Genetic Alterations in TP53 in Recurrent Urothelial Cancer: A Longitudinal Study

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ABSTRACT

Purpose: **Because bladder cancer has a recurrence rate that can be as high as 90% at 2 years, we sought to clarify whether these metachronous tumors are polyclonal or monoclonal in origin. We have examined the genetic alterations of the *TP53* gene in a cohort of patients with urothelial cancer who underwent multiple biopsies at different times and sites because of tumor recurrence and/or progression. We postulated that if tumor cells at different points in the natural history of the disease contain an identical mutation in the *TP53* gene, this pattern could provide evidence for the monoclonality of the recurrent bladder tumors.**

Experimental Design: **Fifty-three biopsy specimens from 13 patients at different times and sites were selected for this study. Microdissection was used to ensure the purity of tumor cells. DNA extraction, PCR, and direct sequencing of exons 5 through 8 of the *TP53* gene were conducted following protocols optimized in our laboratory.**

Results: **We found that specimens from seven patients carried tumor-specific TP53 mutations. The number of lesions in these patients ranged from two to seven, extending from 2 to 4 years. All of the seven patients displayed identical mutations in the different microdissected tumors.**

Conclusions: **On the basis of these data, it appears that the recurrent bladder tumors originate from the same clone.**

INTRODUCTION

Bladder cancer has a recurrence rate that can be as high as 90% at 2 years (1). It is still unclear whether these metachronous tumors are polyclonal or monoclonal in origin. Do they originate independently from a "field cancerization" phenomenon or are they derived from the same parent neoplasm? To answer this question, it is imperative to analyze genes that are abnormal in the early stages of the neoplastic process but not in the preneoplastic urothelium. One of these abnormal genes, *TP53*, is a relatively early event in bladder tumors that cannot be detected in the morphologically normal urothelium.

Understanding the origin of the recurrent tumors has important clinical implications. If the recurrent tumors originate from a field cancerization phenomenon only, then a strategy aiming at "normalizing" the entire urothelium should be sought. Such a strategy will require a better understanding of the biology of those changes. If the recurrent tumors originate from the same parent neoplasm, the clinical implications would be different in two respects: prognosis and treatment. The recurrent tumor will have a similar biological potential as the original tumor and will behave similarly. A high-grade tumor with a high metastatic potential requires an aggressive treatment to prevent the occurrences of similar tumors. A tumor with a low metastatic potential could be managed conservatively. Regarding treatment, if endoscopic resection is chosen as the mode of therapy to address the multifocal synchronous tumors and metachronous recurrences, then adjuvant intravesical therapy will be required.

PATIENTS AND METHODS

Patients. Thirteen patients with synchronous and metachronous urothelial tumors in the bladder, ureter, or renal pelvis were selected for this analysis. Patients whose tumors recurred in the same location less than 3 months after the initial resection were excluded, because in all probability they had tumors incompletely resected.

Slide Preparation. Several 6-μm thick sections were cut from formalin-fixed, paraffin-embedded samples. The sections were stained with monoclonal antibody 1801 as described previously (2). One section was stained with H&E to better identify the morphology of the sample and to serve as control for confirming the accuracy of the microdissection. The stained sections were air dried for 20 min at room temperature and then stored at −20°C until microdissection was performed.

DNA Preparation. Manual microdissection was performed when ample amounts of tissue were present. When the areas were relatively small, the PixCell Laser Capture Microdissection system was applied to ensure purity and adequate transfer (3). Both normal and tumor samples were transferred to tubes containing 100 μl of proteinase K buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1% Tween 20]. Cells were lysed overnight by adding 2–8 μl of proteinase K (20 μg/μl) to the buffer. The proteinase K was inactivated at 100°C for 10 min. The cell lysate was used for PCR.

Oligonucleotides and Thermocycling. All of the *TP53* gene-specific primers were obtained from the DNAgency (Berwyn, PA). Four pairs of primers were used for *TP53* gene amplification, which included exons 5 through 8. The sequences of these primers are: exon 5, 5′-TTC ACT TGT GCC CTG ACT T-3′, 5′-ACC AGC CCT GTC GTC TCT CC-3′; exon 6, 5′-TTG CCC AGG GTC CCC AGG CC-3′, 5′-CTT AAC CCC

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TCC TCC CAG AG-3′; exon 7, 5′-CGC ACT GGC CTC ATC TTG GG-3′, 5′-CAG CAG GCC AGT GTG CAG GG-3′; exon 8, 5′-GCC TCT TGC TTC TCT TTT CC-3′, 5′-CCC TTG GTC TCC TCC ACC GC-3′.

Thermocycling was performed according to a methodology reported previously (4). Briefly, PCR reactions were carried out in a PE 9600 thermocycler (Perkin-Elmer, Norwalk, CT) using a mixture (20 μl) containing the DNA extracted from the microdissected specimens (100 ng), 10 mM Tris-HCl (pH 8.3), 2.0 mM $MgCl_2$, 50 mM KCl, 0.1% Tween 20, 0.2 mM deoxynucleotide triphosphate, 10 pM of each primer, and two units of AmpliTaq Gold polymerase (Perkin-Elmer). Temperature cycles and times for PCR reactions were: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Each PCR reaction was preceded by an 11-min denaturation at 95°C, and the final cycle was followed by a 10-min extension at 72°C. The total number of cycles for PCR amplification was 35 to 50 depending on the sample DNA.

Purification of PCR Product. All of the PCR products were purified before sequencing reaction. PCR products were loaded on a 1.5% agarose gel containing ethidium bromide and run for 30 min at 100 V. Under UV light, the specific PCR product band was cutoff and transferred to an Eppendorf tube. The QIAEX II Gel Extraction Kit (Qiagen, Chatsworth, CA) was used for DNA purification. After purification, the concentration of DNA template was quantitated by agarose gel using 1 μl of purified DNA template together with a standard marker.

PCR-Single-stranded Conformation Polymorphism. PCR-single-stranded conformation polymorphism assays were performed using the sets of primers detailed above and following protocols described previously (5, 6). Briefly, the PCR reactions were performed in 10-μl volumes containing 80 ng to 100 ng of template DNA, 2.2 mCi of [α-^{32}P]dCTP or [α-^{33}P]dCTP (Amersham Life Science Inc., Arlington Heights, IL), 3 mM $MgCl_2$, 100 mM deoxynucleoside triphosphates, 3% DMSO, 0.6 units of TaqI polymerase, and 1 × PCR buffer (Promega, Madison, WI). The annealing temperatures ranged from 55°C to 65°C. The PCR products were denatured and loaded on a nondenaturing 8% polyacrylamide gel containing 10% glycerol and subjected to electrophoresis at room temperature for 12–16 h at 10–12 W. After electrophoresis, the gels were dried and exposed to X-ray film at −70°C for 4–16 h.

Sequencing. ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) was used for the sequencing of PCR products. Briefly, Terminator Reaction Mix (8 μl) plus 3.2 pmol of primer and 1 μl of PCR DNA sample (approximately 50 ng) were mixed in each tube to bring the total volume to 20 μl. Then, the tube was placed in the thermal cycler to perform the sequencing reaction (25 cycles at 96°C, 10 s; 50°C, 5 s; and 60°C, 4 min). Samples were precipitated by ethanol, and the pellet was dried. Automatic sequencing was performed with the ABI 377 (PE Applied Biosystems, Foster City, CA).

Sequencing was performed on normal and tumor tissue and analyzed from both strands to validate laboratory findings. When we observed a mutation in one strand, we always confirmed its presence by sequencing the other strand. In addition, we always confirmed the mutations identified by reanalyzing the original DNA template. To define a mutation, we analyzed each nucleotide peak in the automated sequencing reader and used a rise of 15% signal above the background threshold level as a cutoff for the presence of mutation.

RESULTS

Monoclonality was demonstrated by determining the P53 mutation in exons 5 through 8. Patients whose tumors recurred in the same location less than 3 months after the initial resection were excluded, because in all probability they had tumors incompletely resected. Thirteen patients with a total of 53 specimens remained. PCR-single-stranded conformation polymorphism analysis followed by sequencing was conducted on all of the specimens. We identified 30 T_a, five T_1, nine *cis*, two T_2, four T_3, and three metastatic lesions in pelvic lymph nodes. P53 mutations were detected in seven patients. Each had identical p53 mutations by sequencing (Table 1). The number of lesions in these patients ranged from 2 to 7 over a period up to 4 years. Case 3 displayed a polymorphism with an A to G transition. Cases 2 and 5 showed a mutation at the splice site resulting in a truncated protein. Cases 7, 8, 9, and 11 carried missense mutations.

DISCUSSION

The definition of field cancerization or clonality requires clarification. Field cancerization is a well-known phenomenon described in the nonurological literature, particularly in cancer of the head and neck. This hypothesis predicts that synchronous and metachronous tumors arise from independent genetic events as a result of a diffuse exposure to carcinogens. The literature is replete with examples of synchronous tumors showing a polyclonal process of field cancerization (7–11). Field cancerization implies carcinogen-induced changes throughout the mucosa. The derived neoplasms are genetically distinct. A clonal origin implies that the parent and the recurrent tumors originate from the same clone and have the same early genetic make-up. The field cancerization and monoclonality are mutually exclusive.

A distinction has to be made between field cancerization and field defect. A field defect implies diffuse histopathological changes in the urothelium. A field defect is a morphological term and does not support or disprove a monoclonal or a polyclonal origin of the recurrent tumors. The concept of a field defect is supported by clinical observations. A mapping study by Koss *et al.* (12) showed a diffuse morphological abnormality in the bladder of patients who underwent a radical cystectomy for bladder cancer favoring a field defect. He stated that "bladder cancer is not a local disease, but a local manifestation of a diffuse abnormality of the urothelium" (12). In a prospective surveillance study of patients with bladder cancer, Murphy *et al.* (13) reported a 14% rate of hyperplasia, dysplasia, *cis*, or carcinoma in cystoscopically normal areas remote from the area of the tumor.

Monoclonality, a relative term, depends on the genetic abnormality evaluated. Sidransky *et al.* (14) analyzed the pattern of X-inactivation in 13 tumors from four women with bladder cancer and concluded that they were all derived from the same clone in contrast to colorectal cancer (15). X-inactivation occurs during early embryogenesis. Showing monoclonality in this context indicates that the preneoplastic and neoplastic cascade

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Table 1 The results of sequencing exons 5 through 8 of the *TP53* gene from the lesions (53 biopsy specimens) of 13 patients with recurrent or progressive urothelial cancer

Case	Pathology	Location	Date	P53 mutation analysis			
				Exon 5	Exon 6	Exon 7	Exon 8
1	T_1G_3	Bladder	6/94	wt[a]	wt	wt	wt
	T_aG_3	Bladder	8/94	wt	wt	wt	wt
	T_3G_3	Ureter	12/95	wt	wt	wt	wt
2	N_1	Lymph node retroperitoneum	2/91	wt	wt	wt	Codon 306 splice site intron mutation GAGgta→GAGata
	T_aG_2	Left renal pelvis	9/91	wt	wt	wt	Codon 306 splice site intron mutation GAGgta→GAGata
	cis	Bladder	9/92	wt	wt	wt	Codon 306 splice site intron mutation GAGgta→GAGata
	T_aG_1	Bladder	3/93	wt	wt	wt	Codon 306 splice site intron mutation GAGgta→GAGata
	T_aG_1	Bladder	5/93	wt	wt	wt	Codon 306 splice site intron mutation GAGgta→GAGata
	T_3G_3	Right ureter	10/93	wt	wt	wt	Codon 306 splice site intron mutation GAGgta→GAGata
	T_aG_1	Bladder	1/94	wt	wt	wt	Codon 306 splice site intron mutation GAGgta→GAGata
3	T_1G_3	Bladder	6/97	wt	Codon 213, CGA→CGG, Arg→Arg	wt	wt
	T_1G_3	Bladder	7/97	wt	Codon 213, CGA→CGG, Arg→Arg	wt	wt
	N_1	Pelvic lymph node	7/97	wt	Codon 213, CGA→CGG, Arg→Arg	wt	wt
	cis	Ureter	7/97	wt	Codon 213, CGA→CGG, Arg→Arg	wt	wt
	cis	Bladder	7/97	wt	Codon 213, CGA→CGG, Arg→Arg	wt	wt
4	T_aG_2	Ureter	2/96	wt	wt	wt	wt
	T_aG_1	Bladder	4/97	wt	wt	wt	wt
	T_aG_2	Bladder	8/97	wt	wt	wt	wt
5	T_aG_3	Bladder	3/94	wt	Codon 224 splice site intron mutation GAGgtc→GAGatc	wt	wt
	cis	Ureter	4/94	wt	Codon 224 splice site intron mutation GAGgtc→GAGatc	wt	wt
	cis	Bladder	10/94	wt	Codon 224 splice site intron mutation GAGgtc→GAGatc	wt	wt
	cis	Bladder	5/95	wt	Codon 224 splice site intron mutation GAGgtc→GAGatc	wt	wt
6	T_aG_1	Bladder	6/82	N/A	wt	wt	wt
	T_aG_1	Bladder	6/82	N/A	wt	wt	wt
	T_aG_1	Bladder	7/82	wt	wt	wt	wt
	T_aG_2	Bladder	11/82	wt	wt	wt	wt
	T_aG_1	Bladder	2/83	N/A	wt	wt	wt
	T_aG_1	Bladder	12/83	wt	wt	wt	wt
	T_aG_1	Bladder	4/84	wt	wt	wt	wt
	T_aG_1	Bladder	7/85	N/A	wt	wt	wt
	T_aG_1	Bladder	10/95	wt	wt	wt	wt
7	T_1G_3	Bladder	4/95	Codon 158 CGC→CTC, Arg→Leu	wt	wt	wt
	T_1G_3	Bladder	4/95	Codon 158 CGC→CTC, Arg→Leu	wt	wt	wt
	$T_{3a}G_3$	Bladder	9/95	Codon 158 CGC→CTC, Arg→Leu	wt	wt	wt
8	T_1G_2	Bladder	1/95	Codon 163 TAC→TGC Tyr→Cys	wt	wt	wt
	T_1G_3	Bladder	3/95	Codon 163 TAC→TGC Tyr→Cys	wt	wt	wt
	N_1	Pelvic lymph node	1/97	Codon 163 TAC→TGC Tyr→Cys	wt	wt	wt

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Table 1 Continued

Case	Pathology	Location	Date	P53 mutation analysis			
				Exon 5	Exon 6	Exon 7	Exon 8
9	*cis*	Ureter	6/92	wt	wt	wt	Codon 280 AGA→AAA, Arg→Lys
	T_2G_3	Ureter	10/96	wt	wt	wt	Codon 280 AGA→AAA, Arg→Lys
10	T_2G_3	Ureter	9/81	wt	wt	wt	wt
	cis	Bladder	12/83	wt	wt	wt	wt
	cis	Bladder	9/84	wt	wt	wt	wt
11	T_3G_3	Renal pelvis	5/76	wt	Codon 196 CGA→CAA, Arg→Gln	wt	wt
	T_aG_1	Bladder	6/79	wt	Codon 196 CGA→CAA Arg→Gln	N/A	wt
12	T_aG_1	Bladder	8/93	wt	wt	wt	wt
	T_aG_1	Bladder	2/94	wt	wt	wt	wt
	T_aG_1	Renal pelvis	8/95	wt	wt	wt	wt
13	T_aG_2	Bladder	9/80	wt	wt	wt	wt
	T_aG_2	Bladder	12/80	wt	wt	wt	wt
	T_aG_3	Bladder	8/83	wt	wt	wt	wt
	T_aG_2	Bladder	2/84	wt	wt	wt	wt
	T_aG_3	Bladder	8/84	wt	wt	wt	wt
	T_aG_2	Bladder	12/84	wt	wt	wt	wt

[a] wt, wild type; N/A, not applicable.

of events originated from the same precursor cell, but it does not exclude the possibility that at the preneoplastic stage, different genetic events led to malignant cells with different genotypes. To prove that the frank neoplastic cell is the parent of any recurrence, we had to evaluate a genetic event absent in the preneoplastic cells and present in the neoplastic cells. P53 is the ideal candidate, because we could not demonstrate p53 overexpression in the morphological normal urothelium of patients with bladder cancer.

Thirteen patients with a total of 53 tumors were analyzed. P53 mutations were detected in seven patients, and they all had identical p53 mutations by sequencing. These data strongly suggest that the original tumors persisted and migrated resulting in recurrences. A similar finding has been reported (16) in a patient with metachronous tumors in the renal pelvis, bladder, and vagina in which all of the four tumors had the same p53 mutation.

An alternative explanation for the uniformity of the p53 mutation in our study is the fact that all of the premalignant lesions with the same genetic make-up will result in the same p53 mutation. Because it has been shown that *in vivo* and *in vitro*, nonrandom chromosome losses in stepwise neoplastic transformation do not follow a constant pattern (17), this hypothesis is difficult to support. Similarly, in Barrett esophagus, the genetic changes seen in different lesions do not follow a uniform pattern either. Furthermore, if all of the recurrences result from independent events, it would imply a high rate of mutation. It is known that not all of the premalignant lesions progress to cancer (18). In the colon, it is estimated that only 2.5 adenomatous polyps/1000/year progress to colon cancer (19).

The clinical data pertaining to a field defect in bladder cancer are irrefutable (12, 13). Sidransky *et al.* (14) have clearly shown that bladder tumors originate from a single clone of cells. Simon *et al.* (20) reported the results for comparative genomic hybridization and P53 analysis of muscle invasive tumors, suggesting a monoclonal nature of multifocal tumors. Takahashi *et al.* (21), using microsatellite markers, supported the monoclonal nature of recurrent bladder tumors. In their series, 84% of the recurrent bladder neoplasms were derived from the same progenitor cells (21). Our data and those of others have also shown that recurrences originate from the parent tumor. How can we reconcile in a single model the data put forward? Fig. 1 depicts an initiation event or first hit leading to preneoplastic changes that confer a proliferative advantage to the cells, resulting in partial repopulation of the urothelium. This leads to the field defect phenomenon. A second hit results in the development of a neoplasm with increased motility and decreased cell adhesion. Pagetoid spread, a well-described phenomenon in carcinoma *in situ* of the bladder, reflects the increased motility. The presence of exfoliated cells as detected by urine cytology reflects decreased cell adhesion. These manifestations of the neoplastic cells are responsible for subsequent recurrences which, as we

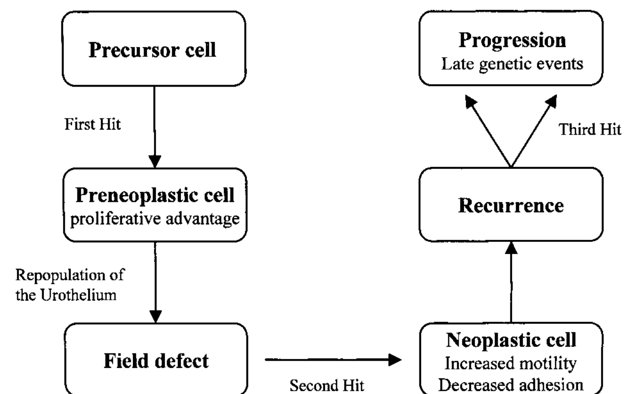

Fig. 1 Neoplastic transformation (see text for full explanation).

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and others demonstrated, have the same early genetic make-up. The recurrent tumors will subsequently develop a third hit with additional genetic changes that will result in tumor progression.

This finding has important clinical implications. It also emphasizes the current limitations of our treatment in eradicating urothelial cancer. We conclude that recurrent urothelial tumors originate from a parent neoplasm that dictates behavior of the recurrence. Endoscopic resection alone is not sufficient to completely eradicate all of residual disease. Adjuvant therapies are needed, particularly for high-grade tumors. We also conclude that field cancerization does not play a role in urothelial neoplasms, but a field defect is the result of seeding or lateral migration of a parent tumor.

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