Identification of p53 Gene Mutations in Bladder Cancers and Urine Samples

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fects hearing and is inherited as an autosomal recessive trait (*23*). The issue of genetic heterogeneity can be resolved by testing additional families with LQT and Jervell and Lange-Nielsen syndrome with the markers described here.

We conclude that a mutation at a single genetic locus on the short arm of chromosome 11 predisposes individuals in this family to ventricular arrhythmias and sudden death. Presymptomatic diagnosis of LQT has previously been difficult because of the normal variability of the QT interval. The existence of genetic markers will greatly improve our ability to identify families and family members who are at risk for sudden death. Attention can then be directed to a search for the gene that causes LQT and to an explanation for the distinctive pattern of ventricular arrhythmias seen in this syndrome.

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Identification of p53 Gene Mutations in Bladder Cancers and Urine Samples

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Although bladder cancers are very common, little is known about their molecular pathogenesis. In this study, invasive bladder cancers were evaluated for the presence of gene mutations in the p53 suppressor gene. Of 18 tumors evaluated, 11 (61 percent) were found to have genetic alterations of p53. The alterations included ten point mutations resulting in single amino acid substitutions, and one 24–base pair deletion. In all but one case, the mutations were associated with chromosome 17p allelic deletions, leaving the cells with only mutant forms of the p53 gene product. Through the use of the polymerase chain reaction and oligomer-specific hybridization, p53 mutations were identified in 1 to 7 percent of the cells within the urine sediment of each of three patients tested. The p53 mutations are the first genetic alterations demonstrated to occur in a high proportion of primary invasive bladder cancers. Detection of such mutations ex vivo has clinical implications for monitoring individuals whose tumor cells are shed extracorporeally.

CARCINOMA OF THE BLADDER IS THE fifth most common cancer in the United States, with an annual incidence of approximately 18 cases per 100,000. The disease prevalence peaks in the seventh decade of life, and over 45,000 cases were detected last year (*1*). Patients with superficial tumors often have recurrences and require careful observation and clinical follow-ups. Those patients with invasive tumors are at risk of dying from their disease, despite radical surgery, radiation, and chemotherapy.

Although genetic alterations are thought to underlie the development of malignancies, no such alterations have yet been identified in a high proportion of invasive bladder carcinomas. The first example of a point mutation in a human tumor (a *ras* oncogene mutation) was identified in a bladder carcinoma cell line (*2*), but similar mutations have rarely been found in the primary bladder tumors subsequently examined (*3*). Differences in the expression of other oncogenes have also been observed in bladder tumors (*4*), but the expression patterns are not necessarily specific for the neoplastic state and have not been associated with mutations.

Cytogenetically, several specific chromosomal losses have been reported to commonly occur in bladder cancers (*5*). The cytogenetic findings have been confirmed and extended by restriction fragment length polymorphism analysis; this analysis revealed frequent losses of chromosomal arms 9q, 11p, and 17p in tumors of this type (*6*). Such chromosomal losses are thought to indicate the presence of putative tumor suppressor genes within the deleted areas (*7*). These suppressor genes presumably exert a negative regulatory effect on neoplastic cell growth. When such suppressor genes are inactivated (by mutation or deletion), the cell acquires a competitive growth advantage compared to other cells, and the neoplastic process progresses.

We suspected that the chromosome 17p deletions in bladder cancers reflected underlying mutations in the p53 suppressor gene; p53 gene mutations have been observed in other human tumor types with chromosome 17p deletions (*8*). To test this idea, we obtained cystectomy or biopsy specimens from 18 patients who had invasive bladder carcinomas. DNA was purified from these specimens (*9*), and exons 5 to 9 were amplified from tumor DNA by means of the polymerase chain reaction (PCR) (*10*). The PCR products were then isolated and subcloned into phage, and pooled clones were sequenced together to evaluate p53 mutations (*11*) (a representative example is in Fig. 1). Eleven of the 18 tumor specimens (61%) contained p53 gene mutations (Table 1). When present, the mutations appeared to be clonal, that is, they were present in the majority of the tumor cells, as

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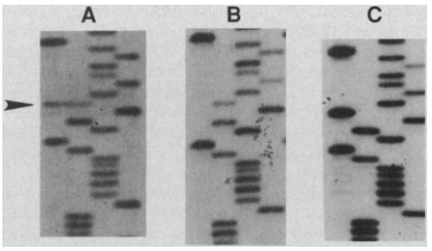

Fig. 1. Gene mutations of p53 in invasive bladder cancer. Sequencing gel of pooled p53 clones (exon 8) of tumor B41 (**A**) showed a C to A transition when compared to DNA clones from normal cells (**B**). Approximately 40% of tumor B41 was composed of nonneoplastic inflammatory cells; this accounts for the residual C band at the position corresponding to the mutation. The identical mutation was seen in a p53 clone from the patient's urine DNA (**C**), identified by oligomer-specific hybridization as described in the text. The methods used for sequencing are detailed in (*10*) and (*11*). In each panel, the lanes represent A, C, G, and T terminations, from left to right.

reflected by the ratios of the normal and mutant bases on the sequencing gels. The most common mutation was a single base pair substitution. Seven tumors had missense mutations at either codons 141, 157, 241, 245, 246, 248, or 282, respectively, resulting in amino acid substitutions. Three other tumors had nonsense mutations resulting in termination at either codons 183, 196, or 298. One tumor, B2A, had a 24–base pair deletion that resulted in a change of one amino acid and a deletion of seven other amino acids.

There was a strong association of p53 gene mutation with chromosomal 17p allelic loss. To assess allelic loss, we digested tumor DNA with appropriate enzymes and analyzed DNA blots with polymorphic probes to chromosome 17p (*12, 13*). Nine of the ten tumors with chromosome 17p allelic loss had a p53 gene mutation (Table 1). Conversely, only one of the tumors without chromosome 17p allelic loss had such a mutation. This difference in the prevalence of p53 mutations in tumors with chromosome 17p loss was statistically significant ($P = 0.017$, Fisher's exact test).

To evaluate the possibility that tumor cells containing p53 gene mutations were shed into the urine, we attempted to identify these mutations in DNA amplified from urine sediments. Even if such mutant p53 genes were present in the urine sediment, we suspected that they might form only a small proportion of the total urine sediment genes, as the sediment is composed of normal bladder epithelium and inflammatory cells, as well as neoplastic cells. The PCR sequencing strategy used for the primary tumors could detect mutations only if the mutations were present in more than 20% of the genes in the analyzed population. Therefore, a technique was used for the urine samples that allowed detection of mutant genes among an excess of wild-type (wt) p53 genes. DNA from the urine sediments was used to amplify p53 exons 5 to 9 by means of the PCR (*14*). The PCR products were then cloned into a bacteriophage vector, and 10^2 to 10^4 clones were transferred to nylon membranes and then hybridized to ^{32}P-labeled oligonucleotide probes (*15*) that were specific for the mutations found in the respective primary tumors. Positively hybridizing plaques indicated the presence of mutant p53 genes (Fig. 2).

This test was applied to three patients with p53 mutations in their primary tumors from whom urine specimens were obtained before surgery. In each of these three cases, the same mutation identified in the primary tumor was also identified in the urine sediment (Fig. 2). In the three cases studied, the percentage of recombinant clones containing mutant p53 alleles was 1% (B47), 3% (B41), and 7% (B40) of the clones containing wt p53 alleles, suggesting that a small but significant percentage of the urine sediment cells contained the mutations.

The specificity of this assay was confirmed in two ways. First, each mutant-specific oligonucleotide was hybridized to 5000 phage clones from normal urine containing only the wt p53 gene (or to an equivalent number of clones from urine containing other mutant p53 alleles), and these hybridizations were consistently negative (example in Fig. 2C). Second, positively hybridizing clones were plaque-purified and sequenced in each of the three cases, and the same mutations were observed in the individual clones (example in Fig. 1C).

Little is known about the exact role of p53 in neoplasia, but accumulating evidence suggests that it acts as a tumor-suppressor gene (*16–19*). The p53 gene product was originally discovered through its interaction with DNA tumor virus antigens (*20*). Binding of mutant p53 gene product to the wt product may produce an inactive oligomeric complex leading to accelerated cell growth (*21*). Transfection of mutant p53 genes has been shown to transform cells in vitro (*22, 23*). Moreover, transfection of wt p53 suppresses this transformation (*19*) and can also suppress the growth of human cells containing endogenous p53 mutations (*24*). Thus, mutant p53 alone may confer a growth advantage on tumors, and a subsequent allelic loss of the remaining wt p53 gene could further diminish negative growth control (*16*).

The discovery of p53 mutations in bladder cancer is consistent with the view that alterations of tumor-suppressor genes play a role in the pathogenesis of many human cancers (7). Although allelic losses have been analyzed in bladder cancers (*6*), few specific gene alterations have been described. The retinoblastoma (RB) tumor-suppressor gene has been implicated in bladder cancer, as it has also been shown to be inactivated in 5 out of 16 bladder cancer cell lines

Table 1. Mutations of p53 in invasive bladder carcinomas. All tumors were transitional cell carcinomas, except B17 and B47, which were poorly differentiated small-cell and poorly differentiated spindle-cell tumors, respectively. NI, noninformative (the probes could not discriminate the paternal from the maternal allele, so that allelic loss could not be evaluated). Subscript on T denotes increasing depth of tumor invasion, from mucosa (1) to pelvis (4); a corresponds to superficial and b to deep muscle invasion in T_3 tumors. Subscript on N denotes extent of nodal involvement with higher numbers indicating more extensive spread (*30*). The higher the grade the more advanced the tumor.

Tumor	Mutated codon no.	Amino acid substitution	17p Allelic loss	Stage	Grade
4940	241	Ser → Cys	Yes	T_3bN_2	IV
5095	141	Cys → Tyr	Yes	T_1N_0	III
S197	183	Ser → Term	Yes	T_3bN_0	III
B2A	236–244	Deletion	Yes	T_3bN_0	III
B17	282	Arg → Trp	NI	T_3aN_0	
B40	245	Gly → Ala	No	T_3bN_1	II
B41	298	Glu → Term	Yes	T_3bN_0	III
3243	196	Arg → Term	Yes	T_3bN_0	IV
4410	248	Arg → Gln	Yes	T_3bN_2	III
T25	246	Met → Val	Yes	T_3aN_0	III
B47	157	Val → Phe	Yes	T_3bN_0	
B23	None		No	T_1N_0	III
4275	None		No	T_2N_0	III
B35	None		No	T_4N_1	III
B38	None		No	T_3aN_0	II
T16	None		Yes	T_1N_0	II
B3	None		NI	T_2N_0	III
B46	None		NI	T_3bN_0	II

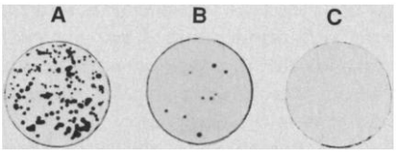

Fig. 2. Identification of p53 gene mutations in urine. Many clones obtained by PCR from the urine of patient B40 (with a grade II tumor) hybridized to an oligomer probe specific for wt p53 (**A**). An oligomer probe specific for mutant p53 (**B**) hybridized to fewer, but still a significant number, of clones from the same source as in (A). Clones containing only wt p53 genes (from PCR products of normal DNA) did not hybridize (**C**) to the mutant-specific probe but did hybridize efficiently (more than 500 hybridizing plaques) to the wt probe (*31*). The methods used for PCR, cloning, and hybridization are detailed in (*10*), (*11*), and (*15*).

(*25*). It will be of interest to determine the frequency of RB tumor-suppressor gene mutations in primary bladder cancer specimens.

The technique we used to identify p53 mutations in urine approaches the limit of sensitivity attainable with PCR. PCR can generate an almost limitless quantity of DNA, and each amplified copy of the p53 gene can be individually assessed by the plaque hybridization assay used. Its specificity, however, is limited by the spurious mutations caused by the misincorporation of bases during amplification. PCR errors can be minimized by modification of the amplification conditions and by the use of enzymes with lower inherent error rates (*26*). In practice, no false positives have been seen with this assay on six normal samples tested, with over 5000 clones per test screened under the conditions specified.

These results have two potential clinical applications. First, patients at high risk for primary bladder cancer (such as patients exposed to occupational carcinogens) could be screened for the presence of neoplastic cells in the urine by analysis of p53 gene mutations. Such screenings are at present limited by the large number of different mutations identified in the p53 gene (Table 1). Furthermore, the timing of p53 mutations in relation to the neoplastic progression of the bladder is not known. Screening for p53 gene mutations would be more useful if these mutations were found to precede the stage at which patients become surgically incurable.

A second application would be in the follow-up of patients with known invasive bladder cancer treated by bladder-sparing strategies, such as partial cystectomy. Such patients could have their tumors analyzed for p53 mutations, and, if found, urine sediments could be periodically retested for the presence of the mutation. Current non-invasive methods for examining voided urine specimens include cytologic examination (*27*). Cytology is useful in certain circumstances, but its limitations have spurred attempts to develop additional, more quantitative techniques. Flow cytometry has shown some promise in this regard, but it often requires a high percentage of abnormal cells (greater than 10%) to establish a diagnosis of neoplasia (*28*). Because of the high sensitivity of the molecular approach outlined here (which allows detection of one mutant cell among over 5000 normal cells), this strategy could prove to be a valuable adjunct to more traditional methods.

Other tumors in which cells are exfoliated (such as those of the lung, colon, or cervix) lend themselves to similar studies. Mutations in p53 occur in at least two of these other tumor types (colon and lung) at a high frequency (*13, 16, 18*), and mutations of other genes could theoretically be monitored in the same way. The use of DNA probes to identify gene mutations in extracorporeal samples is an important area of research with implications for tumor diagnosis and monitoring.

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9. In some cases, multiple sections through the tumor revealed that the neoplastic cells constituted the great majority of the cells within the sample, and tumor homogenates were used for DNA preparation. In the remaining cases, DNA was purified directly from cryostat sections from which areas containing many nonneoplastic cells were physically removed [E. R. Fearon *et al.*, *Nature* **318**, 377 (1985)]. DNA was isolated from tumor sections by SDS–proteinase K treatment and phenol-chloroform extraction, followed by ethanol precipitation. We handled tissue specimens and purified the DNA with disposable equipment in a sequestered area to minimize possible contamination during subsequent amplification procedures.
10. DNA (50 to 500 ng) was amplified with a modification of the procedure described by Kogan and co-workers (*29*). We synthesized 18–base pair oligomers flanking exons 5 to 9 of the p53 gene and used them at a concentration of 350 ng/μl in a 50-μl reaction. Exons 5 to 9 contain over 95% of the p53 mutations found in other human tumor types [reviewed in (*8*)]. The primers were 5′-GTAGGAATTCACTTGTGCCTGACTTG-3′ and 5′-CATCGAATTCTGGAAACTTTCCACTTGAT-3′. Nucleotides, dimethyl sulfoxide, and magnesium concentrations were identical to those described by Kogan and co-workers (*29*). We performed 35 amplification cycles, each consisting of a denaturing step at 95°C for 30 s, annealing at 58°C for 3 min, and extension at 70°C for 2 min. The last cycle was followed by a 5-min extension at 70°C. After amplification, one-tenth of the reaction mixture was separated on a 1.2% agarose gel and the 1.8-kb amplified segment was identified by ethidium bromide staining. Each experiment included at least one reaction devoid of DNA as a negative control.
11. PCR products were purified by phenol-chloroform extraction and ethanol precipitation. They were cleaved with Eco RI and repurified with phenol-chloroform extraction and ethanol precipitation. The 1.8-kb PCR product was recovered from a 1.2% agarose gel by freeze-thawing, followed by centrifugation through a Spin-X column (Costar) [B. Vogelstein, *Anal. Biochem.* **160**, 115 (1987)]. Approximately 50 ng of recovered DNA was then ligated to Lambda ZAP II vector arms (Stratagene) with T4 DNA ligase. We packaged the ligation mixture by using GigaPack Plus extracts (Stratagene) following the manufacturer's protocol. Phage were then used to infect BB4 cells and plated on L-agar plates at a density of 1,000 to 10,000 plaques per plate. Phage were eluted from the plates, and single-stranded phagemids were obtained as described (*13*). We used these phagemids to infect XL1 Blue cells (Stratagene) and obtained double-stranded plasmids that were purified and used for sequencing. We performed T7 sequencing reactions as previously described [R. Kraft, *Biotechnology* **6**, 544 (1988)] with the following primers: 5′-GACTTTCAACTCTGTCTC-3′ and 5′-CTGGGGACCCTGGGCAAC-3′ for exon 5, 5′-GAGACGACAGGGCTGGTT-3′ and 5′-CCACTGACAACCACCCTT-3′ for exon 6, 5′-GAGGCAAGCAGAGGCTGG-3′ and 5′-CCA-AGGCGCACTGGCCTC-3′ for exon 7, 5′-CCTTACTGCCTCTTGCTTC-3′ and 5′-TGAATCTGAGGCATAACTGC-3′ for exon 8, and 5′-TTATGCCTCAGATTCACTTTT-3′ for exon 9. Sequencing gels were exposed to film for 1 to 5 days at room temperature.
12. DNA was analyzed with the Southern blot technique; genomic DNA from tumor or normal cell specimens was digested with appropriate enzymes, separated on 1.5% agarose gels, and transferred to nylon membranes. These blots were hybridized sequentially to chromosome 17p with polymorphic probes as described in (*13*).
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14. Urine specimens were obtained 1 to 6 hours before surgery and were stored at 4°C for 3 to 4 hours before pelleting by centrifugation at 1000*g* for 5 min. DNA was purified from the pellets (sediments) as described in (*9*).
15. Recombinant bacteriophage containing the p53 genes from the PCR reactions described in (*10*) were inoculated at a density of 10^2 to 10^3 phage per 75 cm^2 of petri dish. Bacteriophage were then transferred to nylon membranes, alkaline-denatured, and baked at 80°C for 10 min. We synthesized 18–base pair oligomers corresponding to mutant sequences for each tumor studied, and in each case the mutant base was placed between bases 8 and 10 of the oligomers. Mutant-specific 18–base pair oligomers were 5′-ATGGGCGCCATGAACCGG-3′ for tumor B40, 5′-CCTCACCACTAGCTGCC-3′ for B41, and 5′-GCACCCGCTTCCGCGCCA-3′ for B47. These oligomers were labeled with ^{32}P–adenosine triphosphate and T4 kinase to a specific activity of 5×10^8 dpm. Hybridization with oligomers was performed at 52°C for 6 to 12 hours. After hybridization, the membranes were rinsed in 3× SSC (450 mM sodium chloride, 18 mM sodium citrate, and 1 mM tris, pH 7.2), 0.1% SDS at room temperature for 5 min and then washed at approxi-

mately 15°C below the estimated melting temperature in 3× SSC, 0.1% SDS for 30 min. Final washing temperatures ranged from 54° to 61°C. The filters were then exposed to film at −70°C for 2 to 6 hours. In each case, examples of the hybridizing clones were plaque-purified and sequenced as described in (*11*).
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In Vivo Footprinting of MHC Class II Genes: Bare Promoters in the Bare Lymphocyte Syndrome

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Major histocompatibility complex (MHC) class II genes are coordinately regulated and show tissue-specific expression. With the use of in vivo footprinting, common promoter sites in these genes were found to be occupied only in cells that expressed the genes, in spite of the presence of the promoter binding proteins. In vivo analysis of mutant cell lines that exhibited coordinate loss of class II MHC expression, including several from individuals with bare lymphocyte syndrome, revealed two in vivo phenotypes. One suggests a defect in gene activation, whereas the other suggests a defect in promoter accessibility.

CLASS II MHC GENES CONSTITUTE A multigene family and encode the α and β chains of a group of cell surface heterodimeric glycoproteins that are critical for normal T lymphocyte–mediated immune responses. B lymphocytes constitutively express class II genes, whereas other cell types can be induced to express them (*1*). Class II genes are thought to be coordinately regulated, which would be consistent with the combined loss of expression of all class II genes in individuals with type II bare lymphocyte syndrome (BLS) (*2*) and in experimentally derived mutant B cell lines (*3*). The defect in BLS and these mutant cell lines has been attributed to a trans-acting factor, because the BLS trait and the MHC locus segregate independently (*4*) and class II gene expression can be restored by fusion to a class II MHC–positive cell (*5*). Several complementation groups have been defined by fusion studies (*6*), suggesting that class II gene regulation involves multiple factors.

Attempts to determine the mechanism underlying coordinate regulation have focused on the promoter region of class II genes, which contains motifs conserved in all these genes. This region contains critical cis regulatory elements and confers tissue-specific expression on linked genes in transient transfection assays (*7–9*). Electromobility shift assays have identified factors that are capable of recognizing promoter elements in vitro (*8–11*); these binding activities are present in nuclear extracts of both class II MHC–positive and class II MHC–negative cells, and certain factors have different affinities for the various class II genes in vitro (*11, 12*).

To study the role of the class II gene promoter region in the tissue-specific and coordinate expression of these genes and to gain insight into the BLS defect, we have performed an in vivo genomic footprinting analysis of multiple class II genes by the ligation-mediated polymerase chain reaction (LMPCR), which allows a direct examination of the promoter region (*13*). Because the class II genes are highly polymorphic, we chose to first examine murine genes in cell lines from inbred mice of the H-2^d haplotype, for which the promoter sequences of the four class II genes, E_β, E_α, A_β, and A_α, are available.

We first examined the promoter region of the murine E_β gene in the class II MHC–positive B lymphoma line M12.4.1 and in the class II MHC–negative fibroblast line BALB/3T3 (Fig. 1A). The E_β gene in M12.4.1 cells displayed protected guanine residues on both the coding and noncoding strands in the conserved X and Y boxes. The Y box contains a reverse CCAAT box, and the two guanines on the coding strand were protected. The X box is located a conserved distance upstream of the Y box and had protected residues both within the X box proper, referred to as X_1, and in the adjacent site X_2, which overlaps the 3′ end of the X box and the 5′ end of the interspace between X and Y (*1*). A guanine residue at position −120 on the coding strand was neither protected nor hypersensitive and lies between the protected residues of the X_1 and X_2 sites, demonstrating that these elements are separable. A fourth conserved motif has recently been defined and is called the S box (*1*). No protected residues were observed in the S motif or the surrounding region upstream of the X box, which has variably been called W, Z, or H, and which we will refer to as the S region. A second B lymphoma line, A20, displayed an identical in vivo footprinting pattern to M12.4.1 (*14*). In contrast, no protected or hypersensitive residues were observed in the E_β gene from BALB/3T3 fibroblasts on either the coding or noncoding strands in any of the conserved regions.

The footprint patterns of the three other murine class II gene promoters in M12.4.1 cells on both the coding and noncoding strands were very similar to those of E_β in the same cells (Fig. 1, B, C, and D). As with E_β, BALB/3T3 fibroblasts showed no protection of these genes at any of the sites contacted in M12.4.1 cells, although certain hypersensitivities were observed. A_β had hypersensitive sites in the X_1 site on the

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