CLONAL ORIGIN OF BLADDER CANCER

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Abstract *Background.* Patients with cancer of the urinary bladder often present with multiple tumors, appearing at different times and at different sites in the bladder. This observation has been attributed to a "field defect" in the bladder that allows the independent transformation of epithelial cells at a number of sites. We tested this hypothesis using molecular genetic techniques.

Methods. We examined 13 tumors from cystectomy specimens from four women, using a method that analyzes the pattern of X-chromosome inactivation to determine whether the tumors were derived from the same precursor cell. In addition, we analyzed allelic loss on autosomes to determine whether different tumors had the same genetic alterations. The alterations evaluated included the loss of chromosome 9q sequences (commonly found in superficial bladder tumors) and the loss of 17p and 18q sequences (usually found only in advanced tumors).

Results. For each patient studied, all the tumors had inactivation of the same X chromosome, whereas normal bladder mucosa cells had random patterns of inactivation. Moreover, each tumor that could be evaluated from a given patient had lost the same allele on chromosome 9q, suggesting that the loss of this allele preceded the spread of neoplastic cells elsewhere in the bladder. The losses of chromosome 17p and 18q alleles, which are late events in tumor progression, were not common to different tumors from the same patient.

Conclusions. A number of bladder tumors can arise from the uncontrolled spread of a single transformed cell. These tumors can then grow independently with variable subsequent genetic alterations. (N Engl J Med 1992; 326:737-40.)

MONOCLONALITY is a fundamental characteristic of neoplasia.[1-4] One transformed cell gives rise to daughter cells, all of which exhibit the same genetic change that initially provided the growth advantage to the parent cell. The accumulation of further genetic changes in subsequent daughter cells, each providing an additional growth advantage, has been well documented in human cancers.[5,6] This concept of clonal origin and expansion poses a difficulty for the understanding of neoplasia in organs where several metachronous tumors occur. In patients with bladder cancer, for example, several tumors scattered over the bladder epithelium are often discovered at cystoscopy. Moreover, diffuse areas of dysplasia are often found throughout the bladders of such patients.[7] These clinical observations have given rise to the idea of a "field defect," whereby a carcinogenic insult results in the independent transformation of many epithelial cells.[8] An alternative explanation, however, is that only one transforming event occurred and that the progeny of a single transformed cell spread through the bladder, giving rise to topologically distinct but genetically related tumors. In the current study, we have used molecular genetic techniques in an attempt to distinguish between these possibilities.

METHODS

Bladder specimens from four female patients who each had more than one geographically isolated tumor of the bladder were obtained through cystectomy. The tumors were carefully defined anatomically, and great care was taken to map them with respect to their position after the bladder was opened. If there was any question that tumors were contiguous, they were not evaluated. Patient 1 and Patient 4 each had three tumors, Patient 2 had two well-separated tumors, and Patient 3 had five tumors. Each tumor was sectioned in a cryostat, and nonneoplastic tissue was removed by microdissection.[9] DNA was obtained from the tumor sections and from normal bladder mucosa of the same patients by sodium dodecyl sulfate–proteinase K digestion and extraction with phenol and chloroform, followed by ethanol precipitation, as described elsewhere.[10]

X-Chromosome Inactivation and Allelic Loss

DNA was digested with appropriate restriction enzymes, and the fragments were separated by electrophoresis through an agarose gel and transferred to nylon membranes. For the assessment of X-chromosome inactivation, the membranes were incubated with radioactively labeled hypoxanthine phosphoribosyltransferase (HPRT) or phosphoglycerate kinase (PGK) probes, as described elsewhere.[11] In the studies of allelic loss, Southern blots were hybridized with highly polymorphic probes (EFD126.3 for chromosome 9q,[12] pYNZ22.1 and p144D6 for 17p,[13] p15-65[14] and AC404 for 18q,[15] pYNH24 for 2q,[16] MHZ47 for 13q,[17] YNA13 for 1q,[18] and MS8 for 5q[19]). Allelic loss was assessed by methods described elsewhere.[20]

RESULTS

Four bladder specimens obtained by cystectomy, each containing two to five separate tumors, were examined for X-chromosome inactivation. Inactivation takes place during the first trimester of embryogenesis, and subsequently each somatic cell in a female contains only one active X chromosome.[4,21] In approximately half of such cells, the paternal X chromosome is active; in the other half, the X chromosome inherited from the mother is active. The pattern of X-chromosome inactivation can be assessed by a combined analysis of restriction-fragment–length polymorphism (RFLP) and methylation.[4,11] In female heterozygotes, two restriction fragments are observed on Southern blot assays with X-chromosome RFLP probes. One fragment is derived from the paternal X chromosome, and the other from the maternal chromosome. Because the DNA on the active X chromosome is methylated differently from that on the inac-

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tive X chromosome, endonucleases that cleave DNA only at unmethylated sequences can distinguish the restriction fragments derived from the active chromosome from those derived from the inactive chromosome. For example, the methylation-sensitive restriction endonuclease *Hpa*II, when used to cleave DNA from normal bladder mucosa previously digested with an appropriate RFLP-revealing endonuclease, results in a reduction of both polymorphic alleles by approximately 50 percent, because approximately half the DNA is derived from cells in which the paternal X chromosome is inactive and half from cells in which the maternal X chromosome is inactive (Fig. 1, lanes 1 and 2). In contrast, in a neoplasm, which generally results from the clonal expansion of a single cell, all the cells have inactivation of the same X chromosome. Thus, one of the two restriction fragments will remain uncleaved by *Hpa*II digestion, but the other will be digested completely (Fig. 1, lanes 4 and 6). On the basis of previous analysis of other tumor types,[9,11] bladder cancers would be expected to demonstrate this monoclonal pattern, and this was confirmed in our experiments. A more important question addressed in these studies was whether multiple bladder tumors from the same patient all had inactivation of the same X chromosome. If two tumors were derived from the same precursor epithelial cell, they would have the same inactivation pattern. However, if two tumors were derived from different precursor cells, there would generally be only a 50 percent probability of their having the same inactivation pattern.

An analysis of X-chromosome inactivation of DNA from the normal bladder mucosa of Patient 1 is shown in Figure 1 (lanes 1 and 2). This woman was heterozygous for a *Bam*HI RFLP within the HPRT gene located at chromosome Xq26, so that two fragments, designated as alleles 1 and 2, were observed after *Bam*HI digestion (Fig. 1, lane 1). Subsequent digestion with the methyl-sensitive endonuclease *Hpa*II revealed a normal polyclonal pattern of X-chromosome inactivation — i.e., the reduction in intensity of both alleles was approximately 50 percent, indicating that allele 1 was active in approximately half the cells and allele 2 in the other half (Fig. 1, lane 2). In contrast, analysis of tumors from this patient showed a monoclonal pattern of digestion; each tumor showed a nearly complete loss of allele 1 after *Hpa*II digestion (Fig. 1, lanes 4 and 6). Likewise, Patient 2 was heterozygous for a *Bst*XI RFLP within the PGK gene at Xq13, and two allelic fragments were observed after *Bst*XI digestion (Fig. 2, lane 1). This patient's normal mucosa showed a polyclonal pattern of X-chromosome inactivation after digestion with *Hpa*II (Fig. 2, lane 2). Tumors from Patient 2 showed a monoclonal pattern of digestion, however, with a nearly complete loss of allele 1 after digestion with *Hpa*II (Fig. 2, lanes 4 and 6). Similarly, all five tumors from Patient 3 had inactivation of the same X chromosome (Table 1). In Patient 4, X-chromosome inactivation could not be assessed because the patient was not heterozygous for the RFLPs detected by the HPRT and PGK probes.

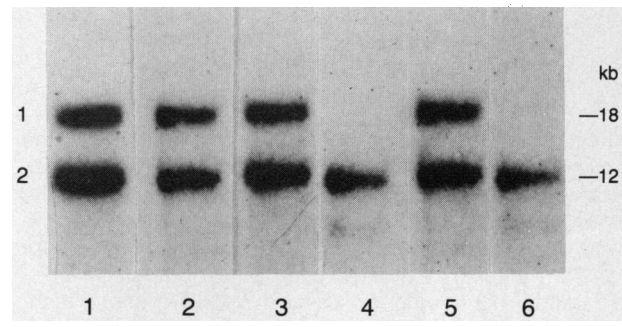

Figure 1. X-Chromosome Inactivation within the HPRT Gene.
DNA samples from normal bladder mucosa from Patient 1 (lane 1) and from tumors A and B from the same patient (lanes 3 and 5) were digested with *Bam*HI and *Pvu*II, revealing a heterozygous RFLP within the HPRT gene (lane 1). After *Hpa*II digestion, both alleles from the samples of normal mucosa were equally diminished in intensity (lane 2), signifying normal polyclonal X-chromosome inactivation. In contrast, both tumors had a monoclonal pattern after *Hpa*II digestion, with complete loss of the 18-kb allele (lanes 4 and 6).

These tumors were then examined for the somatic loss of alleles on chromosome 9q. In this assay, Southern blots of DNA from normal tissue and carcinoma tissue were hybridized to a highly polymorphic probe from chromosome 9q, as shown in Figure 3. DNA from the tumors in Patient 1 showed the loss of the same 9q allele in each tumor. The chromosomal losses appeared to be clonal — that is, they were present in at least 90 percent of the neoplastic cells within each tumor, as assessed by the ratio between the hybridization intensities of the two alleles in the tumors. Similarly, each tumor in Patient 4 showed the loss of the same 9q allele. In Patient 3, sufficient DNA was available for only tumors A and B to be examined, and both were found to have lost the same 9q allele (Table 1).

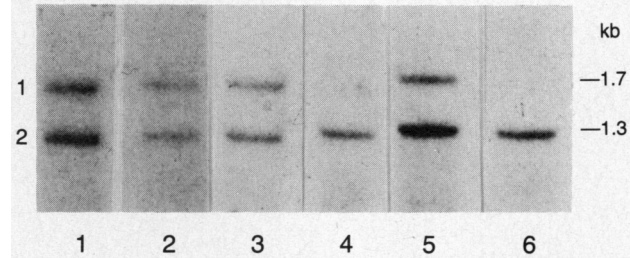

Figure 2. X-Chromosome Inactivation within the PGK Gene.
DNA samples from normal bladder mucosa from Patient 2 (lane 1) and from tumors A and B in the same patient (lanes 3 and 5) were digested with *Bst*XI and *Pst*I, revealing a heterozygous RFLP within the PGK gene (lane 1). After *Hpa*II digestion, both alleles from the samples of normal mucosa were equally diminished in intensity (lane 2), signifying normal polyclonal X-chromosome inactivation. In contrast, both tumors had a monoclonal pattern after *Hpa*II digestion, with complete loss of the 1.7-kb allele (lanes 4 and 6).

Table 1. Results of Clonal Analysis.*

Patient No.	Tumor	Stage/ Grade	X Chromosome Inactivated	Allele Lost						
				9q	17p	18q	2q	13q	5q	1q
1	A	Ta/II	1	1	None	None	None	NI	None	None
	B	T1/III	1	1	None	None	None	NI	None	None
	C	T3/III	1	1	1	None	1	NI	None	None
2	A	T1/II	2	NI	NI	None	None	NI	NI	NI
	B	Ta/II	2	NI	NI	None	None	NI	NI	NI
3	A	T1/III	2	2	2	1	None	None	None	None
	B	T2/II	2	2	2	None	None	None	None	None
	C	T1/I	2	ND	ND	ND	ND	ND	ND	ND
	D	T1/II	2	ND	ND	ND	ND	ND	ND	ND
	E	T1/II	2	ND	None	ND	ND	ND	ND	ND
4	A	T1/II	NI	1	NI	NI	None	NI	None	None
	B	T1/II	NI	1	NI	NI	None	NI	None	None
	C	T1/II	NI	1	NI	NI	None	NI	None	None

*The larger and smaller alleles identified by each RFLP probe are designated as 1 and 2, respectively. NI denotes noninformative (i.e., the probes could not distinguish between maternal and paternal alleles), and ND insufficient DNA for further analysis. The staging and grading classification follows the recommendations of the American Joint Committee on Staging and End Results.[22]

We could not determine the loss of 9q for Patient 2, because the probes we used could not distinguish between maternal and paternal alleles in this patient (i.e., the RFLP was noninformative).

It is evident from Table 1 that in each case in which the RFLP was informative, the same X chromosome was inactivated and the same 9q allele lost. The probability that all tumors from a given patient would have inactivation of the same X chromosome is 0.5^{n-1}, where n equals the number of tumors. The probability that this would be true for all three patients for whom results were informative is $0.5 \times 0.5^4 \times 0.5^2 = 0.5^7$ (i.e., $P<0.01$). The same statistical argument holds for the loss of 9q alleles; there was only a 0.5^5 probability that the 9q results were due to chance. Furthermore, because 9q loss and X inactivation were independent events, the probability that the results for both 9q and the X chromosome were due to chance is the product of the individual probabilities — i.e., $0.5^5 \times 0.5^7$, or <0.0005.

Allelic loss of sequences on chromosome 9 is generally considered an early change in bladder tumorigenesis, whereas the loss of alleles on other chromosomes (particularly 17p and 18q) is generally considered a late event.[23-26] We analyzed the bladder tumors for such late changes in order to determine whether they displayed the uniformity observed in the case of X-chromosome inactivation and 9q loss. The results of these analyses proved quite different. First, in these tumors (which were generally of early grade; Table 1), allelic losses of 17p, 18q, and 2q were much less common than losses of 9q ($P<0.0001$ by Fisher's exact test). Second, in the few cases in which losses were observed, their pattern was not consistent among the tumors in a given patient. For example, in Patient 3, tumor A lost a chromosome 18q allele but tumor B did not (Table 1). Similarly, in Patient 1, tumor C lost a chromosome 17p allele whereas tumors A and B retained both alleles (Fig. 4). In general, tumors in which there was a loss of 17p were of higher grade and stage (Table 1).

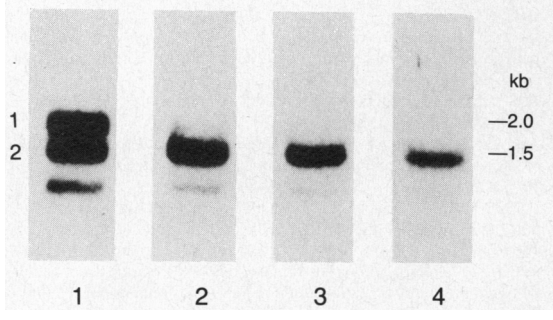

Figure 3. Loss of an Allele from Chromosome 9q.

DNA samples from normal bladder mucosa from Patient 1 were digested with *MspI*, revealing two alleles after Southern blots were hybridized to a polymorphic probe from chromosome 9q (lane 1). The smaller unlabeled band below the blots is a constant (i.e., nonpolymorphic) fragment detected with this probe.[12] DNA samples from tumors A, B, and C of the same patient had clonal loss of the 2.0-kb allele (lanes 2, 3, and 4, respectively).

Discussion

These studies provide strong evidence that a number of tumors of the bladder can arise from a single transformed cell. Every tumor in a given patient had inactivation of the same X chromosome, suggesting that all the tumors arose from the same progenitor cell. By contrast, in patients who inherit a predisposition to tumors of the bowel and parathyroid, the examination of multiple tumors revealed variation in the pattern of X-chromosome inactivation.[27,28] Independent somatic alterations are apparently responsible for the initiation of neoplasia in these tumor types.[27,28]

Autosomal loss of alleles is likewise thought to occur randomly in adult tumors. One would expect an equal probability of losing either a maternal or a paternal allele in these tumors. Because the patients with bladder cancer we studied were all over 50 years of age and their parents had generally died, we could not determine the parental origins of the alleles identified. Recently, data have been presented that suggest a preferential loss of maternal 13q and 11p alleles in embryonal tumors, such as retinoblastoma and Wilms' tumor.[29-32] In adult patients with colon cancer, however, no such preference has been demonstrated (unpublished data). Our finding that all the tumors in individual patients lost the same 9q allele confirms that this loss is an early event in bladder neoplasia, and it strengthens the observation that all the tumors in each patient arose from the same progenitor cell. It would be interesting to see whether recurrent bladder tumors from the same patient, like the con-

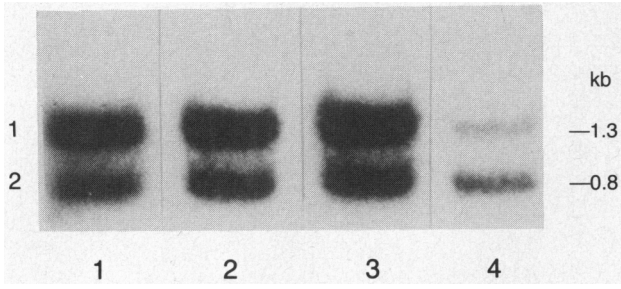

Figure 4. Loss of an Allele from Chromosome 17p.

DNA samples from normal bladder mucosa from Patient 1 were digested with *TaqI*, revealing two alleles after Southern blots were hybridized to a polymorphic probe from chromosome 17p (lane 1). Although the DNA from tumors A and B of the same patient retained both alleles (lanes 2 and 3), the DNA from tumor C lost the 1.3-kb allele (lane 4).

current tumors studied here, are also derived from the same precursor cell.

How does a single progenitor cell lead to multiple bladder tumors? We suggest that a specific genetic alteration (perhaps loss of a 9q allele and the concomitant inactivation of a tumor-suppressor gene on 9q) occurs in a bladder epithelial cell and provides an important growth advantage. During subsequent turnover of the surrounding epithelium, uncontrolled growth leads to the repopulation of surrounding areas by daughters of this cell, either by gradual migration or through intravesicular transfer through the urine. In some cases, this may lead to seeding of the neoplastic cell in areas scattered throughout the bladder. Subsequently, additional rare genetic events (such as 17p and 18q loss) transform a small percentage of such cells to a more invasive state, eventually leading to clinically invasive carcinoma. This situation is analogous in some ways to that of chronic myelogenous leukemia, in which an early event (c-*abl* activation) gives rise to a chronic proliferative state in which all neoplastic cells have the same genetic abnormality.[33] Subsequent events (e.g., p53 inactivation)[34] lead to further proliferation of a subgroup of cells, manifested as blast crisis.

According to this model, all the transformed cells in the bladder of a patient who has cancer are likely to be derived from one original cell, and these cells are at increased risk for subsequent progression. Identification of the putative tumor-suppressor gene at the 9q locus could aid substantially in early diagnosis, because this genetic event appears to precede the spread of tumors to other areas of the bladder. Moreover, these results may have important implications for future therapeutic measures. Because all tumor cells in the bladder appear to share the same initiating genetic alteration, therapy directed against this alteration would not affect just a portion of the transformed cells, but would theoretically have the potential to affect all of them.

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