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GENETIC DETECTION OF BLADDER CANCER BY MICROSATELLITE ANALYSIS OF p16, RB1 AND p53 TUMOR SUPPRESSOR GENES

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ABSTRACT

Purpose: We investigated the incidence of genetic alterations in urine specimens from patients with bladder cancer.

Materials and Methods: A total of 28 cytological urine specimens were assessed for microsatellite alternations, and 15 microsatellite markers were located on p53, RB1 and p16 regions. In 15 patients DNA from tumor specimens was also available.

Results: Loss of heterozygosity was detected in 26 of 28 patients (93%) in at least 1 microsatellite marker. Allelic losses were found in 18 patients (64%) for the p16 locus, in 8 (29%) for the RB1 locus and in 17 (61%) for the p53 region. In contrast, no microsatellite alterations were found in the normal group without evidence of bladder cancer. In 11 cases genetic alterations in the cytological urine specimens were not detectable in the corresponding tumor specimen, suggesting heterogeneity of bladder cancer.

Conclusions: The detection of loss of heterozygosity in cytological urine specimens may be a prognostic indicator of early detection of bladder cancer. Our results suggest that microsatellite analysis of urine specimens represents a novel, potentially useful, noninvasive clinical tool to detect bladder cancer.

KEY WORDS: microsatellite repeats; bladder neoplasms; genes, p53; genes, p16

Bladder cancer represents approximately 4% to 5% of new cancer cases per year in the United States. It is the fourth most common malignancy in men, accounting for 6% to 10% of all cancer cases, and the eighth most common cancer in women. The male-to-female ratio is 4:1. The increased rate of bladder cancer in males is not fully explained by gender differences, smoking habits or occupation. Bladder cancer affects mainly middle age and elderly patients with an average age at diagnosis of 65 years. The risk of developing bladder cancer before the age of 75 years is 2% to 3% for males and 0.5% to 1% for females.[1,2,3]

Although the etiology and pathogenesis of bladder cancer remain unknown, it is well established that bladder cancer can result from industrial exposure to aromatic amines and benzidine derivatives as well as from α- and β-naphthylamine secreted into the urine of tobacco smokers.[4] Oncogenes and tumor suppressor genes, such as H-ras, p21WAF1 and p53, have been shown to be mutated in bladder tumors from workers exposed to aromatic amines,[5,6] while genetic polymorphisms of detoxifying genes increase the risk of bladder cancer development in smokers.[7] Experimental studies in animals have demonstrated that artificial sweeteners (cyclamates, saccharin) can also cause invasive urothelial carcinoma. The role of caffeine and other substances in bladder carcinogenesis is unclear.[8,9] Many of these nongenotoxic compounds, such as sodium saccharin, increase cell proliferation in the bladder and deregulation of the cell cycle.[10]

Conventional approaches for clinical staging include bimanual investigation with the patient under general anesthesia, various imaging techniques, including excretory urography, abdominal or endovesical ultrasound, computerized tomography, magnetic resonance imaging and cystoscopy, urine cytology and flow cytometry. However, during urine cytology 50% of low grade or superficial carcinomas may be missed. Cystoscopic examination remains the gold standard for detecting bladder cancer and is always indicated in suspicious cases or during followup. However, its cost is relatively high and usually general anesthesia is required. The radiological techniques have limited sensitivity and fail to detect lesions, such as carcinoma in situ.[11–13] Accurate clinical staging is essential for better therapy selection in patients with bladder tumors. Thus, the development of a reliable method with high sensitivity and specificity is needed to assess patients at risk for bladder cancer.

Multiple genetic alterations affect urothelial tumorigenesis. Loss of chromosome 9 heterozygosity and p53 gene mutations represent 2 distinct pathways of tumor progression. Loss of heterozygosity of chromosome 9 is an early event in the tumorigenesis of papillary transitional cell carcinomas (34%), whereas it was observed in only 12% of urothelial dysplasia and carcinoma in situ.[14,15] Mutations in the p53 gene were commonly seen (50% to 65%) in carcinoma in situ and invasive tumors but in only 3% of papillary transitional cell carcinomas.[16,17] Also, the implication of RB1 tumor suppressor gene in bladder cancer has been documented.[18,19]

We investigated the incidence of genetic alterations at the level of microsatellite DNA using microsatellite markers located at p16, RB1 and p53 loci. Cytological urine specimens and the corresponding tumor biopsies, when available, were studied. Loss of heterozygosity was detected in 26 of 28 samples (93%) in at least 1 microsatellite marker. The presence of loss of heterozygosity in cytological urine specimens suggests that microsatellite analysis of urine specimens may be a potential noninvasive tool to detect bladder cancer.

MATERIALS AND METHODS

Specimens and DNA extraction. A total of 28 urine samples were obtained from patients with histologically confirmed bladder cancer who presented with symptoms of hematuria to the Department of Urology, University Hospital of Herak-

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lion Greece. Urine samples were collected before surgical resection in disposable 50 ml. centrifuge tubes and centrifuged at 5,000 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in 40 ml. normal saline and centrifuged. Pellets obtained after the second centrifugation were stored at −80C until DNA extraction. A corresponding tissue biopsy from the tumor was also available in 15 patients. Tumor biopsies were obtained by transurethral resection and stored immediately at −80C until DNA extraction. Also, 10 healthy individuals without evidence of bladder cancer served as the normal control group. Urine specimens obtained from all 28 patients and 10 controls were distributed in blind fashion for microsatellite analysis.

Informed consent was obtained from all patients who participated in the study. The clinical features of the patients with bladder cancer are presented in the table. Mean age at diagnosis was 68.8 years (range 55 to 82) and the majority of specimens (24 of 28) were obtained from male patients. In all cases fresh patient blood was collected in tubes containing ethylenediaminetetraacetic acid and stored at 4C to serve as a source for normal DNA. DNA from frozen biopsies, urine samples and peripheral blood lymphocytes was extracted using standard methods.[20]

Polymerase chain reaction (PCR) amplification and microsatellite analysis. We used 15 highly polymorphic markers for microsatellite analysis of the specimens. These markers were selected to map the known tumor suppressor genes p16, RB1 and p53 at 9p21, 13q13-14 and 17p13, respectively. The microsatellite markers tested were selected to map the chromosomal regions of the p16, RB1 and p53 tumor suppressor genes, known to be involved in oncogenesis of the bladder. Loss of p16 function due to deletion or mutation of the gene has been reported,[21-23] while allelic imbalance of chromosomal arm 9p is common in tumors and urothelial hyperplasia of the bladder.[24,25] The p53 gene is mainly implicated in the development of bladder cancer via point mutations,[26,27] whereas simultaneous alterations in RB1 and p53 or RB1 and p16 may function in a cooperative or synergistic manner to promote tumor progression.[18,19] All microsatellite markers were comprised of dinucleotides.

PCR analysis was performed as described previously.[20] A total of 7 μl. of the PCR product were electrophoresed in a 10% polyacrylamide gel and silver stained. Gels were scanned, and the intensity of the bands corresponding to the microsatellite alleles was quantitated by a commercially available image analysis system. The analyses were performed twice and the results were highly reproducible. The electrophoretic patterns of the amplified marker segments in the normal and pathological tissues were compared. Loss of heterozygosity was defined as a decrease of at least 50% in intensity of 1 allele relative to the other as determined after comparison of tumor and normal DNA. In a few cases, although loss of heterozygosity occurred, PCR amplification of polymorphic microsatellite alleles resulted in an allelic imbalance due to the presence of a contaminating band in decreased levels. This band corresponds to the deleted allele, which is present in the normal tissue. Generally, this contamination is attributed to the presence of normal DNA derived from either peripheral blood or adjacent normal tissue.[28] Analysis of all samples was performed in a blinded manner without knowledge of clinical status.

To assess the sensitivity of our loss of heterozygosity method, we prepared samples of various tumor-to-normal ratios from a tumor specimen with known loss of heterozygosity. The analysis showed that loss of heterozygosity was detectable in 1:16 tumor-to-normal dilution.

Histopathological and clinical data of 28 patients with bladder cancer

Pt. No.	Age	Sex	Stage	Grade
1	72	M	T1	I–II
2	65	M	T1	I–II
3	55	M	Tα	I
4	59	M	T2	III
5	76	F	T3α	III
6	81	M	T1	I–II
7	72	F	T3α	III
8	73	M	T3β	III
9	73	M	Tα	I
10	59	M	T1	III
11	64	M	Tα	I
12	80	M	T1	I–II
13	57	M	Tα	I
14	68	M	T1	I–II
15	60	M	T2	III
16	76	M	Tα	I
17	82	M	T1	I–II
18	74	M	T1	III
19	72	M	Tα	I
20	68	F	Tα	I
21	65	M	Tα	I–II
22	70	M	T2	III
23	58	M	T1	I–II
24	66	M	Tα	I
25	71	M	Tα	I
26	70	M	Tα	I–II
27	75	F	Tα	I
28	66	M	T1	I–II

RESULTS

We tested DNA of normal and cytological urine specimens from 28 patients with bladder cancer for the presence of microsatellite alterations. In addition, the same genetic analysis was done in the corresponding tumor biopsy from 15 patients and 10 normal controls who also underwent microsatellite analysis. A panel of 15 dinucleotide repeat microsatellite markers was used, which mapped p16, RB1 and p53 loci. At the end of genetic analysis the code was broken, and we established that all microsatellite alterations were restricted to patients with bladder cancer. Loss of heterozygosity in the urine of 26 of 28 patients (93%) in at least 1 microsatellite marker was detected. Allelic imbalance was found in 18 (64%) patients at the p16 locus, in 8 (29%) at the RB1 locus and in 17 (61%) at the p53 locus. Representative examples of specimens exhibiting loss of heterozygosity are shown in figure 1.

The graphic representation of allelic losses is presented in figure 2. Of the specimens 64% exhibited genetic alterations using microsatellite markers at the p16 locus, including 43%

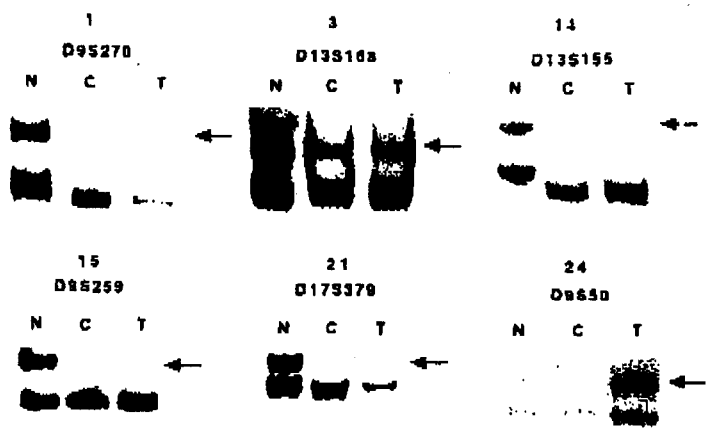

FIG. 1. Loss of heterozygosity in urine and tumor biopsy (*T*) of individuals with bladder cancer. Loss of heterozygosity is seen in cytological (*C*) and tumor samples except in case 24 in which allelic deletion is limited to cytological sediment. In cases 1, 14, 15 and 21 loss of 1 allele is almost complete indicating large proportion of cancer cells in urine specimen, while in case 3 loss is incomplete indicating higher proportion of normal cells. *N*, normal.

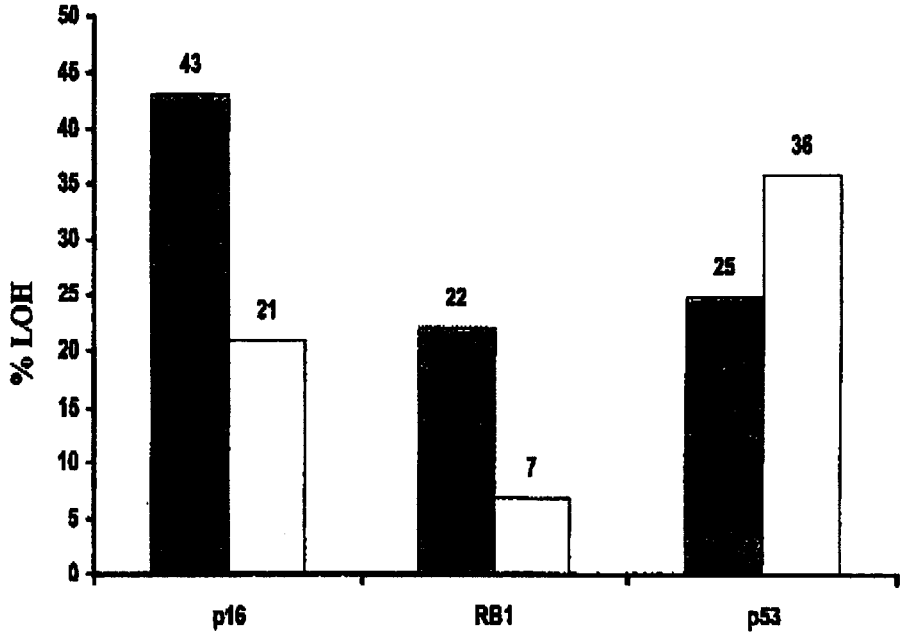

FIG. 2. Graphic representation of microsatellite analysis at p16, RB1 and p53 loci in bladder cancer shows loss of heterozygosity (*LOH*) detected in urine specimens (shaded column), and in urine and corresponding tumor biopsy (unshaded column).*

detectable in urine samples and 21% in urine and the corresponding tumor biopsy. Similarly, 29% of the specimens showed microsatellite alterations at the RB1 locus with 22% in urine and 7% in urine and tumor specimens. Finally, 25% of genetic alterations in urine and 36% in urine and tumor samples comprised the 61% of genetic lesions at the p53 locus. In healthy controls the lymphocyte and urine DNA displayed the same electrophoretic patterns, which indicated absence of microsatellite alterations.

In most cases the genetic change in urine DNA was identical to that identified in the primary tumor. However, in 11 cases (9 patients) genetic alterations found in the cytological urine specimens were detectable only in those specimens but not in the corresponding tumor biopsy. Patients who exhibited microsatellite alterations in urine specimens exclusively had early, moderate or late stages and grade I, II or III disease. These data further substantiate the heterogeneity of bladder tumors and polyclonal origin of bladder cancer cells. In all cases loss of heterozygosity in at least 1 locus and loss of the identical allele were shared between the urine sample and primary tumor.

Disease was missed in cases 2 and 19 on microsatellite analysis, which displayed no alterations at any of the 15 tested markers. These 2 tumors were T1, grade I–II and stage Ta, grade I, respectively. No correlation was observed between genetic alterations and histological and clinical parameters of the patients.

DISCUSSION

We studied genetic alterations in 28 patients with bladder cancer by microsatellite analysis of cells exfoliated in urine and corresponding tumor biopsies when available. Genetic analysis allowed detection of allelic imbalance in 26 (93%) of these patients. Even tumors of early stage and low grade displayed multiple genetic alterations. The exclusive type of microsatellite alteration of these specimens was loss of heterozygosity, which is consistent with previous reports.[29,30] There was no evidence of microsatellite instability or generation of new alleles in the specimens, although this genetic event has been reported in bladder cancer.[29,31] This finding implies that loss of heterozygosity is associated with neoplastic cell transformation and, consequently, altered cytological appearance of the cell.

Bladder cancer is the second most common urological tumor and, thus, early detection of bladder cancer cells is critical for early diagnosis and successful treatment of the disease. Diagnostic methods, such as endoscopy and biopsy of the lower urinary tract, are expensive procedures, can cause discomfort to the patient and are sources of infection. Our results provide support for the use of microsatellite alterations as a genetic marker for early diagnosis of bladder cancer. Furthermore, PCR based microsatellite analysis of the cytological urine specimens can be used as a noninvasive, inexpensive and easily performed analytical method to diagnose bladder cancer. We achieved a 93% diagnostic sensitivity using 15 microsatellite markers but as new markers are identified the approach can be expanded and improved. Previous studies have proposed microsatellite analysis as a diagnostic tool for the diagnosis of bladder cancer with sensitivity ranging from 83% to 91% using 20 microsatellite markers.[29,30,32,33] Mao et al reported genetic alterations in 19 of 20 patients (95%) using 13 markers.[31] Of significance is the specificity achieved by microsatellite analysis for bladder cancer detection as genetically altered alleles were restricted to tumor cells.

Allelic deletions were detectable in cancers of all stages. Small lesions showed microsatellite alterations at a level similar to that of more invasive lesions and, thus, molecular analysis appears promising as a method of detecting early lesions. In addition, early detection of bladder tumors will be beneficial not only for surgical treatment, but also for further followup of patients after surgical resection. In addition to definitive resection, diagnosis of early stage bladder cancer by microsatellite analysis may be of major importance for chemopreventive approaches to disease progression. The 2 patients whose microsatellite abnormalities could not be identified from the urine specimens had small low grade tumors. The use of a larger number of markers may further increase the sensitivity of the test.

In 11 cases loss of heterozygosity in cytological urine specimens was not detectable in the corresponding tumor biopsies. This finding can be explained by a lack of cancer cells in the biopsies, a presence of heterogeneous clones in the tumor or urine specimens that contained more advanced tumor cell clones which were not sampled by the small biopsy of the

* Readers should contact the authors directly for the detailed pattern of allelic losses.

tumor.[31] An alternative explanation could be the survival of more tumor cells than normal cells[34] during storage, and one cannot exclude the possibility of cancer cell exfoliation from the outer surface of the tumor into the urine due to loss of adhesion.[35]

Genetic alterations have also been detected in body fluids, such as plasma[36,37] and sputum,[38,39] and, thus, may be considered attractive candidates for the development of strategies for early diagnosis of cancer along with other molecular methods that require no invasive or painful techniques. We detected bladder cancer in 93% of our cases and demonstrated that microsatellite analysis can be a powerful tool for the detection of primary bladder cancer. Our results also suggest the potential application of routine microsatellite urinalysis for bladder cancer as we provide evidence of its high sensitivity for detecting tumors of all stages and demonstrate its potential clinical use. Additional microsatellite markers may further improve the sensitivity of this test. Finally, in addition to diagnosis, microsatellite analysis may provide molecular information regarding tumor progression, prognosis and metastasis.

CONCLUSIONS

Our results show that microsatellite analysis of cytological urine specimens may be used for early detection of bladder cancer. Molecular analysis with the set of markers tested in our study reliably detected tumors of all stages and grades with 93% sensitivity and 100% specificity. This noninvasive and painless procedure is a potential clinical tool for detection and screening of bladder cancer. Moreover, a nonradioactive PCR based assay makes this method feasible for routine use without requiring expert interpretation.

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