Molecular Evidence Supporting Field Effect in Urothelial Carcinogenesis

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Abstract  Purpose:  Human urothelial carcinoma is thought to arise from a field change that affects the entire urothelium. Multifocality of urothelial carcinoma is a common finding at endoscopy and surgery. Whether these coexisting tumors arise independently or are derived from the same tumor clone is uncertain. Molecular analysis of microsatellite alterations and X-chromosome inactivation status in the cells from each coexisting tumor may further our understanding of urothelial carcinogenesis.

Experimental Design:  We examined 58 tumors from 21 patients who underwent surgical excision for urothelial carcinoma. All patients had multiple separate foci of urothelial carcinoma (two to four) within the urinary tract. Genomic DNA samples were prepared from formalin-fixed, paraffin-embedded tissue sections using laser-capture microdissection. Loss of heterozygosity (LOH) assays for three microsatellite polymorphic markers on chromosome 9p21 (IFNA and D9S1771), regions of putative tumor suppressor gene p16, and on chromosome 17p13 (TP53), the p53 tumor suppressor gene locus, were done. X-chromosome inactivation analysis was done on the urothelial tumors from 11 female patients.

Results:  Seventeen of 21 (81%) cases showed allelic loss in one or more of the urothelial tumors in at least one of the three polymorphic markers analyzed. Concordant allelic loss patterns between each coexisting urothelial tumor were seen in only 3 of 21 (14%) cases. A concordant pattern of nonrandom X-chromosome inactivation in the multiple coexisting urothelial tumors was seen in only 3 of 11 female patients; of these 3 cases, only one displayed an identical allelic loss pattern in all of the tumors on LOH analysis.

Conclusion:  LOH and X-chromosome inactivation assays show that the coexisting tumors in many cases of multifocal urothelial carcinoma have a unique clonal origin and arise from independently transformed progenitor urothelial cells, supporting the “field effect” theory for urothelial carcinogenesis.

Urothelial carcinoma is the most common malignancy of the urinary bladder, ureter, and renal pelvis with ~63,000 new cases occurring each year in the United States and accounting for nearly 13,000 cancer-related deaths each year (1). The development of multifocal tumors in either a synchronous or metachronous manner in the same patient is a common characteristic of this type of malignancy (2–5). The multiple coexisting tumors have often arisen before clinical symptoms become apparent and the separate tumors may or may not share a similar histology (3). Two theories have been proposed to explain the frequent occurrence of urothelial tumor multifocality. One theory suggests that the multiple tumors are of monoclonal origin, arising from a single malignant transformed cell which proliferates and spreads throughout the urothelium either by intraluminal spread with secondary implantation at different sites within the urinary tract or by intraepithelial migration. The second theory explains tumor multifocality as developing secondary to a field-cancerization effect precipitated by carcinogens causing independent genetic alterations at different sites within the urothelial lining and leading to the development of multiple, genetically unrelated tumors.

The issue of monoclonal versus oligoclonal origin of multifocal urothelial carcinomas is clinically important because an understanding of patterns of early tumor development must be considered in the development of appropriate treatment and surgical strategies and in the genetic detection of recurrent or residual tumor cells in posttreatment urine samples (6–10). There continues to be no consensus on which of the theories is most important in the development of multifocal urothelial carcinoma. Whereas many studies have suggested a monoclonal origin for multifocal urothelial carcinoma (9, 11–21), other studies have clearly shown an independent origin for some multicentric urothelial tumors (13, 15, 19, 20, 22–28). In this study, molecular analysis of microsatellite alterations...
and X-chromosome inactivation status in separate urothelial carcinomas from the same patient was used to assess the molecular genetic relationships among the multiple coexisting tumors.

Materials and Methods

Patients. Eleven women and 10 men with multifocal urothelial carcinomas of the urinary tract underwent surgical excision of their tumors from 1993 to 2003. The patients had a mean age of 69 years (range, 44-86 years). All patients had two or more urothelial carcinomas. The 2004 WHO bladder tumor classification criteria were used for grading (29). The tumors were classified as high-grade in 15 patients and low-grade in 6 patients. Pathologic staging was done according to the 2002 tumor-lymph node-metastasis classification system (30). Six patients had stage pT1 lesions; seven patients had pT2 lesions; four patients had pT3 lesions; and four patients had pT4 lesions. The mean diameter of the largest tumor was 2.6 cm (median, 2.0 cm; range, 1.2-6.0 cm).

Tissue samples and microdissection. Archival surgical materials from 21 patients with urothelial carcinoma of the urinary bladder (11 female patients and 10 male patients) having two or more separate tumors within the urinary tract accessioned from 1993 to 2003 were retrieved from the surgical pathology files of the Department of Pathology and Laboratory Medicine of the Indiana University School of Medicine (Indianapolis, IN), the Department of Pathology of Case Western Reserve University (Cleveland, OH), and the Department of Pathology of Corboda University (Corboda, Spain). All multifocal tumors were at least 1 cm apart in the urinary tract. This study included a total of 58 separate urothelial carcinomas, including 37 tumors of the urinary bladder, 12 tumors of the ureter, and 9 tumors from the renal pelvis. Eight patients had multifocal urothelial carcinomas only in the urinary bladder; nine patients had multifocal tumors involving both the urinary bladder and the upper urinary tract (ureter and/or renal pelvis); and four patients had multifocal tumors only in the upper urinary tract.

Histologic sections were prepared from formalin-fixed, paraffin-embedded tissue and were stained with H&E for microscopic evaluation. Laser-assisted microdissection of the separate tumors was done (Fig. 1) using a PixCell II Laser Capture Microdissection system (Arcturus Engineering, Mountain View, CA), as previously described (31–33). Approximately 400 to 1,000 cells of each tumor were microdissected from the 5-μm histologic sections. Normal tissue from each case was microdissected as a control.

Detection of loss of heterozygosity. The dissected cells were deparaffinized with xylene and ethanol alcohol. PCR was used to amplify genomic DNA at three specific loci on two different chromosomes: 9p21-22 (D9S171 and IFNA) and 17p13 (TP53). Previous studies have shown that loss of heterozygosity (LOH) at these loci occurs frequently in urothelial carcinoma (11, 34). D9S171 and IFNA include region of the putative tumor suppressor gene p16. There is no significant difference in the frequency of chromosome 9 alterations between upper and lower tract tumors (20). Alterations of chromosome 9 are one of the earliest and most frequent events in papillary urothelial carcinogenesis (23, 35). The TP53 locus corresponds to the gene encoding the p53 protein. Mutations of the p53 gene are the most common genetic abnormalities in cancer (36). PCR amplification and gel electrophoresis were done as previously described (32, 33, 37). The criterion for allelic loss was complete or nearly complete absence of one allele in tumor DNA (32, 33, 37–43). PCR products were separated by electrophoresis at 80 W for 2 hours. The bands were visualized after autoradiography with Kodak X-OMAT film (Eastman Kodak Company, Rochester, NY) for 8 to 16 hours.

Analysis of X-chromosome inactivation. The cases were considered to be informative if two AR alleles were detected after PCR amplification in normal control samples that had not been treated with HhaI. Only informative cases (i.e., those without a skewed pattern of X-chromosome inactivation after being treated with HhaI in normal control samples) were included in the analysis. In tumor samples, nonrandom X-chromosome inactivation was defined as a complete or a nearly complete absence of an AR allele after HhaI digestion, which indicated a predominance of one allele. Tumors were considered to be of the same clonal origin if the same AR allelic inactivation pattern was detected in each separate tumor. Tumors were considered to be of independent origin if alternate predominance of AR alleles after HhaI digestion (different allelic inactivation patterns) was detected in each tumor (Fig. 2; ref. 41).

Results

Seventeen of 21 (81%) cases of multifocal urothelial carcinomas showed allelic loss in one or more of the separate tumors (Table 1). The number of specific loci lost in a single tumor ranged from one to two with none of the tumors showing LOH at all three loci. The frequency of allelic loss in all of the informative cases of urothelial carcinoma was 33% (19 of 58) with D9S171, 25% (13 of 51) with IFNA, and 33% (19 of 58) with TP53. The frequency of allelic loss in the informative cases of urothelial carcinoma of the urinary bladder was 30%
Among the 21 patients in our study, eight (cases 1-8) had multifocal urothelial carcinomas only in the urinary bladder without involvement of the upper urinary tract (ureter and/or renal pelvis). Of these eight patients, five had different allelic loss patterns among the multifocal tumors, suggestive of independent clonal origin. X-chromosome inactivation analysis was done on two of the five cases with a variable LOH pattern and showed a random pattern of X-chromosome inactivation in tumors of both cases. One case (case 3) had an identical LOH pattern in each tumor. Two cases (cases 5 and 8) did not show LOH at any of the loci examined.

Among the 21 patients in our study, nine (cases 9-17) had coexisting tumors of the upper urinary tract and the urinary bladder. Of these nine patients, five had different allelic loss patterns among the multifocal tumors, with four of five having tumors with a random pattern of X-chromosome inactivation and one of five having tumors with a concordant pattern of nonrandom X-chromosome inactivation. Two cases (cases 12 and 17) had an identical LOH pattern at the three loci examined and a concordant pattern of nonrandom X-chromosome inactivation, consistent with a monoclonal origin. Two cases (cases 9 and 16) showed no LOH at any of the loci examined. X-chromosome inactivation analysis was done on case 9 and noninformative results were obtained.

(11 of 37) with D9S171, 27% (8 of 30) with IFNA, and 41% (15 of 37) with TP53. The frequency of allelic loss in the informative cases of urothelial carcinoma of the upper urinary tract (ureter and renal pelvis) was 38% (8 of 21) with D9S171, 24% (5 of 21) with IFNA, and 19% (4 of 21) with TP53.

The allelic loss patterns at the three loci examined were variable among many of the multifocal urothelial carcinomas that were analyzed, consistent with independent origin. In 5 of 21 patients, the allelic loss pattern was different among all of the coexisting tumors, suggestive of independent origin. Eleven of 21 patients had two or more separate tumors with identical allelic loss patterns, suggestive of monoclonal origin; however, 8 of these 11 patients had additional coexisting urothelial carcinomas displaying an alternate LOH pattern. Four of 21 patients did not have LOH at any of the three loci examined in any of the coexisting tumors.

The coexisting tumors from the 11 female patients were examined by X-chromosome inactivation analysis. One case yielded noninformative results. A discordant or random pattern of X-chromosome inactivation was seen in 7 of 10 informative cases. A concordant pattern of nonrandom X-chromosome inactivation in the multiple coexisting urothelial tumors was seen in 3 of 10 informative cases (cases 12, 15, and 18). Of these three cases, only one (case 12) displayed an identical allelic loss pattern in all of the tumors on LOH analysis.
Among the 21 patients in our study, four (cases 18-21) had multifocal urothelial carcinomas only in the upper urinary tract without involvement of the urinary bladder. All of these four patients had different allelic loss patterns among the multifocal tumors, suggestive of independent clonal origin. X-chromosome inactivation analysis was done on two of the four cases. One case (case 19) showed a discordant pattern of nonrandom X-chromosome inactivation, consistent with an independent, oligoclonal origin for these multifocal tumors (Fig. 3). One case (case 18) showed a concordant pattern of nonrandom X-chromosome inactivation.

Discussion

Urothelial carcinomas are frequently multicentric, occurring at multiple separate sites within the urinary tract (4, 5). Urothelial tumor multifocality is often present before the onset of clinical symptoms (3), and patients with multiple, separate tumors are at higher risk for recurrence, progression, and cancer-related death (4, 5, 44). Although numerous studies have been done to address the clonal nature of multifocal urothelial carcinoma, no consensus currently exists on whether these lesions are monoclonal in origin or whether they arise independently. Two explanations have been proposed to explain tumor multifocality within the urothelium: the monoclonal theory and the "field-effect" theory. Whereas the two theories are not mutually exclusive, it is unknown which mechanism is more important in leading to urothelial tumor multifocality. Detailed characterization and comparison of genetic alterations in the cells of separate tumors may provide information about the clonal evolution of multifocal cancer. LOH has been shown at various chromosomal loci in urothelial carcinomas. The chromosomal regions where LOH has been detected are thought to contain specific genes, the disruption of which leads to either neoplastic transformation or progression. In this study, we examined 21 patients with two or more coexisting urothelial carcinomas \( (n = 58) \), using LOH analysis and X-chromosome inactivation analysis, to assess tumor clonality. We found evidence for the independent origin of multifocal urothelial carcinomas in the majority of patients with multifocal urothelial carcinomas.

The monoclonal theory suggests that all of the coexisting tumors are derived from a single progenitor cell that has undergone malignant transformation. Multifocality then arises secondary to either intraluminal shedding of tumor cells with secondary implantation at different urothelial sites or secondary to intraepithelial migration (45). The possible role of intraluminal spread of tumor cells in tumor multifocality is supported by the finding that later recurrences often occur downstream from the site of the original tumor. Patients with...
upper urinary tract tumors frequently develop future tumors in the urinary bladder; however, patients with urinary bladder tumors are less likely to develop future tumors in the upper urinary tract (46, 47). In addition, urothelial carcinoma cells in animal models have been shown to be capable of adhering to urothelium and subsequently growing into tumors, especially when the urothelium has been previously injured (48–50). Tumor cells may be implanted in other urothelial sites secondary to cystoscopic manipulation (51, 52). The field-cancerization theory, which has been shown to be important in the development of multicentric squamous cell carcinomas of the head and neck (53–55), suggests that multifocal urothelial carcinomas arise secondary to numerous independent mutational events at different sites within the urothelial tract as a consequence of external cancer-causing influences. In support of the field effect theory is the frequent finding in patients with bladder cancer of genetic instability in normal-appearing bladder mucosa (56) and of abnormalities in the surrounding urothelium, such as dysplasia or carcinoma in situ (57). In addition, the application of the carcinogen N-butyl-N-(4-hydroxybutyl)nitrosamine to the bladders of chimeric C3H/HeN-BALB/c mice resulted in the formation of multiple urothelial tumors of oligoclonal origin in 30% of cases (58). Determining the molecular mechanisms responsible for tumor multifocality may further our understanding of urothelial carcinogenesis.

Whether multifocal urothelial carcinomas are clonally related is of clinical importance. Because tumor multifocality is associated with poorer outcomes in patients with urothelial carcinoma (4, 5, 44), an understanding of how tumor multifocality arises may aid in assessing prognosis in these patients. The detection of genetic alterations in urine samples has been

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Table 1. LOH and X-chromosome inactivation analysis of multifocal urothelial carcinomas

NOTE: ▲, both alleles present; ▼, loss of lower allele; ▼, loss of upper allele.

Abbreviations: UC, urothelial carcinoma; NI, noninformative.
used in patients for the diagnosis and follow-up of urothelial tumors (6–9). If multifocal tumors are of a common clonal origin, molecular genetic findings specific to a patient’s urothelial carcinoma could be used to identify recurrent, residual, or multifocal disease. However, multiple independent tumor clones secondary to field-cancerization might not be detected using the same molecular diagnostic techniques. In addition, determining the clonal origins of multifocal urothelial carcinomas may affect the development of new therapeutic options, such as gene therapy, which might be more effective against a monoclonal population of tumors than against multifocal tumors that arise independently and that are genetically unrelated (10).

A variety of specific molecular genetic alterations have been reported in urothelial carcinomas. Most low-grade, noninvasive papillary urothelial carcinomas and some high-grade lesions are characterized by the loss of chromosome 9 (23, 59, 60). By contrast, p53 gene mutations and loss of chromosome 17p are primarily seen in high-grade, invasive urothelial carcinomas (23, 61, 62). A model of urothelial carcinogenesis has been proposed in which loss of chromosome 9 is the initial step leading to a low-grade papillary lesion with invasion and progression characterized by later mutations of the p53 gene. In urothelial carcinoma in situ, p53 gene mutations occur earlier (23, 35). Because of the frequent genetic alterations of chromosomes 9 and 17 in urothelial carcinomas, we chose microsatellite markers on these chromosomes for our clonality analysis. A number of molecular diagnostic techniques, including LOH analysis, X-chromosome inactivation analysis, mutation analysis, comparative genomic hybridization, fluorescence in situ hybridization, and classic cytogenetics, have been employed for clonality studies of multicentric urothelial carcinomas. Although the majority of previous clonality studies have shown multifocal urothelial carcinomas to be monoclonal in origin (9, 11–21), with a minority of the multifocal tumors being oligoclonal (20), no consensus currently exists about which mechanism for multifocality is clinically and biologically most important. Other studies have emphasized the role of oligoclonality and field cancerization in the development of multifocal urothelial tumors (20, 22–28), especially in early-stage disease.

The two theories proposed to explain urothelial tumor multifocality are not mutually exclusive. Indeed, it has been suggested that oligoclonality is more common in early lesions with progression to higher stages leading to the overgrowth of one clone and pseudomonoclonality (20, 45). Thus, early or preneoplastic lesions may arise independently with a specific clone undergoing malignant transformation, which subsequently spreads through the urothelium by either an intraluminal or intraepithelial mechanism producing multifocal tumors of monoclonal origin. Our data suggest this notion in that the case with the most convincing evidence for oligoclonality (case 19) is made up of three low-grade, stage pT1 tumors. We did, however, find evidence for oligoclonality in many patients with higher-stage lesions as well, which is likely due to more than one population of cells undergoing malignant transformation without dominance and overgrowth of a single clone. In our study, many patients (8 of 21) had two tumors with identical allelic loss patterns and a third or fourth tumor with a different LOH pattern, providing evidence that both the monoclonal and field effect theories may both be of importance in bladder carcinogenesis.

Whereas most of the previous studies have focused only on multifocal urothelial carcinomas of the urinary bladder, the current study also examines tumors of the upper urinary tract, including the ureter (n = 12) and renal pelvis (n = 9). Two previous studies, by Takahashi et al. (15) and Hafner et al. (20), examined simultaneous and consecutive urothelial carcinomas of the bladder and the upper urinary tract and found a higher frequency of multiple tumor clones, consistent with the field-cancerization effect (63). In this study, we also found evidence for independent origin of tumors among the nine patients in our study with coexisting urothelial carcinomas of the urinary bladder and upper urinary tract. However, a similar proportion of patients with multifocal disease, limited either to the urinary bladder or to the upper urinary tract, yielded data suggestive of oligoclonal tumor origin as well.

The most consistently informative marker of the clonal composition of neoplastic disorders in females is the nonrandom pattern of X-chromosome inactivation (41, 64, 65). In our study, our results are complicated by the frequent finding of random X-chromosome inactivation patterns in many cases.
Several studies have shown that random X-chromosome inactivation may be observed in up to 50% of invasive cancers (66–70). For example, in a study by Buller et al. (66), nonrandom X-chromosome inactivation was observed in only 53% of patients with invasive ovarian cancer. There are a number of possible explanations for the persistence of biallelic bands after digestion by a methylation-sensitive restriction enzyme in tumor samples (clonal cell populations). These include (a) incomplete digestion of DNA samples prepared from formaldehyde-fixed, paraffin-embedded tissues; (b) contamination of normal tissues; (c) the presence of X chromosome aneuploidy; (d) reactivation of inactive X-chromosome–linked genes (71, 72); (e) variable methylation patterns at the CpG sites of the AR locus; and (f) the coexistence of multiple tumor subclones of independent origins (40). Indeed, divergent tumor subclones have been previously shown to exist in different regions of a single urothelial carcinoma in seven of nine informative cases (42).

As mentioned previously, we cannot entirely exclude monoclonality based solely on the LOH data in some cases of multifocal urothelial carcinoma. Indeed, results consistent with a monoclonal origin of multiple tumors were seen in at least one case on both LOH analysis and X-chromosome inactivation analysis (case 12). Urothelial carcinomas have been shown to be morphologically and genetically heterogeneous with various subpopulations displaying unique genetic alterations (42, 73). It is possible that one tumor resulted from a clonal metastasis from a specific, unsampled subpopulation of tumor cells within another tumor. Alternatively, different allelic loss patterns among multifocal tumors may represent clonal divergence after intraluminal spread rather than true oligoclonality. For example, because p53 gene mutations are a late event in papillary urothelial neoplasms, cases of multifocal papillary urothelial carcinoma in which the multiple tumors displayed different LOH patterns at only the TP53 locus (cases 13 and 14) may be monoclonal in origin with subsequent genetic alterations accumulating after intraluminal seeding.

In conclusion, our data suggest that in some cases of multifocal urothelial carcinoma, each tumor may arise independently, consistent with the field-cancerization theory for multicentric urothelial carcinogenesis. We find evidence supporting an oligoclonal origin for multifocal urothelial carcinomas in the majority of cases (14 of 21). This finding is clinically important as an understanding of early tumor development and spread must be considered in the development of appropriate treatment and surgical strategies and when molecular diagnostic techniques are used in the detection of recurrent or residual disease. Whereas tumor multifocality seems to be an oligoclonal phenomenon in the majority of our cases, we do find support for the monoclonal hypothesis in some cases. In addition, our data suggest that both field-cancerization and monoclonal tumor spread may coexist in the same patient.

References

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