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p53 Mutations in Human Bladder Cancer

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Mutations in the tumor suppressor gene *p53* play an important role in carcinogensis and tumor progression. To assess the status of *p53* from genomic DNA from bladder cancer samples a two stage polymerase chain reaction was employed. The technique provided material for subsequent detection of mutations by Single Strand Conformation Polymorphism (SSCP) analysis followed by DNA sequence analysis. SSCP analysis of exons 5 to 9 of *p53* was performed using fragments from PCR end-labeled with ^{32}P followed by autoradiography using an electrophoresis system with temperature control. This SSCP method improved resolution of mutations in exons 5, 7, and 8 and the sharpness of bands in exons 6 and 9. Bands with altered migration patterns were excised from the dried SSCP gels, reamplified by PCR, and sequenced. Mutations in conserved exons 5, 6, 7, 8, and 9 of the *p53* gene were analyzed from bladder tumor biopsies. Our results are consistent with the literature in that mutations in *p53* are predominantly found in high grade bladder cancer (Odds Ratio = 4.05, Fisher Exact $P = 0.104$); however, the results were not statistically significant due to small numbers. Eight of 35 (23%) tumor samples examined showed mutations in *p53* (including two double mutations). Six of 13 (46%) grade III and IV tumors had *p53* mutations vs. 2 of 17 (12%) grade I and II tumors. Normal individuals carried no *p53* mutations. We found no correlation between pack years of smoking and mutation in *p53*. The spectrum of mutations confirmed a high proportion of G:C C:G transversions as well as the occurrence of double mutations.

Key words: PCR, SSCP, tumor suppressor gene *p53*, bladder cancer

INTRODUCTION

Molecular techniques like polymerase chain reaction (PCR) and DNA sequencing permit the routine analysis of mutation at the DNA sequence level. The resultant "mutational spectra" have led to the conclusion that the nature and location of DNA alterations reflect the original deposition and repair of DNA damage. Recent studies indicate that *p53* mutations are common in many forms of human malignancies [Hollstein et al., 1991; Levine et al., 1991], including bladder cancer [Hollstein et al., 1991; Sidransky et al., 1991; Brash et al., 1991; Fujimoto et al., 1992; Spruck et al., 1993]. The overwhelming majority of *p53* mutations represent somatically acquired events. The analysis of mutant and wild type *p53* alleles in a wide variety of tumors and cell lines has established P53 as a tumor suppressor protein. Mutations in the *p53* gene can play a crucial role in tumorigenesis and tumor progression and information about the nature of the change in the DNA sequence may provide clues to the cause of the mutation.

The genomic sequence of *p53* spans over 20 kb. The coding region of the *p53* gene in fish, birds, amphibians, and mammals shows five conserved blocs [Soussi et al., 1990]. Four of these domains (2 to 5) are contained within exons 5 to 8 of the human *p53* gene and it is within this region that a large majority of the identified mutations have been found. The real advantage of the human *p53* gene in these studies lies in the remarkable number of different sites available for mutational analysis. Many independent mutations, comprised largely of missense point mutations, have been identified [Hollstein et al., 1991]. The high number of different mutational events in a manageable biomarker makes this target very interesting for the purposes of molec-

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ular epidemiology. The techniques we applied to the analysis of mutations in the *p53* gene from human bladder cancer were the polymerase chain reaction (PCR) [Saiki et al., 1985] and SSCP analysis [Murakami et al., 1991; Orita et al., 1989; Suzuki et al., 1991].

MATERIALS AND METHODS

Subject and Specimen Collection

Thirty-eight tissue samples from 37 subjects were available for assay. The 37 subjects had been selected as possible cases based on screening tests for bladder cancer, i.e., positive cytology, positive quantitative fluorescence image analysis, or other indications of bladder tumors, for a case-control study of bladder cancer in male smokers. They answered a questionnaire that contained questions about their smoking habits and prior history of treatment for bladder cancer. None of the subjects had a prior treatment for bladder cancer other than the local excision of a prior tumor.

Bladder cancer specimens were obtained during biopsy or treatment. Upon collection by the surgical pathologist, tumors were covered with cryo-preservation OCT compound (optimum cutting temperature compound, Miles Inc., Elkhart, IN) and snap frozen in an isopentane bath immediately after removal. Histologic grading of the tumors was performed in accordance with WHO guidelines [Mostofi, 1973]. Pathology reports were not available on one subject. Tissue samples from three subject did not contain malignant cells.

DNA Templates and DNA Extraction Procedure

DNAs used for the analysis of the *p53* gene were derived from control cell lines and bladder cancer biopsies. Tumor biopsies were equilibrated to 0°C, the OCT compound removed mechanically, and the biopsies homogenized in TE buffer with a Dounce tissue homogenizer. Cell lines used included: retinoblastoma cell line Y79 (ATCC HTB 18) (Y79 contains an RFLP in exon 4, codon 72 of the *p53* gene [Murakami et al., 1991]); lymphoblastoid cell line GM1 (wild type for *p53* exons 5 to 9; unpublished results), and colo 320 DM (ATCC CCL 220); (colo 320 DM contains a point mutation in exon 7, codon 248 of *p53* [Murakami et al., 1991]). DNA from homogenized tissue or cell suspensions was extracted by proteinase K digestion and phenol extraction as described [Goelz et al., 1985]. Concentration of extracted and purified DNA was determined by fluorescence measurements using Hoechst H33258 stain.

Performance of the PCR

A Perkin Elmer 9600 series PCR machine and Taq polymerase was used for PCR. Amplification of *p53* from genomic DNA (from neoplastic and normal cells) was performed by a two-stage PCR protocol as described [Kusser et al., 1993]. In the first round, a 1841 bp DNA fragment spanning exons 5 to 9 is amplified from genomic DNA. In the second round, the individual exons with their adjoining splice sites are amplified from this fragment. This method can be applied to the PCR of *p53* from subnanogram amounts of DNA.

5′-End-Labelling of PCR Fragments With γ-[^{32}P]ATP

PCR fragments were purified using PrimErase Quick columns (Stratagene Cloning Systems, La Jolla, CA). For end-labeling, 1 pmol purified PCR product, 2 units of T_4 polynucleotide kinase, and 1 μCi γ-[^{32}P]ATP (4500 Ci/mmol) were mixed in kinase buffer and incubated at 37°C for 90 min. The reaction was stopped by addition of 2 volumes SSCP dye (95% deionized formamide, 0.05% bromophenol blue, 0.05% xylene cyanole, 20 mM EDTA, 0.5% SDS, 10 mM Tris/HCl pH 8.0).

Single Strand Conformation Polymorphism Analysis

For standard SSCP runs, gels (34 cm × 40 cm, 0.4 mm thick) were prepared with Mutation Detection Enhancement gel plus 10% glycerol as gel matrix (MDE gel, Hydrolink, obtained from AT Biochem, Malvern, PA) using a BaseAce vertical sequencing apparatus (Stratagene). After end-labeling, 2 volumes of SSCP dye were added to the samples. Samples were denatured at 95°C for 10 min, placed immediately into liquid nitrogen for 15 sec and transferred to crushed ice. Two microliters to 4 μl were loaded on the gel within 10 min after transfer on ice. A 1-kb marker (Gibco BRL, Gaithersburg, MD) and undenatured controls (dissolved in TE buffer pH 7.5, 0.05% bromophenol blue, 0.05% xylene cyanole, 20% glycerol) were loaded on the gel without heating. Electrophoresis (running buffer: 0.5× TBE, 4 mM EDTA: for 2 L: 100 ml 10× TBE, 8 ml 0.5 M EDTA pH 8.0) was performed using a Stratatherm cold temperature controller combo (Stratagene). Gels were dried on Whatman No. 3 filter paper and exposed to X-ray film at −80°C for 1 to 48 hr with an intensifying screen. Using artificial mixtures of wild-type and mutant DNA, the mutant fragment could be detected by the SSCP technique when it comprised 10% or more compared to the wild type DNA fragment (data not shown).

Elution of Putative Mutant Bands From Dried SSCP Gels for PCR and Sequencing

Dried gels were marked with radioactive ink on three corners and exposed. Bands to be sequenced were identified by their altered migration patterns compared to wild type controls and excised from the gel using a scalpel. The gel slice (dried onto Whatman No. 3 paper) was transferred to a 1.5 ml tube and eluted with 50 μl TE buffer pH 7.5. Ten microliter of the eluted material was reamplified by PCR using the second round primers described above (Performance of the PCR) and the PCR protocol.

Direct Sequencing With Vent Polymerase Based Cycle Sequencing

New England Biolabs CircumVent Thermal Cycle Sequencing kit was used for sequencing of double stranded DNA. The PCR fragments were purified by low melting point agarose gel electrophoresis and the sequencing reaction was performed using γ-[^{32}P]ATP or fluorescein end-labeled primers as described in the manufacturers instructions. Reactions were run on manual sequencing gels (γ-[^{32}P]ATP) or an automated laser fluorescent DNA sequencer (A.L.F., Pharmacia, Baie d'Urfé, Québec) for fluorescein end-labeled primers. Sequences of the exons were read and compared with the published wild type sequences in order to detect mutations in the coding regions. The non-coding exon-intron junctions (15 bases on each side) were also analyzed for the presence of mutations. Mutations found in the first round were confirmed by repeating the PCR-SSCP method followed by DNA sequencing.

RESULTS

We analyzed 38 tissue samples from 37 subjects including three specimens without bladder tumors. One of these three specimens did not form a PCR product and the remaining two specimens carried no *p53* mutations. Among the 34 patients who had tumors (32 had transitional cell carcinoma [TCC] and two had TCC in situ), we found eight specimens with *p53* mutations that resulted in either an amino acid change or a stop codon. All of the 37 subjects had a history of smoking. Indeed, all but three had smoked more than 20 pack-years.

The grading of one tumor was ambiguous and hence not included in the analysis and the pathology report was miss-

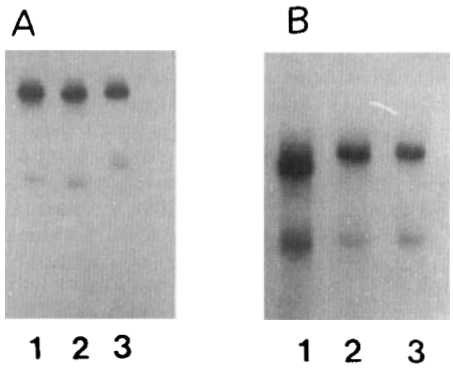

Fig. 1. ^{32}P end-labeled exons were prepared and loaded onto MDE gels as described in Materials and Methods. **A:** SSCP analysis using Stratatherm cold box: 1) mutation in p53 exon 8:G→C@ 14508 AGA to ACA Arg to Thr codon 280; 2) mutation in p53 exon 8:G→T@ 14487 CGT to CTT Arg to Leu codon 273; 3) control tissue, wild type in 053 exon 8. The gel was run at set point 20°C for 4 hr at 50 Watts. **B:** SSCP analysis in fan-cooled box: 1) mutation in p53 exon 8:G→C@ 14508 AGA to ACA Arg to Thr codon 280; 2) mutation in p53 exon 8:G→T@ 14487 CGT to CTT Arg to Leu codon 273; 3) control tissue, wild type in p53 exon 8. The gel was run at room temperature for 16 hr at 8 Watts.

ing on another specimen. Five of 12 (42%) specimens from subjects with high grade tumors showed mutations while only 3 of 20 (15%) specimens from subjects with low grade tumors had a *p53* mutation (Odds Ratio = 4.05, Fisher Exact $P = 0.104$). The Fisher exact test revealed no significant association between subjects with mutation in *p53* and age, total smoking (pack-years), or recent smoking history (i.e., smoking within the last 5 years) (data not shown). However, the power to detect differences was small.

In a standard PCR, 10 ng genomic DNA template from tumor tissue biopsies, control biopsies, and control cell lines (Y79, colo 320 DM and GM1, see Materials and Methods) were amplified using a two stage PCR protocol [Kusser et al., 1993]. The PCR protocol described here worked effectively, and as little as 0.07 ng of genomic DNA could be amplified [Kusser et al., 1993]. Exons 5 to 9 were end-labeled using γ-^{32}P ATP and γ-^{33}P ATP. For the detection of mutations, these fragments were analyzed by SSCP. The size of the amplified exons (200 to 250 bp) was ideal for the subsequent analysis by SSCP. The SSCP results showed that different exons require different running conditions for optimal results. For the SSCP method, polyacrylamide with 6% glycerol as described by Murakami et al. [1991] and MDE Hydrolink gel were used as gel matrices. MDE gel supplemented with 10% glycerol was found to provide generally better resolution than the standard 6% polyacrylamide gels with or without glycerol.

For the electrophoresis procedure of the DNA fragments in non-denaturing MDE gels, two methods were compared (Fig. 1): (1) conventional SSCP analysis at room temperature in a fan-cooled box as described by Orita et al. [1989]: two to 4 μl of the labeled exon were loaded on the gel and electrophoresis was performed for 16 to 18 hr at 8 Watts (constant current) and room temperature for 16 hr for exons 5, 7, 8, and 9. Exon 6 was run at 4°C under the same conditions. (2) SSCP analysis in a Stratatherm cold temperature controller: the cold box was cooled with dry ice 15 min before prerun. The gel was prerun at the experimental conditions of subsequent electrophoresis of samples for 20 min, gel temperature at this time was 18–20°C. Two to 4 μl of the labeled exon were loaded on the gel and electrophoresis was performed at a temperature set point of 20°C and 50 Watts for 4 hr for all exons. Under our laboratory conditions it was sufficient to load the dry ice chambers of the cold box to 50% capacity.[1] Under these conditions, the temperature during the run was 18–20°C.

Samples with altered migration behavior were excised from the dried gels and reamplified by PCR. Sequencing was performed using ^{32}P end-labeled primers in a cycle sequencing protocol with Vent polymerase [Ellingboe and Gyllensten, 1992]. The published DNA sequences of exons 5 to 9 and the point mutation in exon 7 of the colo 320 DM cell line (8) were confirmed in the control templates Y79, GM1, and colo 320 DM (data not shown).

The mutations in *p53* from tumor biopsy samples detected in eight of 34 tumor samples examined (24%) are shown in Table I. In addition, three tumor samples showed a *Taq*I polymorphism in exon 6 of *p53* [Serra et al., 1992].

DISCUSSION

The SSCP method used here combined the MDE gel matrix with a temperature-controlled electrophoresis apparatus. The method resulted in improved separation of wild type and mutant fragments. Exons 5 to 9 produced sharper bands and the running time of the SSCP analysis was reduced from 16 hr at 8 Watts to 4 hr at 50 Watts by using the cold box system. Snap cooling of the samples in liquid nitrogen after heat denaturation successfully prevented the single stranded DNA fragments from reannealing. The elution of DNA fragments from dried gels with TE buffer worked well as the subsequent PCR yielded the proper fragment in all cases. We noted, however, that some fragments which showed altered migration pattern in the SSCP gels exhibited a wild type DNA sequence in subsequent analysis. Mutations detected were confirmed by repeating the PCR-SSCP technique followed by DNA sequencing to exclude the possibility of PCR-induced DNA sequence changes.

The low percentage of *p53* mutations found in the tumor samples (24%) was most likely a reflection of the tumor grade of the samples analyzed. More than half of the samples were grade II or lower. Studies in bladder cancer have shown that *p53* mutations are specifically linked to invasive

[1]Substitution of the dry ice with 10 to 12 cooling elements (e.g., as used in the shipments of biochemicals) chilled to −80°C was also adequate to provide sufficient cooling capacity for the 4 hr SSCP run.

TABLE I. Mutations in the *p53* Gene Exon 5 to 9 From Human Bladder Cancer

Case no.	WHO Grade classification	Exon	Codon	Target	Base change	Amino acid change
1050	III	5	151	cCc	C → G	Pro to Arg
1044	III	7	249	agG	G → C	Arg to Ser
		8	280	aGa	G → C	Arg to Thr
1053	III	7	248	Cgg	C → T	Arg to Trp
1089	III	7	249	agG	Deletion of G	Frameshift
1061	III	8	299	ccC	C → A	silent
		8	300	cCa	C → T	Pro to Leu
1112	I	8	273	cGt	G → T	Arg to Leu
1043	I	9	316	Cag	C → T	Gln to STOP
1086	II	9	316	cAg	A → C	Gln to Pro

TABLE II. Nature of the *p53* Gene Mutations Found in Bladder Cancer

Mutation	Number found	Percent of total
Transitions		
GC to AT	3	30
AT to GC	0	0
Total	3	30
Transversions		
AT to TA	0	0
AT to CG	1	10
GC to TA	2	20
GC to CG	3	30
Total	6	60
Deletions	1	10
Of above mutations:		
Mutations at GC base pairs	8	80
Mutations at AT base pairs	1	10
Double mutations	2	20

aggressive bladder cancer of grade III [Fujimoto et al., 1992]. The results of this study confirm this finding; the mutations in *p53* were correlated with advanced bladder cancer of grade III. However, our results were not statistically significant due to small numbers. We analyzed 34 tumor samples and 10 mutations were detected in eight tumor biopsy samples (including two double mutations). Five of 12 grade III tumors had *p53* mutations vs. 3 of 20 grade II or lower. Two mutations were detected in exon 9, both in codon 316. Exon 9 is not always included in the analysis of mutations in *p53*. Based on our results we recommend the analysis of exon 9 as well. Exon 9 of *p53* is relatively short (74 bp) and can be conveniently included with the two-stage PCR used in this study.

The spectrum of mutations is shown in Table II. Of interest is the high proportion of G:C→ C:G transversions and the occurrence of double mutations. Both features were also found in the analysis of *p53* mutations in bladder cancer from patients who smoke cigarettes [Spruck et al., 1993]. The origin of G:C→ C:G transversions remains speculative. A possible connection to oxidative DNA damage has been suggested [Spruck et al., 1993] and indeed a high frequency of this generally rare transversion event has been induced by the singlet oxygen generating mutagen methylene blue plus light [McBride et al., 1992]. It is perhaps also noteworthy that *o*-anisidine-induced transversions at G:C base pairs in bladder tissue of transgenic *lacI* mice (BigBlue mouse) were three times higher when compared to the spectrum of spontaneous *lacI* mutations in the same tissue (Glickman et al., manuscript in preparation). In our opinion, several possible contributing factors in addition to oxidative damage should be considered. Little is known about the environment of the (pre)cancerous cells within which the *p53* mutation must take place. Oxidative damage, endogenous factors, and bladder specific metabolism may contribute, as defects in a carcinogen-metabolizing gene have been identified as bladder cancer risk factors [Bell et al., 1993]. The tumor metabolic environment may also be related to the high frequency of double mutations recovered in this and other studies. Because of their very low probability to occur, the presence of two *p53* mutations in one tumor suggests a possible positive selection effect for these mutations under certain circumstances or the possible occurrence of a more general genetic instability in these tumor cells [Gonzalez-Zulueta et al., 1993].

In conclusion, the results from this study confirm the link of *p53* mutations with advanced tumor grade in bladder cancer, a high proportion of G:C→C:G transversions, and the occurrence of double mutations in human bladder cancer. The two-stage PCR protocol and the temperature-controlled SSCP method using the MDE gel matrix provided a sensitive tool for the analysis of mutations in *p53* exons 5, 6, 7, 8, and 9. Because mutations were also found in exon 9, we recommend that it be included in the analysis of *p53* mutations from tumors.

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