

Genetic and Epigenetic Alterations in Normal Bladder Epithelium in Patients with Metachronous Bladder Cancer

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Abstract

Mechanisms for multifocal bladder carcinogenesis remain unclear. To see whether normal mucosa had already acquired genetic or epigenetic changes, we examined loss of heterozygosity (LOH) at 10 microsatellite loci and methylation of the p16^{INK4} CpG island in multiple tumors and pathologically normal mucosa in six patients with bladder cancer. Either LOH or methylation was detected in 77% of samples of normal epithelium, and LOH detected in samples of normal epithelium was also observed in most tumor samples. This result indicated that a population of cells in morphologically normal epithelium possessed genetic or epigenetic aberrations in common with bladder cancer, which might provide a ground for multiple tumorigenesis.

Introduction

Transitional cell carcinoma of the urinary bladder features metachronous or synchronous multiple tumors. Approximately 70% of patients with an initial diagnosis of superficial transitional cell carcinoma of the urinary bladder develop recurrent disease, and invasive lesions appear in 15% of patients within a short period (1). Sidransky *et al.* (2) and Mao *et al.* (3) demonstrated that synchronous bladder cancers are of clonal origin, based on an identical pattern of X chromosome inactivation and microsatellite alteration. Clonal origin of metachronous urothelial tumors is shown by the identical mutation of the p53 tumor suppressor gene (4). These findings indicate that bladder cancer originates from a progression of genetic changes in a monoclonal fashion and that multiple tumors of either the synchronous or metachronous type are derived from micrometastatic foci that have migrated from the original site, rather than the occurrence of a polyclonal mutation. Recently, several lines of evidence suggest that at least some of the genetic aberrations found in invasive cancers are already present in morphologically normal epithelium. In aerodigestive cancers, multiple tumors and morphologically normal mucosa had a polyclonal p53 mutation (5). LOH² has been detected in morphologically normal lobules adjacent to breast cancers (6). These reports indicated that clonal genetic abnormalities might be detectable before any phenotypic abnormalities are evident in epithelia. If a single mutant progenitor cell clone expands to populate widespread areas of the bladder epithelia and subsequently progresses to cancer, the recurrence rate would be high after local treatment. To test this hypothesis, we analyzed the patterns of several genetic and epigenetic changes in synchronous and metachronous bladder cancers and histologically normal mucosa to determine whether genetic aberrations found in invasive bladder cancers are also present in the same patient in the morphologically normal epithelium. For this purpose, we ex-

amined LOH patterns in a panel of microsatellites whose alterations are frequently seen in bladder cancer. In addition to genetic instability that causes LOH, epigenetic factors are the responsible mechanisms that drive the evolutionary process of cancer (7). To determine whether epigenetic alterations occur in cancers and histologically normal mucosa, methylation of the promoter region of the p16 tumor suppressor gene, which is frequently observed in bladder cancer (8), was also examined.

Materials and Methods

Patients and Tissue Samples. Bladder tumor and morphologically normal epithelium samples were obtained from six patients (five males and one female; mean age at the time of diagnosis, 64.7 years; age range, 54–76 years) who developed metachronous bladder tumors and were subsequently treated by total cystectomy between 1990 and 1997 at the University of Tokyo Hospital. No patients had occupational hazards for exposure to known carcinogens for bladder cancer, a history of habitual smoking, or a familial history of urothelial cancer. Twenty-three operations (15 transurethral resections/biopsies, 6 total cystectomies, and 2 excisions of metastatic sites) were performed on these patients. In two patients (cases 2 and 5), the pathology of the cystectomy specimen resulted in pT0 after TUR. The profiles of patients are described in Table 1. Tumors were staged according to the TNM (tumor-node-metastasis) classification (9), and grades were classified according to the WHO classification (10). Progression of tumor stage or grade occurred in all patients with recurrent tumors. Nineteen tumors and 13 normal epithelium samples were obtained from archival formalin-fixed paraffin-embedded tissue. On nine occasions (seven TUR-Bt and two cystectomies), tumor samples and normal epithelium samples were obtained during the same operation. As a control, specimens of bladder epithelium were obtained from 10 patients during surgery for nonneoplastic prostate pathology. Histological examination of these samples showed morphologically normal bladder epithelium, and urine cytology in these patients was negative. The mean age of these patients with normal bladder mucosa was 69.6 years (age range, 57–81 years).

Microdissection and DNA Extraction. Microdissection and DNA extraction were performed as described previously (11). Briefly, serial 5- μ m sections were cut from archival formalin-fixed paraffin-embedded tissues, mounted on UV-treated acetate sheets, and deparaffinized. Sections were stained with H&E to confirm the histopathological diagnosis. A section of morphologically normal epithelium was obtained from the paraffin block that did not contain cancer tissues to avoid potential contamination of cancer cells. Microdissected samples were incubated overnight in 0.1 M Tris-HCl (pH 8.0) and 2 mM EDTA containing 400 μ g of proteinase K at 37°C. Normal lymph nodes obtained by pelvic lymph node dissection or from ovaries without invasion (patient 6) resected at cystectomy served as a constitutive control for each patient. In the healthy control, DNA extracted from patient leukocytes was used as control DNA in the analysis.

Microsatellites. After DNA extraction, LOH was assessed with the following polymorphic markers: (a) D3S1038 (chromosomal locus, 3p26.1–25.2; size of the amplified product, 115 bp); (b) D3S1435 (chromosomal locus, 3pter-24.2; size of the amplified product, 154 bp); (c) D3S1274 (chromosomal locus, 3p12; size of the amplified product, 128 bp); (d) D4S243 (chromosomal locus, 4p32.3; size of the amplified product, 173 bp); (e) ANK1 (chromosomal locus, 8p12–11.2; size of the amplified product, 107 bp); (f) IFNA (chromosomal locus, 9p22; size of the amplified product, 138 bp); (g) D9S118 (chromosomal locus, 9q31–34; size

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² The abbreviations used are: LOH, loss of heterozygosity; MSP, methylation-specific PCR; TUR-Bt, transurethral resection of bladder tumor; TUR, transurethral resection.

Table 1 Clinicopathological characteristics

Case	Sex	Age (yrs)	Operation	Date (mo/yr)	Sample	Tumor grade	pT	LN	Prognosis
1	M	54	TUR-Bt ^a	12/1989	1T1	G2>3	pT1	(-); 0/10	
			Total cystectomy	2/1990	1T2	G3	pT3a		
2	M	76	PLND	3/1992	1T3	LN metastasis		(-); 0/18	Dead (7M)
			TUR-Bt	7/1990	2T1	G2	pT1		
			TUR-B	1/1991	2T2	G2	pT2a		
3	M	60	Total cystectomy	2/1991	2N	NE	pT0	(+) ; 2/13	NED (101M)
			TUR-B	5/1990	3T1	G3	pT1		
			Total cystectomy	5/1990	3T2	TCC>AC,G3	pT3a		
			Resection of LN metastasis	11/1990	3N	NE			
4	M	63	TUR-Bt	1/1995	4T1	TCC>Ac,G2	pTa	(-); 0/4	NED (29M)
			TUR-B	2/1996	4N1	NE			
			TUR-B	6/1996	4N2	Atypical			
			TUR-Bt & B	10/1996	4T2	G2-3	pTa		
			Total cystectomy	2/1997	4N3	NE			
5	M	72	TUR-Bt & B	4/1995	5T1	G2	pTa	(-); 0/4	NED (31M)
			TUR-Bt & B	10/1996	5N1	NE			
			TUR-Bt	12/1996	5T2	G2>3	pT1		
			Total cystectomy	1/1997	5N2	NE			
6	F	63	TUR-Bt & B	4/1995	5T3	G3>2	pT1	(-); 0/4	NED (31M)
			TUR-Bt & B	2/1996	5N3	NE	pT0		
			TUR-Bt & B	4/1996	6T1	G2	pTa		
			TUR-Bt & B	2/1996	6N1	NE			
			TUR-Bt & B	4/1996	6T2	G1-2	pTa		
			TUR-Bt & B	6/1997	6N2	NE			
			Total cystectomy	6/1997	6T3	G1	pTa		
7	M	63	TUR-Bt & B	4/1996	6N3	NE		(+); 2/2	Dead (19M)
			TUR-Bt & B	6/1997	6T4	G2-3	pT2a		
			TUR-Bt & B	6/1997	6N4	NE			
			Total cystectomy	6/1997	6T5	G3>2	pT4		

^aTUR-Bt transurethral resection of bladder tumor; TUR-B, transurethral biopsy of bladder mucosa; PLND, pelvic lymph node dissection; NE, normal epithelium; TCC, transitional cell carcinoma; AC, adenocarcinoma; LN, lymph node; NED, no evidence of disease.

of the amplified product, 69 bp); (h) ABL1 (chromosomal locus, 9q34.1; size of the amplified product, 89 bp); (i) MS34 (chromosomal locus, 13q11–12.1; size of the amplified product, 169 bp); and (j) D16S310 (chromosomal locus, 16q22.1; size of the amplified product, 162 bp). We selected these genomic loci because they frequently show LOH in urothelial cancer (12), and their amplified products were small enough in size to have definite PCR results. Primer sequences and the annealing temperature for PCR were retrieved from the newest update of Genome Database.³ Primers labeled with FITC were synthesized commercially (Grainer Japan, Tokyo, Japan). DNA was amplified by PCR in a final reaction volume of 25 μ l containing 200 μ M of each deoxynucleotide triphosphate, 2 mM Tris-HCl (pH 8.0), 10 mM KCl, 0.01 mM EDTA, 0.1 mM DTT, 0.05% Tween 20, 0.05% NP40, 5% glycerol, 6 pmol of each primer, and 1 unit of Ex. Taq DNA polymerase (Takara Shuzo Co., Tokyo, Japan). PCR cycling conditions using the cold start technique on the DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT) were identical for all primers used: (a) an initial denaturation at 94°C for 2 min; (b) 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C to 60°C for 1 min, and extension for 1 min at 72°C; and (c) a final extension at 72°C for 7 min. Aliquots of PCR products (3 μ l), which were diluted with an equal volume of loading dye (15% Ficoll and 10 mg/ml blue dextran), were denatured at 94°C for 10 min and electrophoresed in 8% Long Ranger (FMC BioProducts, Rockland, ME) gel containing 7 M urea on DNA sequencer DSQ 500S (Shimadzu Corp., Kyoto, Japan) for 3–5 h at 21 W. The relative allelic dosage was quantified using densitometry with an image analysis software program (DSQ 500S; Shimadzu Corp.). LOH was scored in informative cases when a >50% reduction in the ratio of signals from tumor alleles to signals from the corresponding normal (control) alleles in the adjacent lane was observed. Analysis of all samples was carried out in a coded fashion.

Methylation Analysis of p16^{INK4} CpG Island. Methylation status of the p16^{INK4} CpG island was assessed by MSP developed by Herman *et al.* (13). This assay entails an initial modification of DNA by sodium bisulfite, converting all unmethylated (but not methylated) cytosine to uracil and subsequent

amplification with primers specific for methylated *versus* nonmethylated DNA. Briefly, DNA (1 μ g) in a volume of 50 μ l was denatured by NaOH (final concentration, 0.2 M) for 10 min at 37°C. For samples with nanogram quantities of human DNA, 1 μ g of salmon sperm DNA (Stratagene, La Jolla, CA) was added as a carrier before modification. Freshly prepared hydroquinone (30 ml of 10 mM hydroquinone; Sigma-Aldrich) and sodium bisulfite (520 μ l of 3 M sodium bisulfite; Sigma) at pH 5.0 were added and mixed, and samples were incubated at 50°C for 16 h. Modified DNA was purified using the Wizard DNA purification resin according to the instructions provided by the supplier (Promega, Madison, WI) and subsequently used for MSP analysis using primer pairs p16-M and p16-U as described elsewhere (13). The PCR mixture contained 1 \times GC buffer I (Takara Shuzo Co.), 0.4 mM deoxynucleotide triphosphate mixture, 300 ng of sense and antisense primers, and 156 ng of bisulfite-modified DNA or 50 ng of unmodified DNA in a final volume of 50 μ l. Reactions were hot-started at 95°C for 5 min before the addition of 2.5 units of LA Taq (Takara Shuzo Co.). Amplification was carried out in a thermal cycler for 35 cycles (30 s at 95°C, 30 s at 60°C, and 30 s at 72°C), followed by a final 4-min extension at 72°C. Each of the PCR products (20 μ l) was loaded directly onto 2.5% MetaPhor Agarose gels, stained with ethidium bromide, and visualized directly under UV light. Methylation-specific primers produce a product of 234 bp, and a product of 151 bp is obtained by nonmethylated-specific primers (13).

Results

Table 2 shows the pattern of LOH in each sample. The ambiguous or nonreproducible results were included in the noninformative category in Table 2. Overall, LOH was seen in 20.2% (33 of 163 informative tests) in tumors and 13.3% (14 of 105 informative tests) in the normal epithelium. The lost alleles showing LOH were identical in each individual locus in the same patient. Fig. 1a shows a typical result of LOH. In five of six cases, we detected LOH in both cancers and morphologically normal epithelium. Furthermore, 12 of 14 (86%) LOHs seen in the normal epithelium (case 1, 3pter-24.2, 8p12–11.2, and 9q31–34; case 2, 3p12; case 3,

³ Available on the World Wide Web at <http://www.gdb.org/>.

Table 2. LOHs and p16^{INK4} methylation

CASE#1						
	MICROSATELLITE	LOCI	1T1	1T2	1N	1T3
1	D3S1038	3p26.1-25.2	△	△	△	△
2	D3S1435	3pter-24.2	●	●	●	ND
3	D3S1274	3p12	○	○	NI	○
4	ANK1	8p12-11.2	●	●	●	●
5	IFNA	9p22	○	●	○	●
6	D9S118	9q31-34	○	●	●	●
7	ABL1	9q34.1	○	○	NI	○
8	D4S243	4p32.3	NI	○	ND	○
9	MS34	13q11-12.1	○	○	ND	NI
10	D16S310	16q22.1	△	△	ND	△
Methylation of p16 ^{INK4} CpG-island			U	U	U	U

CASE#4					
4T1	4N1	4N2	4T2	4N3	4T3
○	○	○	○	○	○
○	○	○	○	○	○
○	○	○	○	○	○
△	△	△	△	△	△
○	○	○	○	○	○
○	○	○	○	○	○
○	○	○	○	○	○
○	○	○	○	○	○
○	○	○	○	○	○
△	△	△	△	△	△
U	M	M	M	M	M

CASE#2					
	MICROSATELLITE	LOCI	2T1	2T2	2N
1	D3S1038	3p26.1-25.2	●	○	○
2	D3S1435	3pter-24.2	NI	NI	NI
3	D3S1274	3p12	●	●	●
4	ANK1	8p12-11.2	●	●	○
5	IFNA	9p22	○	○	○
6	D9S118	9q31-34	△	△	△
7	ABL1	9q34.1	●	●	○
8	D4S243	4p32.3	●	○	○
9	MS34	13q11-12.1	○	○	○
10	D16S310	16q22.1	NI	●	○
Methylation of p16 ^{INK4} CpG-island			U	U	U

CASE#5					
5T1	5N1	5T2	5N2	5T3	5N3
●	●	●	●	●	●
NI	NI	NI	NI	NI	NI
○	NI	○	○	○	○
○	○	○	●	NI	○
●	●	●	●	●	●
○	○	○	○	○	○
○	○	○	○	○	○
NI	NI	NI	NI	NI	NI
○	NI	○	NI	NI	○
△	△	△	NI	NI	△
M	U	U	U	U	U

CASE#3						
	MICROSATELLITE	LOCI	3T1	3T2	3N	3T3
1	D3S1038	3p26.1-25.2	●	●	●	●
2	D3S1435	3pter-24.2	NI	NI	NI	NI
3	D3S1274	3p12	○	○	○	○
4	ANK1	8p12-11.2	△	△	△	△
5	IFNA	9p22	○	○	○	○
6	D9S118	9q31-34	●	●	●	●
7	ABL1	9q34.1	○	○	NI	○
8	D4S243	4p32.3	△	△	ND	△
9	MS34	13q11-12.1	NI	NI	NI	NI
10	D16S310	16q22.1	NI	○	ND	○
Methylation of p16 ^{INK4} CpG-island			U	M	U	M

CASE#6									
6T1	6N1	6T2	6N2	6T3	6N3	6T4	6N4	6T5	
○	○	○	○	○	○	○	○	○	○
NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
○	○	○	○	○	○	○	○	○	○
△	△	△	△	△	△	△	△	△	△
○	○	○	○	○	○	○	○	○	○
○	●	○	○	○	○	○	○	○	○
○	○	○	○	○	○	○	○	○	○
○	○	○	○	○	○	○	○	○	○
○	○	○	○	○	○	○	○	○	○
○	○	○	○	○	○	○	○	○	○
○	○	○	○	○	○	○	○	○	○
△	△	△	△	△	△	△	△	△	△
U	U	NI	U	U	U	NI	U	NI	

○heterozygote △homozygote ●LOH NI; non-informative ND; not determined
M; Methylated p16^{INK4} CpG-island
U; Unmethylated p16^{INK4} CpG-island

3p26.1–25.2 and 9q31–34; case 5, 3p26.1–25.2 and 9p22; case 6, 9q31–34) were maintained in the corresponding tumors. Methylation of the p16^{INK4} CpG island was detected in 37.5% of cancer samples (6 of 16 informative tests) and in 17.6% of normal epithelium (3 of 17 tests; seen only in case 4). Fig. 1b shows a typical result of methylation of p16^{INK4} CpG island.

Changes in chromosomal deletions during tumor progression were demonstrated in two cases (cases 1 and 2). In these cases, recurrent tumors showed heterogeneity in the LOH pattern compared with that of the primary cancer (case 1, 1T1, 1T2, and 1T3; case 2, 2T1 and 2T2).

In case 5, morphologically normal epithelium obtained from four

different sites in the bladder was examined on LOH and showed identical results (Fig. 2). Two cases (cases 4 and 6) did not demonstrate an association of genetic aberrations between normal epithelium and cancer. In case 6, LOH detected in normal mucosa (6N1) was not seen in the tumors. Case 4 did not show LOH in both carcinomas and normal epithelium. However, in this case, methylation at the p16^{INK4} CpG island was detected in the recurrent tumor and morphologically normal epithelium (Fig. 1b). In biopsies of bladder epithelium obtained from patients with nonneoplastic prostate disease, neither LOH in 97 total informative loci nor methylation at the p16^{INK4} CpG island was detected.

These data suggest that (a) some unique and presumably significant

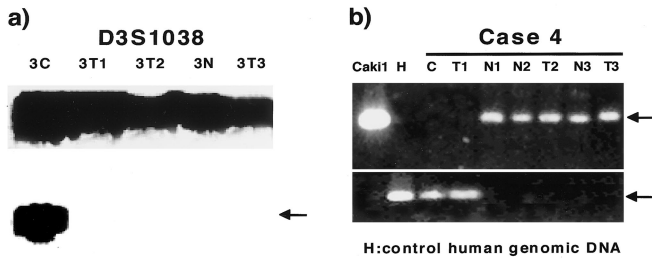


Fig. 1. *a*, typical results of LOH at D3S1038 in tumor samples and morphologically normal epithelium in case 3. 3C shows normal heterozygotes in control DNA. An arrow indicates LOH. *b*, methylation status of the p16^{INK4} CpG island in case 4. *Top*, MSP in sodium bisulfite-treated samples. A product of 234 bp (arrow) amplified by methylation-specific primers (13) indicates positive for methylation of the p16^{INK4} CpG island. Caki-1, a renal cell carcinoma cell line that had methylation of the p16^{INK4} CpG island, 4N1, 4N2, 4N3, 4T2, and 4T3 are positive for methylation. *Bottom*, non-MSP. Human control genomic DNA, 4C, and 4T1 had products of 151 bp (arrow) amplified by nonmethylation-specific primers (13), indicating the unmethylated status of p16^{INK4} CpG island.

combinations of LOH were retained in the progression from morphologically normal epithelium to advanced cancer, which was characteristic of clonal expansion, and (b) epigenetic changes such as methylation at the p16^{INK4} CpG island could occur as early as in morphologically normal epithelial cells.

Discussion

Among the loci with LOHs observed in the morphologically normal bladder epithelium in this study, 3p (14) and 8p (15) have been considered late markers because they have been associated with invasive cancers. Reported cases showed that LOH in 3p is quite rare in pTa tumors (16). Late markers feature the specific chromosomal deletion for invasive cancers in the stepwise genetic changes of carcinogenesis. However, previous studies examined the genetic aberration in invasive cancers in a single time frame, and a chronological analysis from superficial cancer to invasive cancer has not been performed. We saw in this study that morphologically normal epithelium possessed LOHs at loci of late markers, and those LOHs were retained through the metachronous tumors. Thus, our preliminary data turned out to contradict the proposed pathway for stepwise chromosomal changes in the progression of bladder cancer (16). Along with the genetic changes occurring in a population of clustered normal cells, we noticed that epigenetic change, methylation of a putative tumor suppressor gene, could also take place in normal mucosa. To our knowledge, this is the first demonstration that epigenetic changes in a putative tumor suppressor gene were seen in normal epithelium in multiple metachronous cancers.

Schmidt and Mead (17) introduced the concept of patch size in the argument of clonal origin of tumors. A patch is regarded as a group of cells or structures that share a common genotype. Tsai *et al.* (18) examined the monoclonality of normal urothelium using the X chromosome inactivation analysis. They were able to demonstrate individual “patches” of monoclonal cells that covered an area as large as 120 mm² containing about 2 × 10⁶ cells. This suggests that only 200–300 stem cells participated in the formation of the bladder epithelium. They speculated that each of the daughter cells that covered the large area of bladder epithelium derived from a stem cell would have the same genetic predisposition to tumorigenesis as the stem cell itself. In case 5, four samples of normal epithelium obtained from four different portions of the urinary bladder (5N3) showed the same LOHs. A possible explanation is that a single stem cell with LOH detected in normal epithelium might have a growth advantage to form a larger patch that is predisposed to cancerization (19). Apart from genotypic changes, Rao *et al.* (20) showed that changes in

phenotypic biochemical markers, such as the expression of G-actin, in bladder cancer were already seen in the normal mucosa. On the basis of these data, we speculate that a progenitor mutant clone would exist in the morphologically normal epithelium, and it might have a growth advantage to form a larger patch, where subsequent genetic changes could transform “normal” epithelia to cancer. Metachronous cancers can occur in the patch derived from a progenitor mutant clone. However, the pattern of genetic or epigenetic aberrations may encounter heterogeneity along the pathway of evolution from the progenitor clone that disguises multiple tumors as of polyclonal origin. To further explore this issue, discovery of a proper marker gene for a stem cell of bladder epithelium is awaited.

It could be argued that LOHs or epigenetic change of the p16^{INK4} CpG island observed in the normal epithelium does not contribute significantly as a gatekeeper gene to the progression to cancerization. However, the following findings refute such an argument: (a) chromosomal deletions detected in this study have been reported as being relevant to the progression to invasive cancer (14, 15); (b) LOHs detected in the normal epithelium were unique for each patient and largely retained in the cancer; and (c) the p16^{INK4} CpG island is a putative tumor suppressor gene, and its deletion or methylation has been reported in various cancers (7). Additional studies are warranted to see whether patients with LOH or epigenetic changes in normal bladder epithelium are more likely to have a tumor recurrence than patients whose normal epithelium is not genetically aberrant.

In conclusion, in the present study, we demonstrated chromosomal deletions and methylation of the promoter regions of putative tumor suppressor gene in morphologically normal bladder epithelium from patients with bladder cancer. The existence of genetic/epigenetic changes in morphologically normal bladder mucosa might be a ground for multiple carcinogenesis.

Acknowledgments

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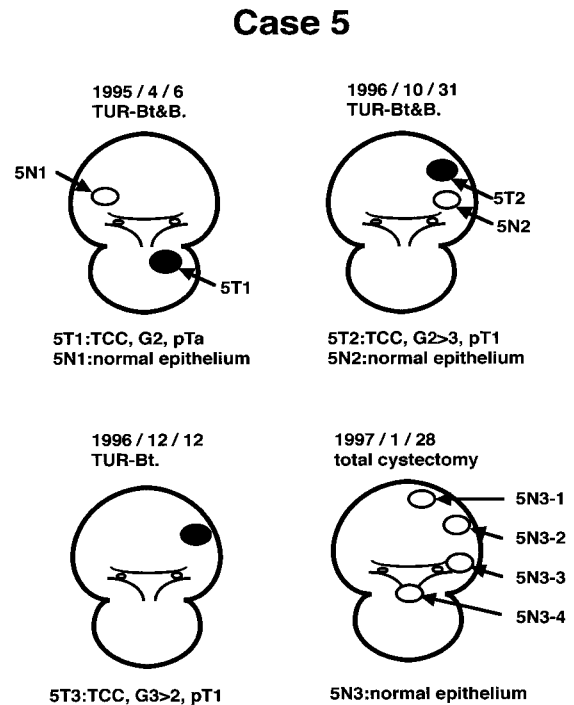


Fig. 2. Mapping of the samples analyzed in case 5. Sites inside the bladder for the individual samples are shown. Normal epithelia from four different sites were examined in 5N3.

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