Partial Allelotype of Carcinoma in Situ of the Human Bladder

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Abstract

Carcinoma in situ (CIS) of the urinary bladder is an aggressive lesion that frequently progresses to an invasive tumor, yet the underlying molecular changes in this lesion are largely unknown. In this study, we microdissected 31 cases of CIS and examined them for loss of heterozygosity (LOH) on 13 chromosomal arms. Twenty-nine microsatellite markers were chosen for this analysis based on their location in regions previously shown to be frequently lost in primary transitional cell carcinoma of the bladder. LOH of chromosome 9 was a frequent event in these samples, occurring in 77% of these lesions, with 19 of 31 cases showing deletion on the 9p arm (61%) and 17 of 28 cases displaying LOH on 9q (61%). Fine mapping at 9p21 demonstrated that CIS also displayed a high frequency of homozygous deletion surrounding the p16INK4A locus, like superficial papillary tumors, the other form of noninvasive lesion found in the bladder. However, loss of 14q (70%) was frequent in CIS yet extremely rare in papillary lesions (9%). Other chromosomal arms showing frequent LOH included 8p (65%), 17p (60%), 13q (56%), 11p (54%), and 4q (52%), whereas slightly lower frequencies of loss were observed for 1q (36%), 4p (32%), 3p (31%), 18q (29%), and 5q (20%). CIS lesions already possess many of the genetic alterations displayed by invasive transitional cell carcinomas, potentially accounting for the aggressive nature of these lesions.

Introduction

Although the occurrence of bladder cancer is common in the Western world, relatively few studies have addressed the molecular changes underlying progression of this tumor (1–4). Current models of carcinogenesis propose that tumors develop through progressive stages from noninvasive lesions to invasive and metastatic tumors (5, 6). Underlying this clinicopathological progression is the accumulation of multiple genetic changes in oncogenes and tumor suppressor genes. A number of specific genes and chromosomal loci have been implicated in the development of invasive TCCs by molecular and cytogenetic techniques (4, 7–13). However, little is known of the frequency of occurrence of many of these genetic changes in earlier, noninvasive lesions in the bladder.

The natural history of development of invasive TCC is as yet ill-defined. Clinical data suggest that these tumors may develop by different molecular pathways, arising de novo, or originating from either superficial papillary tumors (T tumors) or CIS (14, 15). Several observations suggest that the precursor of many solid, invasive tumors may be the CIS lesion. The noninvasive papillary transitional cell carcinoma most often occurs as a low-grade tumor that typically has multiple superficial recurrences. A minority of patients (10–20%) develop invasive disease. In contrast, CIS are high-grade lesions that follow a more aggressive course, with invasive disease developing in 60% of cases within 5 years and 80% of cases within 10 years of presentation (16). Furthermore, most cases of invasive TCC do not have a history of papillary carcinoma (17). Finally, patients with a noninvasive papillary carcinoma have a higher risk of developing invasive disease when there is associated urothelial atypia or CIS (18).

A recent article by Spruck et al. (1) suggests that specific molecular defects in papillary TCC and CIS lesions may be responsible for the different growth and behavioral patterns of these two lesions. They report that LOH of chromosome 9 is an early event in the generation of papillary TCC but not in CIS. In contrast, allelic losses of chromosome 17p and p53 mutations were infrequent occurrences in papillary lesions but were commonly seen in CIS and invasive tumors.

To further examine the genetic alterations in bladder CIS, we microdissected 31 lesions and screened for allelic loss on 13 chromosomal arms using 29 microsatellite markers. These arms contained regions that frequently showed LOH in studies of bladder TCCs. These areas of loss are thought to target critical tumor suppressor genes since the loss can unmask a “recessive” mutant allele. We were aided in the choice of these markers by recent fine mapping studies for several of the chromosomal arms (4p, 4q, 8p, 9p, 9q, and 14q) that localized the region of loss in TCC (19–22). Our data show that CIS lesions display high levels of chromosomal loss for each of the chromosomal arms assayed, including 9p and 9q. In most cases, LOH frequencies are similar to those previously reported for invasive TCCs, but differ at critical loci when compared to less aggressive papillary tumors.

Materials and Methods

Tissue Dissection and DNA Extraction. The study included 31 archival cases of CIS from patients diagnosed at the Johns Hopkins Hospital between 1990 and 1995. Eighteen of the cases were radical cystectomies. Nineteen patients had TCC present concurrent with the CIS, although not on the tissue block being dissected. DNA was isolated from the paraffin-embedded specimens by microdissecting areas identified as CIS from serial hematoxylin and eosin-stained sections. In all cases, the normal genomic DNA was obtained by dissecting stroma underlying the urothelium.

The dissected tissues were placed in a buffered solution containing SDS proteinase K at 48°C and was spiked twice a day for 72 h with fresh proteinase K. The samples were then extracted twice with saturated phenol-chloroform and the DNA precipitated with ethanol.

Allelotyping. DNA was analyzed for LOH by using the microsatellite markers from Research Genetics (Huntsville, AL) listed in Table 1. LOH at the

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3 The abbreviations used are: CIS, carcinoma in situ; LOH, loss of heterozygosity; TCC, transitional cell carcinoma; HPNCC, hereditary nonpolyposis colon cancer.

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p53 locus was detected by using the dinucleotide repeat polymorphism previously described by Jones and Nakamura (23). In addition, we used four highly informative microsatellites (D9S1747, D9S1748, D9S1751, and D9S1752) to fine map the 9p21 region in these CIS. These markers were cloned in this laboratory from a P1 contig of a critical region surrounding p16NK4A and extending from IFN-α to p15 (19).

Prior to amplification, T4 polynucleotide kinase (New England BioLabs) was used to end label 100 ng of one primer from each microsatellite with [γ-32P]ATP (20 mCi; Amersham). PCR amplifications of DNA from normal and CIS tissue were performed as previously described (24). The PCR reaction mixtures were subjected to 35 cycles of amplification consisting of denaturation for 30 s at 95°C, annealing for 60 s at 50–58°C, and extension for 60 s at 70°C, with a final extension for 5 min at 70°C (4). The PCR products were separated on 7% urea-formamide-polyacrylamide gels and visualized by autoradiography, as described previously (25). For informative cases, allelic loss was scored when the signal intensity of one allele was at least 50% decreased from the signal intensity of the corresponding allele.

Results

To investigate the frequency of LOH on chromosome 9, DNA samples from 31 CIS lesions were analyzed with seven microsatellite markers on the p arm and 3 on the q arm. The 9p markers were localized to 9p21, in a critical region of loss flanked by IFN-α and D9S171, and included four new dinucleotide repeat polymorphisms that had been isolated from a P1 contig within this region (19). The markers chosen for the q arm spanned the q arm. Representative results are shown in Fig. 1. Allelic data are presented in Table 1.

LOH on chromosome 9 was a frequent occurrence in the CIS samples. Twenty-four (77%) of the 31 CIS specimens showed partial or entire deletion of chromosome 9. In total, 19 (61%) of these 24 samples demonstrated deletions on the p arm and 17 (61%) displayed LOH on the q arm. Five lesions showed LOH at all informative loci, indicating probable loss of an entire chromosome 9 (monosomy). In the remaining 19 cases, LOH was present at some but not all informative loci, indicating selective hemizygous and/or homozygous deletion on this chromosome including 7 tumors with LOH confined to 9p and 5 cases to the q arm.

The use of the four new microsatellite markers in this analysis allowed us to fine map the 9p21 region and identify very small changes. Homozygous microdeletions at 9p21 were present in 9 (47%) of the 19 CIS specimens with 9p21 LOH. Homozygous deletion was identified by apparent retention of heterozygosity in closely spaced markers that are flanked by a large region of LOH. The apparent retention is attributed to the amplification of normal cells by fluorescence in situ hybridization analysis previously (19). The highest frequency of homozygous deletion occurred with markers D9S1747 and D9S1748, the closest flanking distal and proximal markers to p16, respectively. Taken together these data suggest a high rate of microdeletion (homozygous and hemizygous) in a critical region surrounding p16 in bladder CIS lesions.

We then examined the frequency of LOH on other chromosome arms. Since we had a limited amount of DNA from these samples, we chose those chromosomal arms and markers previously reported to show a high frequency of LOH in studies of bladder TCC. The analysis included 19 microsatellite markers representing 11 chromosomal arms. Table 1 lists the primers used and indicates the number of informative cases and the percentage of cases showing LOH for each chromosomal arm. In all cases, at least 70% informativity was obtained for each arm assayed.

A wide range was observed in these CIS samples for the number of chromosomal arms showing LOH. For example, one CIS retained the entire chromosome 9, while another CIS retained only a small portion of it.
heterozygosity for all informative markers (although it was informative for only one locus, D9S736, on 9p), whereas 3 lesions displayed LOH of loci on 11 of the 13 arms tested, including 9p and 9q. The average number of arms showing LOH in the CIS samples was six. The most frequent regions of loss occurred on chromosome arms 14q (70%), 8p (65%), and 17p (60%), with a high level of loss also observed for loci on 13q (56%), 11p (54%), and 4q (52%). Slightly lower frequencies of LOH occurred for 11q (36%), 4p (32%), 3p (31%), 18q (29%), and 5q (20%).

During this study, it also became apparent that microsatellite alterations occurred at a low rate in some CIS lesions. These samples demonstrated additional bands or "shifts" on the polyacrylamide gel, most likely due to addition or deletion of DNA within the repetitive DNA sequences. Six (19%) of the CIS lesions displayed alterations at more than one of the 29 markers assayed with 4 (13%) of these samples displaying alterations in at least 3 markers.

Discussion

One of the most frequent genetic changes identified in bladder TCCs is LOH on chromosome 9. Evidence suggests that this is an early event, since it is present in tumors of all grades and stages, occurring in approximately 70% of bladder tumors tested (8, 9). Thus, the recent report of a reduced level of LOH for chromosome 9 in CIS compared to papillary or invasive TCC was potentially significant (1). However, the data presented in this article do not support this difference in frequency. LOH on chromosome 9 occurred in the majority of the CIS lesions, with both arms being deleted with a similar frequency. This discrepancy may be partially explained by the use in our study of a large number of markers within the critical region of loss on 9p, thus allowing us to detect very small homozygous and heterozygous lesions. Of interest is the observation that 47% of the CIS lesions had homozygous deletion for loci within the 9p21 region. This is the first report of such homozygous deletions in CIS. Cairns et al. (19) have recently shown that homozygous deletions represent the predominant mechanism of inactivation at 9p21 in invasive bladder tumors, occurring in 126 of 178 (71%) tumors with LOH in this region. The putative tumor suppressor gene p19(AR) (CDKN2/MTS-1) that encodes an inhibitor of cyclin-dependent kinases is contained within the deleted region in many tumors (19).

Our data do support the observation of Spruck et al. (1) that LOH at 17p occurs at a higher frequency in CIS than rates reported for papillary lesions. Sixty percent of the CIS samples in this study displayed deletion on 17p. This frequency is similar to that observed for bladder tumors of high grade or stage (9, 26). The most likely target for this LOH is the suppressor gene p53. Spruck et al. (1) reported that 65% of CIS had a p53 mutation compared with 3% of superficial papillary tumors and 51% of carcinomas that had invaded muscle. Since p53 is involved in checkpoint control after DNA damage to a cell (27, 28), alterations in p53 in CIS could lead to the accumulation of further genetic changes and the progression to invasive TCC.

In addition to this high frequency of LOH on 17p, the CIS lesions have accumulated multiple, complex genetic alterations. As a group these lesions show frequencies of loss on many chromosomal arms that are similar to those reported for high-grade (grade 3) and/or invasive TCCs (17; Refs. 2, 9, 20, 22, 26, 29–31).4 Although most of the suppressor genes that are being targeted by these LOH are unknown, 13q LOH is involved in the loss of the retinoblastoma gene (Rb1), an event that has been reported to occur in 56% of muscle-invasive samples (11). Allelic loss at 18q has been reported to occur in 33% of TCCs, more often in association with an advanced stage of tumor development (31). Another chromosomal arm of interest is 8p.

Knowles et al. (22) have recently reported a deletion mapping study of chromosome 8 that suggests the presence of a suppressor gene(s) for urothelial cancer within a region defined by the loci NEFL and PIAT (8p21–q11.2). Only 11% of superficial papillary tumors showed chromosome 8 LOH compared to 53% of grade 3 muscle-invasive tumors.

The data show that CIS are heterogeneous in their genetic makeup, showing a wide variation in the pattern of allelic loss. However, LOH at 14q is common in CIS, present in 70% of these lesions. Although we have identified two regions of loss on 14q, 18 of the 21 CIS lesions (3 were noninformative) with 14q loss targeted the distal region of 14q32.1 bordered by D14S51 and D14S267 (20). Moreover, these lesions often excluded the proximal region of 14q13, suggesting that a putative tumor suppressor gene in this distal region is critical for the progression of CIS lesions. 14q LOH occurred only in 9% of Ta tumors and was significantly associated with progression, occurring in 41% of invasive TCCs (17; Ref. 20). However, distal 14q loss occurred in only 15–20% of T1 lesions. These data suggest that CIS lesions are not necessarily the precursor to Ta or invasive tumors, although some CIS lesions may progress by such a pathway. Although it is not clear what tumor suppressor gene resides on 14q, the high frequency of 14q loss (higher than in invasive lesions) suggests it may be an important prerequisite for development of CIS lesions.

Also of interest in this study is the presence of microsatellite alterations in some CIS. Widespread genomic microsatellite instability is a rare phenomenon usually observed in tumors from HNPCC kindreds (32, 33). The occurrence of occasional microsatellite alterations in dinucleotide repeats are not uncommon in many types of cancer. A previous study of microsatellite instability in the bladder showed that this was a relatively rare event in bladder cancer occurring in only 6 of 200 cancers (34). It should be noted that all six of these tumors were low stage (T0–Ta), suggesting that these alterations can occur early in bladder tumorigenesis. Microsatellite alterations have also been seen early in other tumors such as the stomach, colon, and Barrett's associated esophageal cancer (35, 36). Although mismatch repair defects account for the widespread instability of HNPCC tumors, the mechanism underlying much less widespread alterations is still unknown. However, it is possible that subtle defects in repair may play an important role in a subset of CIS by increasing genomic instability during DNA replication or repair.

We have shown that CIS lesions are complex, possessing many of the genetic changes associated with highly invasive tumors. Although 9p loss and homozygous deletions at p16 are shared, as a group CIS differs significantly from superficial papillary lesions, the other form of noninvasive bladder tumor. It remains to be resolved which, if any, of the indicated deletions are integral to the development of CIS lesions. Moreover, the critical molecular changes that precede the invasion of CIS are still not known but a loss of a putative tumor suppressor gene on chromosome 14q may be critical for the progression of a CIS lesion. Further characterization and comparison of critical genetic changes in both papillary and CIS lesions should provide a rationale for the development of a comprehensive molecular progression model for TCC of the human bladder.

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References


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