Two Molecular Pathways to Transitional Cell Carcinoma of the Bladder

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ABSTRACT

Noninvasive transitional cell carcinomas of the bladder can have two distinct morphologies suggesting they contain different genetic alterations. Papillary transitional cell carcinomas (Ta tumors) are often multifocal and only occasionally progress, whereas flat tumors (carcinomas in situ, CIS), frequently progress to invasive disease. We examined 216 bladder tumors of various stages and histopathologies for two genetic alterations previously described to be of importance in bladder tumorigenesis. Loss of heterozygosity of chromosome 9 was observed in 24 of 70 (34%) Ta tumors but was present in only 3 of 24 (12%) CIS and dysplasia lesions (P = 0.04). In contrast, only 1 of 36 (3%) Ta tumors contained a p53 gene mutation compared to 15 of 23 (65%) CIS and dysplasias (P < 0.001), a frequency comparable to that observed in muscle invasive tumors (25 of 49; 51%). The presence of p53 mutations in CIS and dysplasia could explain their propensities to progress since these mutations are known to destabilize the genome. Analysis of several tumor pairs involving a CIS and an invasive cancer provided evidence that the chromosome 9 alteration may in some cases be involved in the progression of CIS to more invasive tumors, in addition to its role in the initiation of Ta tumors. However, the CIS and secondary tumor were found to contain different genetic alterations in some patients suggesting divergent progression pathways. Bladder carcinogenesis may therefore proceed through two distinct genetic alteration pathways responsible for generating superficial tumors with differing morphologies and pathologies.

INTRODUCTION

Tumor progression is believed to occur through the multistep accumulation of genetic alterations (1–3). Individual cells in a preexisting tumor have been hypothesized to sustain additional genetic alterations resulting in clonal expansion and ultimately a domination of the tumor population by cells exhibiting uncontrolled growth (4). Sidransky et al. (5) demonstrated that recurrent invasive glioblastomas harbored p53 gene mutations that were present in only a small subset of cells of original low grade astrocytomas, unequivocally showing genetic progression within individual tumors. Models of genetic progression have been postulated for both colorectal (6) and astrocytoma tumors (7). Although the total accumulation of genetic defects may be more important than the order of their occurrence (6), a linear ordering of these defects has been assigned in both cases for the progression from low stage to invasive tumor based primarily on the compilation of data obtained for individual tumors in a large number of patients. Relatively few studies have addressed the genetic defects involved in bladder tumor progression (8, 9).

Clinical data indicate that invasive TCCs of the bladder may not arise through a single progression pathway. These tumors may develop either from the relatively infrequent progression of a superficial papillary tumor (10, 11) or may arise de novo (12). CIS, a flat superficial lesion, may act as the most common precursor to invasive bladder cancers since it shows a high propensity to progress to invasive disease (13).

A number of specific genes and chromosomal loci have been implicated in bladder tumorigenesis. LOH of chromosomes 9 (14, 15), 11, 17 (16), and 18 (9) have been observed in TCCs. Loss of chromosome 9 has been found in TCCs of all stages, whereas loss of chromosome 17p is predominantly observed in invasive tumors (14). The same chromosome 9 allele was observed to be lost in multifocal tumors of varying stage obtained from individual patients, suggesting this event occurs early in bladder tumor progression (17). Reznikoff et al. (18) have also shown that chromosomes 3, 5, 6, 9, and 11 are involved in the in vitro transformation of SV-40-immortalized urothelial cells following 3-methylcholanthrene treatment. Alterations of the p53 tumor suppressor gene (19–21) and retinoblastoma gene (22–24) have also been observed mostly in invasive TCCs.

We have examined the two types of noninvasive bladder cancers for the frequencies of occurrence of some of these defects present in invasive cancers to determine whether there is a molecular basis for their differing morphologies and pathologies. When these data are combined with results obtained for multiple tumors in nine individual patients, they show not only direct evidence for molecular progression but suggest two molecular pathways in bladder tumorigenesis.

MATERIALS AND METHODS

Tumor Selection. Our total analysis included 216 TCCs obtained from patients diagnosed at the Kenneth Norris Jr. Comprehensive Cancer Center in Los Angeles, California (n = 116) and the Herlev Hospital, University of Copenhagen, Denmark (n = 100). Tumors were histopathologically graded according to Bergkvist et al. (25) and staged according to the tumor–nodes–metastasis staging system (26). TCCs analyzed in this study included: papillary low grade (Ta grade I-II; n = 48); papillary high grade (Ta grade III-IV; n = 7); lamina propria invasive (T1; grade III-IV; n = 25); muscle invasive (T2-T4; grade III-IV; n = 42); CIS (Tis; n = 21); and dysplasias (n = 5). All dysplasias were classified as moderate to severe. Results were combined with those reported previously for chromosome 9 loss (14, 16) and p53 mutational status (19, 21). High molecular weight DNA was prepared from fresh frozen tumor specimens and matching blood samples by proteinase K digestion and phenol–chloroform extraction as described (27). DNA was also isolated from archival, paraffin-embedded specimens by microdissecting tumor and normal tissues from hematoxylin and eosin-stained tissue sections as described (21).

p53 Mutation Analysis. Mutations in the p53 tumor suppressor gene were detected by SSCP analysis (28). Our analysis of p53 mutations consisted of 82 new tumors and 54 tumors previously reported (19, 21). Exons 5–8 of the p53 gene were individually amplified by two serial PCR reactions. Exons 5–8 of the p53 gene were analyzed because previous reports have shown that these exons harbor the majority of inactivating mutations in a diverse variety of human tumors (29). Procedures used for PCR, SSCP, and for direct DNA sequencing analysis were reported previously (21). Oligonucleotide primers used for primary PCR amplifications included: PE5LT, 5'-TCAACTCT- GTCTCTCCTCT; PE5RT, 5'-UAACCCCGCTCCACCCAG; PE6RT, 5'-CAGCCCCGATCTCCACTGAT; PE8RT, 5'-TTAACCCTCCTCCACAGA- GA; PE7LT, 5'-AGGCGACTGGCCTCAGTCT; PE7RT, 5'-TGGCA-
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GGGGTGCAAGTGGC; PE8LT, 5'-TTCTACTGCTCTTGCTTGT; and PE8RT, 5'-AGGGCATACGTGCCACCTTTGGG. Secondary PCR primers included: PX5(5L), 5'-GGAAATGCTTCTGCGTAATCTC; PX5(5R), 5'-TCATGTCGACTGTGCTTGTA; PX5(5L), TACAACGTCGAGCAGCCTAGA; PX5(5R), 5'-GGGAATTGCCACGAGCTGTCACC; PX6L, 5'-GGAAATGCTCAGTGCTGCTGTA; PXS(3)RT, 5'-TACAAGCAGTCACAGCA

Allelic Loss Analysis. The presence of allelic loss on chromosome 9 was examined in 146 bladder carcinomas. Sixty-three tumors reported previously (14, 16) were included in the final analysis. Fifty-four new tumors were analyzed by Southern blotting to determine chromosome 9 loss as described (16). Allelic losses were assigned for tumors demonstrating deletions of one or more of the following loci using the following probes and restriction enzymes: LAMPP2 (D9S29), TaqI; MCOA12 (D9S16), MspI+HpaII; ASSG3 (ASS), PstI; MCT136 (D9S10), MspI+HpaII; MCT96.1 (D9S14), Rsal; KKA50 (D9S31), PstI; EFD 126.3 (D9S7), MspI+HpaII, or PstI; and MCT 112 (D9S15), MspI+HpaII. Allelic loss of chromosomes 9 and 17 were also examined in 92 paraffin-embedded archival tumor specimens by PCR amplification of dinucleotide repeat polymorphisms. Loci examined included D9S59 and D9S63 on chromosome 9q (30) and D17S5B on chromosome 17p (31). Although there is evidence for a putative tumor suppressor gene(s) involved in bladder tumorigenesis on both chromosome arms 9p and 9q, our analysis of chromosome 9q should closely approximate LOH of chromosome 9 due to the low frequency of observed partial chromosome 9 loss (32). Archival specimens which showed allelic loss were confirmed by repeating the analysis to prevent the possibility of allelic underrepresentations in the PCR reaction.

RESULTS

A typical result of the deletion and mutational analysis performed on 216 bladder tumors of various stage is shown in Fig. 1. The muscle invasive (T3) tumor analyzed contained a wild-type p53 sequence and had lost a chromosome 9 allele. The preneoplastic dysplasia (dys) sample obtained from the same patient maintained both chromosome 9 alleles but contained a p53 gene mutation.

Table 1 summarizes the molecular genetic defects found in 26 CIS and urothelial dysplasias in addition to 122 other tumors analyzed in this study, combined with results obtained previously (14, 16, 19, 21). Differences were observed in the frequencies of LOH of chromosome 9 and p53 mutations in CIS compared to other superficial (T1) carcinomas. Allelic losses of chromosome 9 were detected in 34% of low and high grade superficial papillary tumors. This was significantly higher than the 12% frequency observed in CIS and dysplasias (χ², 2-sided; P = 0.04). Although this analysis included three dysplasia specimens which can include a significant amount of contaminating normal urothelium cells which might alter LOH detection, we are confident of the microdissected tumor purity based on p53 mutation detection by SSCP and sequencing (Fig. 1b) or antibody staining for the p53 protein (data not shown). Loss of a chromosome 9 allele was observed in 59% of tumors invading the lamina propria and 58% of tumors invading muscularis propria. p53 mutations were observed in 65% of CIS and dysplasias, whereas only 1 such mutation was seen in 36 superficial papillary tumors examined. This difference in the frequency of mutations was highly significant (P < 0.001). The frequency of p53 mutations in CIS was comparable to that observed in invasive tumors. p53 mutations were detected in 32% of tumors which had invaded the lamina propria and in 51% of carcinomas which had invaded muscle.

The results obtained from the molecular analysis of tumors from selected patients with urothelial dysplasias or CIS are given in Table 2. Mutations in the p53 gene were present in the tumors of three of four evaluable patients who had CIS or dysplasia as the only tumor present in the bladder and had no previous history of bladder cancer. Although only one of the three mutations detected by SSCP was verified by direct DNA sequencing, our previous experience with >80 TCCs showed that SSCP shifts always corresponded to a mutation visible on a sequencing gel (21). Mutations in the p53 gene in these patients were present in most cases without detectable allelic losses on chromosomes 9 or 17, suggesting that such mutations may occur early in CIS establishment. Overall, allelic loss of chromosome 17 was observed in only 4 of 15 (27%) CIS and dysplasias. The presence of p53 mutations without 17p loss in many of these samples could reflect a transdominant mechanism of action of the mutant p53 protein.

One or more additional tumors which presented either simultaneously or concurrently with a CIS were available for analysis in nine patients (Table 2). The data for patients 7 through 10 who had multiple tumors were consistent with the second tumor having evolved from the CIS. The presence of identical point mutations in each of the two tumors of patients 8, 9, and 10, respectively, provided strong evidence that the tumors were clonally related. Furthermore, the subsequent allelic loss of chromosome 9 in the invasive tumors of patients 8 and 9, respectively, showed that the p53 mutation had occurred prior to 9 loss, supporting Nowell's hypothesis for the clonal evolution of tumors (4).

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>Loss of chromosome 9</th>
<th>p53 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 (Papillary tumors)</td>
<td>24/70 (34%)</td>
<td>1/36 (3%)</td>
</tr>
<tr>
<td>CIS + dysplasia (Flat tumors)</td>
<td>3/24 (12%)</td>
<td>15/23 (65%)</td>
</tr>
<tr>
<td>T1 (Invasive into lamina propria)</td>
<td>19/32 (59%)</td>
<td>9/24 (32%)</td>
</tr>
<tr>
<td>T2-T4 (Invasive into muscle)</td>
<td>45/83 (58%)</td>
<td>25/49 (51%)</td>
</tr>
</tbody>
</table>

* Table includes a compilation of previously reported data (Refs. 14, 16, 19, 21) in addition to the analysis of 148 new tumors.

Fig. 1. Loss of chromosome 9 allele and p53 gene mutation in multiple tumors obtained from patient 14. (a) Chromosome 9 alleles are detected by a PCR-based technique directed to dinucleotide repeat polymorphisms. The invasive (T3) tumor from patient 14, resected in 1983, has lost the upper allele which is present in the normal (N) and the dysplasia specimen (Dys) resected in 1988. (b) Autoradiogram of DNA sequencing gel showing a G→A mutation at codon 245 (arrow) in the dysplasia specimen which is not present in the normal tissue or T3 tumor.
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Table 2 Summary of molecular genetic defects in tumors of selected CIS patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumor stage</th>
<th>Year resected</th>
<th>Previous history</th>
<th>Chromosome loss</th>
<th>p53 Mutated codon</th>
<th>Possible progression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9</td>
<td>17p</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>CIS</td>
<td>1988</td>
<td>None</td>
<td>●</td>
<td>○</td>
<td>● WT Mut5</td>
</tr>
<tr>
<td>2</td>
<td>CIS</td>
<td>1988</td>
<td>None</td>
<td>○</td>
<td>○</td>
<td>● Mut6 193</td>
</tr>
<tr>
<td>3</td>
<td>CIS</td>
<td>1990</td>
<td>None</td>
<td>○</td>
<td>○</td>
<td>● Mut6 253</td>
</tr>
<tr>
<td>4</td>
<td>CIS</td>
<td>1990</td>
<td>None</td>
<td>○</td>
<td>○</td>
<td>● Mut7 257</td>
</tr>
<tr>
<td>5</td>
<td>CIS</td>
<td>1990</td>
<td>None</td>
<td>●</td>
<td>●</td>
<td>WT</td>
</tr>
<tr>
<td>6</td>
<td>Dys</td>
<td>1990</td>
<td>None</td>
<td>○</td>
<td>○</td>
<td>○ WT Mut8</td>
</tr>
</tbody>
</table>

Patients with secondary CIS (previous or concomitant tumor)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumor stage</th>
<th>Year resected</th>
<th>Previous history</th>
<th>Chromosome loss</th>
<th>p53 Mutated codon</th>
<th>Possible progression</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>CIS</td>
<td>1987</td>
<td>None</td>
<td>●</td>
<td>○</td>
<td>WT Mut7</td>
</tr>
<tr>
<td>8</td>
<td>CIS</td>
<td>1991</td>
<td>None</td>
<td>○</td>
<td>●</td>
<td>Mut6 193</td>
</tr>
<tr>
<td>9</td>
<td>CIS</td>
<td>1990</td>
<td>T2 tumors since 1978</td>
<td>●</td>
<td>○</td>
<td>Mut7 253</td>
</tr>
<tr>
<td>10</td>
<td>CIS</td>
<td>1989</td>
<td>T4 tumors since 1987</td>
<td>○</td>
<td>●</td>
<td>Mut7 257</td>
</tr>
<tr>
<td>11</td>
<td>T1</td>
<td>1990</td>
<td>None</td>
<td>●</td>
<td>●</td>
<td>WT</td>
</tr>
<tr>
<td>12</td>
<td>T1</td>
<td>1988</td>
<td>None</td>
<td>○</td>
<td>○</td>
<td>WT</td>
</tr>
<tr>
<td>13</td>
<td>T4</td>
<td>1987</td>
<td>T2 tumors since 1984</td>
<td>●</td>
<td>●</td>
<td>WT</td>
</tr>
<tr>
<td>14b</td>
<td>T3</td>
<td>1983</td>
<td>Invasive tumor in 1979</td>
<td>●</td>
<td>●</td>
<td>WT Mut5</td>
</tr>
<tr>
<td>15</td>
<td>Dys</td>
<td>1988</td>
<td>None</td>
<td>○</td>
<td>○</td>
<td>● Mut7 245</td>
</tr>
<tr>
<td>16</td>
<td>CIS</td>
<td>1990</td>
<td>None</td>
<td>●</td>
<td>●</td>
<td>Mut8 132</td>
</tr>
</tbody>
</table>

* The T2 and higher stage tumors were all grade III with the exception of case 13 which was grade II. When the dates at which the tumors were excised are the same, the tumor samples were obtained concurrently. Allelic loss is indicated by (●), retention of heterozygosity by (○); –, not informative or not done. Mutations in the indicated exons of the p53 gene were scored by SSCP analysis of exons 5–8; WT, wild-type or no mutation detected. The possible direction of progression is indicated by the arrows. Although it is not always clear that this pathway had been taken, it is clear that the reverse pathway could not have occurred. Dys, dysplasia.

Analysis of the data for the remaining five patients in Table 2 was inconsistent with the progression of the CIS to the secondary tumor. A number of possibilities exist which could explain the genetic relationships between these tumors. The CIS could have evolved from the secondary tumor diverging genetically from a precursor cell or cells which did not contain any of the three marker defects studied, or the two tumors may have arisen independently. The two tumors from patients 14 and 15 were particularly instructive in determining these relationships. The invasive tumor in patient 14 showed allelic loss of chromosome 9 whereas the dysplasia specimen, resected 5 years later, had no such loss (Fig. 1a). Since losses of chromosomal material are irreversible, the dysplasia could not have evolved from the invasive tumor. Similarly, the presence of a mutation at codon 245 of the p53 gene in the dysplasia but not the invasive tumor (Fig. 1b) makes it highly unlikely that the dysplasia was the precursor of the invasive tumor. Since the two tumors were resected 5 years apart, the result might be indicative of two separate transforming events. The two tumors in patient 15 contained unique p53 mutations, indicating that they had either diverged after the transformation of a single precursor cell or were unrelated to one another arising from two independent transforming events.

DISCUSSION

Current concepts for the molecular basis of tumor progression rely heavily on the well-studied models of colorectal (6) and astrocytoma (7) tumorigenesis in which genetic defects are acquired in a relatively linear, cumulative fashion. It is generally assumed that invasive carcinomas arise from the progression of noninvasive adenomas in colorectal tumorigenesis and that p53 gene mutations most often occur after other alterations, such as APC and K-ras mutations and allelic loss of chromosomes 5q and 18q (33). Our findings with bladder cancer clearly do not fit this model since two distinct molecular pathways seem to exist for the formation of noninvasive bladder cancers (Fig. 2). The timing and ordering of the defects may therefore determine the phenotypes and progressive potentials of the tumors. p53 gene inactivation appears to occur early in CIS and dysplasia tumorigenesis (right side pathway, Fig. 2). This is evident in that p53 mutations were found in CIS lesions obtained from patients with no previous history of bladder cancer, and many of these had no detectable loss of either chromosome 9 or 17. Conversely, alterations involving chromosome 9, the proposed location of an unidentified bladder tumor suppressor gene(s), may be sufficient for superficial papillary growth in tumors (left side pathway, Fig. 2).

Progression of either type of superficial tumor to invasion may require the subsequent acquisition of either the chromosome 9 or p53 defect, respectively, as was evident in patients 8 and 9. The CIS tumors in these patients harbored p53 gene mutations, but neither had lost chromosome 9, whereas the invasive tumors each had lost a chromosome 9 allele and contained the identical p53 point mutation present in the respective CIS. The invasive tumor in each case, therefore, most probably resulted from the spreading of a transformed CIS cell(s) to another location in the bladder. The acquisition of the chromosome 9 defect in this transplanted cell population following clonal expansion and possibly other genetic alterations then resulted in an invasive tumor (bottom, right side pathway, Fig. 2). Analysis of these patients unequivocally demonstrated molecular progression and suggested that CIS was the precursor of the invasive tumors. In addition, these data suggest that the chromosome 9 defect is likely involved in both superficial papillary tumor initiation and progression of CIS to invasive tumors. The timing of p53 inactivation in the rare progression of superficial (T3) tumors to lamina propria invasive (T1) and muscle invasive (T2–T4) tumors was not investigated in the present study. The
Fig. 2. Proposed model for TCC progression based on a summary of chromosome 9
differences in the molecular defects observed in the two types
of superficial tumors, as well as in multiple tumors isolated from
individual patients, support this model for a divergent molecular pro-
gression in bladder tumorigenesis. The CIS or dysplasia and invasive
tumors isolated from patients 14 and 15 had distinctly different ge-
netic alterations. These tumors arose either from a precursor neoplas-

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