers and nonsmokers were compared using a computer program for the analysis of mutational spectra data described in Cariello *et al.* (16). This program permits the comparison of independent

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[3] The abbreviations used are: TCC, transitional cell carcinoma; BPDE, benzo(*a*)pyrene diol epoxide; LOH, loss of heterozygosity.

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Table 1 *p53* mutations present in patients with TCC

Patient	Stage/grade	Mutation	Target[a]	Codon	Amino acid change	Exon	LOH[b]	Smoking status[c]
10	pT1/G2	A→T	Aga	65	Arg→Stop	4	LOH	S
533	pT3/G3	C→T	Cag	104	Gln→Stop	4	NI	?
382	pT1/G2	G→C	aaG	132	Lys→Asp	5	LOH	NS
420	pTx/G3	G→A	cGc	175	Arg→His	5	LOH	S
388	pT3/G2	G→T	Gaa	198	Glu→Stop	6	NI	?
439	pT1/G2	C→T	tCc	241	Ser→Phe	7	LOH	NS
316	pT2/G2	G→A	gGc	244	Gly→Asp	7	NI	NS
394	pT1/G2	G→A	cGt	273	Arg→His	8	NI	Pipe
305	pT1/G2	G→A	aGa	280	Arg→Lys	8	No LOH	NS
385	pT1/G2	G→A	aGa	280	Arg→Lys	8	NI	NS
448	pT2/G3	G→C	aGa	280	Arg→Thr	8	NI	exS
419	pTa/G3	C→T	Cgg	282	Arg→Trp	8	LOH	NS
429	pT2/G3	G→A	Gag	285	Glu→Lys	8	No LOH	exS
		G→C	aGa	280	Arg→Thr[d]	8		

[a] Mutated base, upper case letter.
[b] LOH was assayed using informative polymorphisms at codons 72 and 213. NI, noninformative.
[c] S, smoker; NS, nonsmoker; exS, ex-smoker; Pipe, pipe smoker; ?, smoking status unknown.
[d] An additional mutation was found in the urine sediment, arising from a urothelial tumor of unknown origin.

mutational spectra, taking into account specific types of mutational alteration and specific bp position within the target sequence. $P \leq 0.05$ indicates that the spectra are most likely not drawn from the same population.

Results

Fresh tumor material was obtained over a 1-year period from a total of 28 patients suffering from grade 2–3 TCC of the bladder. The material came from both primary and recurrent tumors. Thirteen of 28 tumor samples (46%) were found to contain *p53* mutations (Table 1). This is similar to the frequency of *p53* mutation seen in other studies of high grade TCC (3–6). In one case (patient 429), an additional mutation was found in the exfoliated cells from a urine sample. This mutation (codon 280) presumably originated from an unidentified urothelial tumor and has therefore been included in our analysis. Sequencing of normal DNA obtained from patients confirmed that none of the mutations were polymorphisms or germ-line mutations.

Of the 13 patients with *p53* mutations, 6 were heterozygous with respect to a polymorphism at codon 72 (CGC/CCC), and 1 was heterozygous with respect to a polymorphism at codon 213 (CGA/CGG) when normal DNA was sequenced. LOH was observed on DNA sequencing gels in tumors from five of these seven informative patients (Table 1). In contrast, of the 15 patients with no detectable *p53* mutation, 9 were found to be informative (8 at codon 72 and 1 at codon 213), but only 1 displayed LOH at the *p53* locus. In cases in which heterozygosity persisted, we could not rule out the possibility that contamination with normal tissue was responsible.

Nine of the 14 characterized mutations were GC→AT transitions. Three of these were at CpG sites (codons 175, 273, and 282), which are common sites of mutation in human malignancy. The remaining five mutations were all transversions (three GC→CG, one GC→TA, and one AT→TA alteration). Four of the mutations were found at codon 280, two of which were GC→AT, and two of which were GC→CG.

Statistical Analysis. The smoking status of each of the patients in the study was obtained through interview. The *p53* mutations of patients classified as regular smokers or nonsmokers (never smoked) were added to those from previously published studies (Table 2). The mutational spectra from the smoking and nonsmoking groups were compared using the program described by Cariello *et al.* (16). Five separate analyses were carried out on the same set of data, and an average value of $P = 0.077 \pm 0.012$ was obtained for the probability that the two mutational spectra were derived from the same populations.

Table 2 Comparison of *p53* mutational spectra from TCCs of the bladder in smokers and nonsmokers[a]

Mutation	Nonsmoker (%)	Smoker (%)
GC→AT[b]	16 (44)	10 (24)
CpG[c]	4 (11)	8 (19)
GC→TA	3 (8)	6 (14)
GC→CG	10 (28)	8 (19)
AT→GC	2 (6)	6 (14)
AT→TA	1 (3)	2 (5)
AT→CG	0	0
Fs/In/Del[d]	0	2 (5)
Total	36	42

[a] Data were compiled from this study and four previous studies (5, 6, 37, 38).
[b] GC→AT mutations at CpG sites are not included.
[c] CpG, GC→AT transitions at CpG sites.
[d] Fs/In/Del, frameshift, insertion, and deletion mutations.

Discussion

Mutation Hot Spots in Bladder Tumors. The most striking feature of this relatively small group of mutations is the occurrence of four mutations at codon 280 (AGA), which included two GC→AT transitions (AGA→AAA) and two GC→CG transversions (AGA→ACA). This hot spot was first observed by Spruck *et al.* (5), but until now, it has not been confirmed in any other studies of bladder cancer. Fig. 1 shows the distribution of *p53* mutations in TCC of the bladder from a number of published studies, including the mutations from this study. These data were extracted from the database of *p53* mutations compiled by Hollstein *et al.* (17) and from three other studies (18–20). A total of 217 mutations distributed among 101 codons have been described for TCC. It can be seen that there are several mutational hot spots, with a particular concentration between codons 273 and 285. Twelve percent of the mutations occur at four CpG sites (codons 175, 248, 273, and 282),

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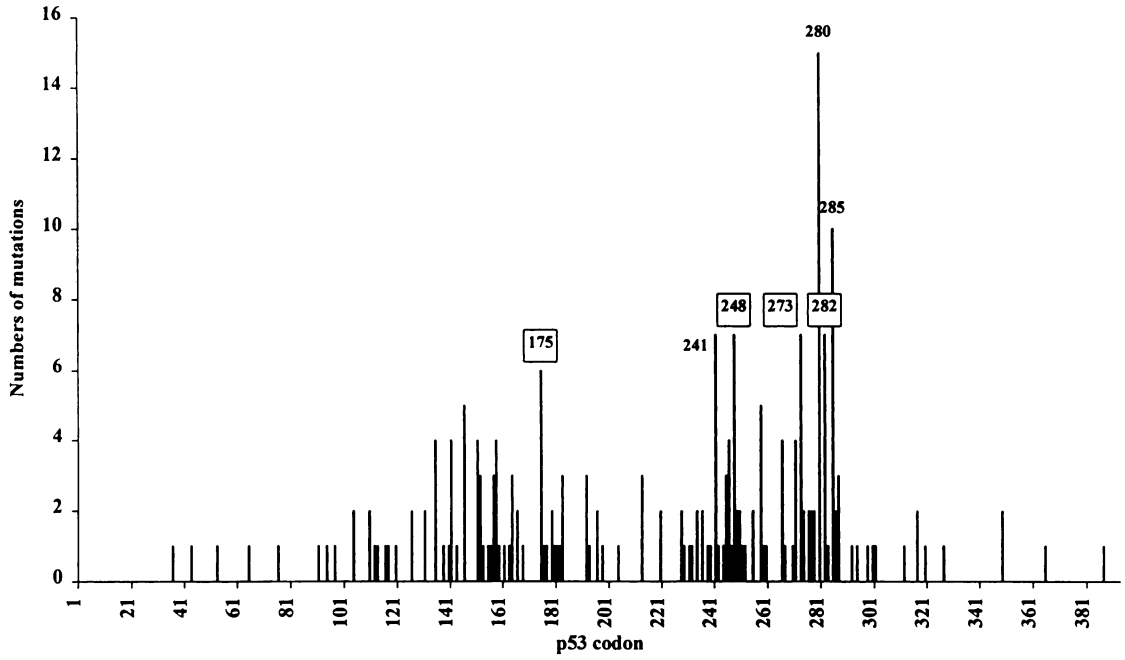

Fig. 1. Distribution of *p53* mutations in TCC of the bladder by codon position. The mutations are taken from the studies listed in the *p53* mutation database compiled by Hollstein *et al.* (17) and from three other studies (18–20) as well as this one. The seven codons with the most mutations are labeled; those at CpG sites are *boxed.*

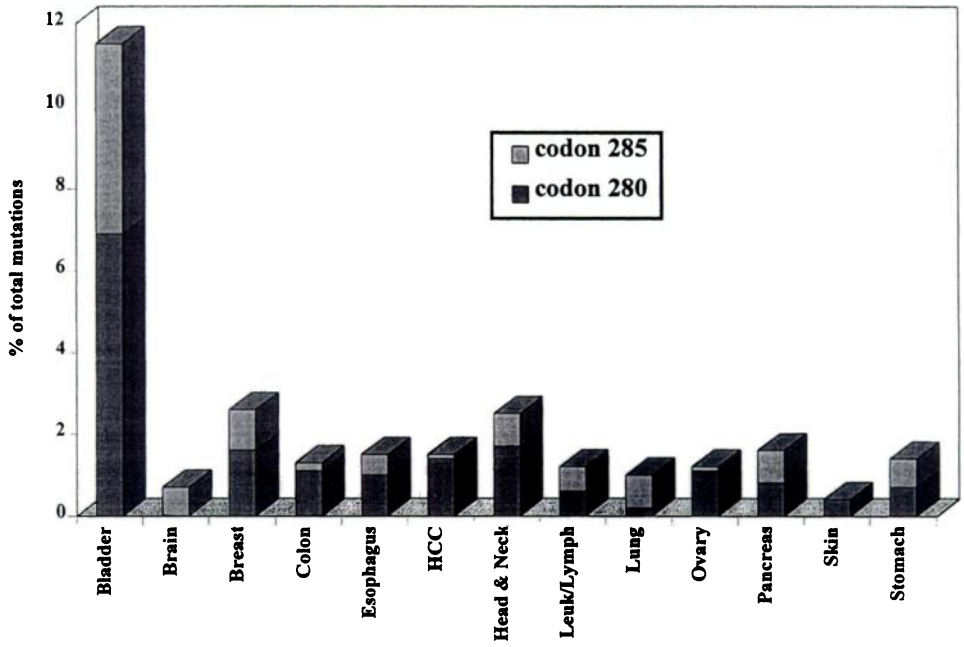

Fig. 2. Proportion of *p53* mutations at codons 280 and 285 in various human tumors. The number of mutations found at codons 280 and 285 in various human tumors is expressed as a proportion of the total number of *p53* mutations characterized in each tumor type. The data were obtained from the *p53* mutation database compiled by Hollstein *et al.* (17) and from three other studies (18–20). The results of this study are also included.

whereas nearly 15% occur at three non-CpG hot spots, including the two most common sites of mutation (codons 280 and 285).

The significance of the codon 280 and 285 hot spots can be seen by comparing the frequency of mutation at these sites in a variety of human malignancies (Fig. 2). In this analysis, the numbers of mutations at codons 280 and 285 have been expressed as a percentage of the total number of characterized mutations for a variety of tumor types. Nearly 12% of bladder cancer mutations occur at these two hot spots. The next highest

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frequency is less than 3%, which is seen in breast cancer and head and neck tumors. The codon 280/285 hot spot would therefore seem to be specific to TCC of the bladder.

Bladder-specific mutational hot spots may exist in the *p53* gene for a variety of reasons. They may be a reflection of the interaction between bladder carcinogens and the *p53* sequence; hot spots for mutation may represent sites of preferential DNA damage. Alternatively, there may be a tissue-specific biological selection that results in a bias toward mutations at particular sites; codon 280 and 285 mutations may confer a stronger selective growth advantage on bladder urothelial cells than other mutations. A third explanation is that bladder cells may lack some repair capability that repairs damage in the 280/285 region of the *p53* gene more efficiently than that in other regions.

There are a number of documented links between preferential sites of damage and *p53* mutation in human tumors. The distribution and type of mutations in skin tumors associated with sun exposure correlate well with the known sequence specificity of UV light mutagenesis (15). In addition, sites of frequent mutation in skin tumors seem to correlate with the sites of slowest repair of UV photoproducts (21). Other examples include sites of deamination at CpG sequences in colorectal cancer (codons 175, 245, 248, 273, and 282; reviewed in Ref. 22), aflatoxin in hepatocellular cancer (also reviewed in Ref. 22), and benzo(*a*)pyrene damage hot spots in lung tumors of smokers (23).

Different mutations of *p53* are known to affect its transforming and suppressor functions to varying extents (24). Mutations in different domains can affect the biological properties of the resulting protein in different ways, depending on the cellular background (25). It is possible, therefore, that certain mutations have a stronger selective advantage in bladder urothelium than others. There is no evidence for this to date. In cell transformation studies, the p53 Thr^{280} mutation is dominant over wild type but has only moderate oncogenic activity (26). Overall, it would seem that the spectrum of *p53* mutations seen in different tumors generally reflects the known mutational specificities of suspected etiological agents, *e.g.*, UV light (15, 27) and tobacco smoke carcinogens (23, 28, 29). Nevertheless, the relative contribution of mutational specificity and biological selection of mutations to the final mutational spectrum in bladder cancer will need to be addressed by functional studies in appropriate cell lines using specific *p53* mutations.

Epidemiological Clues from Bladder Hot Spots. All but 1 of 25 mutations occurring at the 280/285 hot spot in the *p53* database involve mutations of GC bp. Of a total of 223 mutations in TCC of the bladder, 168 (75%) involve mutations of GC bp. In 108 of 168 (64%) cases, the guanine is on the nontranscribed strand. These observations are consistent with transcription-coupled repair of guanine adducts on the transcribed strand, resulting in a bias toward mutation at guanine adducts on the nontranscribed strand (30, 31). Guanines are favored sites of adduction by a wide range of mutagens such as nitrosamines, 4-aminobiphenyl, polycyclic hydrocarbons, and oxygen radicals.

The two hot spots at codons 280 and 285 involve mutation of the first guanine within AGAG sequences occurring as part of extended homopurinic runs in the nontranscribed strand, GGGAGAGA (codon 280, *underlined*) and AGAGGAAGAGAA (codon 285, *underlined*). Six other AGAG sequences are present within the *p53* coding sequence, and three of these sites lie within extended homopurinic runs at codons 14, 287, and 315. No base substitution mutations have been described at codons 14 or 315 of *p53*. However, three mutations involving GC→AT or CG substitutions of the first guanine in an AGAG sequence have been found at codon 287, and two of these were from bladder tumors. Thus, more than 12% of all *p53* mutations in TCCs of the bladder are GC→AT or CG alterations occurring at the first guanine of an AGAG sequence within a homopurinic run. These observations could prove useful in discovering the identity of an important bladder carcinogen. For example, Mazur and Glickman (32) found that BPDE, a polycyclic hydrocarbon occurring in tobacco smoke, has a marked specificity for inducing mutations at $AG(G)_nA$ sequences in Chinese hamster ovary cells. A study of BPDE mutagenesis in the *supF* gene in *Escherichia coli* found a similar hot spot for GC→CG mutations at the first guanine within an AGAG sequence (33). However, the same mutation hot spot was also induced by hydrogen peroxide (34).

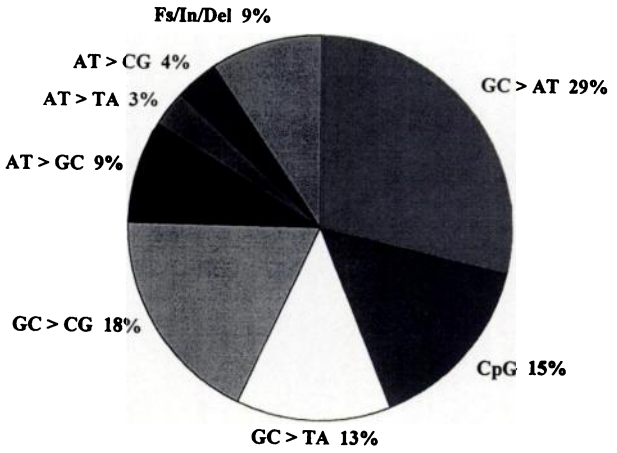

Fig. 3. Mutational spectrum of *p53* mutations in TCC of the bladder. The data are taken from the studies listed in the *p53* mutation database compiled by Hollstein *et al.* (17), from three other studies (18–20), and from this study (n = 223).

Smoking *versus* Nonsmoking. Mutations at CpG sites are thought to arise by an endogenous deamination mechanism. Therefore, the relatively low proportion of mutations at CpG sites in TCC bladder tumors (15%, compared to approximately 50% of colon and germ-line mutations) is consistent with an important role for exogenous mutagenic agents in bladder carcinogenesis. Obvious candidates for such agents can be found in tobacco smoke, which is a risk factor in bladder cancer. An analysis of *p53* mutations in human lung tumors shows a bias toward GC→TA transversions; 42 and 35% of mutations in small cell and non-small cell lung carcinoma, respectively, are of this type, compared to 16% in human tumors in general. A comparison of mutations from lung tumors of smokers and nonsmokers shows a significantly lower frequency of transitions and a significantly higher frequency of GC→TA transversions in smokers (22). The GC→TA transversion is induced in model systems by a number of known tobacco carcinogens such as benzo(*a*)pyrene and 4-aminobiphenyl. However, no particular bias toward GC→TA transversions (13% of total) is seen within bladder tumors (Fig. 3). A relatively high proportion of mutations are GC→CG transversions (18%), which are induced in model systems by aromatic amines such as 4-aminobiphenyl (35). Furthermore, DNA adducts derived from 4-aminobiphenyl have been detected in human exfoliated bladder cells (36).

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Epidemiological information in the form of smoking history was available for most of the patients in this study (Table 1). It was possible, therefore, to combine our data with that from four other studies (5, 6, 37, 38) in which *p53* mutations from smoking and nonsmoking bladder cancer patients were compared. Using a computer program designed by Cariello *et al.* (16), in which the distribution of mutations is taken into account as well as types of mutation, it would seem that the two spectra are not significantly different from each other (P = 0.077, average of five analyses). This confirms the result of an earlier analysis based on lower numbers (16).

The presence of no significant differences between the two spectra would suggest that similar mutagenic processes are involved in *p53* mutagenesis within the bladder urothelium of smokers and nonsmokers. This conclusion is undermined somewhat by the presence in one study of double base substitutions in bladder tumors from four smokers (5). Double mutations of *p53* are often observed in animal studies in which abnormally high levels of carcinogenic exposure are involved (29, 39). However, it is possible that *p53* mutagenesis in bladder cells is due to carcinogens in cigarette smoke to which nonsmokers are also exposed. The lack of any significant difference in the frequency of GC→TA transversions in smokers and nonsmokers suggests that different carcinogenic components of tobacco smoke may be involved in bladder carcinogenesis compared to lung and possibly rules out BPDE as a bladder carcinogen. However, there are a number of alternative carcinogenic agents present in tobacco smoke to which nonsmokers are also exposed, including nitrosamines, 4-aminobiphenyl, and free radicals. The codon 280/285 hot spot occurs in both smokers and nonsmokers, which supports the suggestion that it may be induced by some carcinogenic exposure common to both groups. The lack of any significant difference in mutational spectra between smokers and nonsmokers may suggest that tobacco-specific urothelial carcinogens are promoting agents rather than mutagenic agents.

Studies of *p53* mutational spectra have clearly demonstrated the usefulness of such data in understanding the molecular epidemiology of certain types of cancer. Additional studies on bladder tumors, together with an analysis of mutagenesis at the codon 280 and 285 hot spots, may help to provide valuable clues to the identity of important bladder carcinogens.

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