Evaluation of Microsatellite Analysis in Urine Sediment for Diagnosis of Bladder Cancer

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ABSTRACT

Alterations at microsatellite DNA markers in cells exfoliated in urine have been correlated to the presence of bladder cancer. To check the feasibility of such noninvasive analysis to routinely diagnose bladder cancers, we have developed a highly sensitive method using fluorescent PCR to search for DNA microsatellite alterations in urine sediment compared with a blood paired sample. One hundred eighty-three patients were included in our study. This population comprised 136 bladder cancers (64 pT1 stages), the complement representing controls and other benign or malignant diseases. Results of the analysis at 17 loci in a blinded study were compared with cystoscopy and/or pathology. The high reproducibility of this technique and the analysis of 26 control patients allowed us to determine for each microsatellite a cutoff characterizing a significant allelic imbalance. For bladder cancer detection, the overall sensitivity of the test was 84%. Using this procedure, we identified alterations in 81%, 84%, 91%, and 100% of pT1, pT2, pT3, and >pT3 stages, respectively. This corresponds to 79%, 82%, and 96% sensitivity for grades I, II, and III, respectively. Interestingly, for routine purposes, we observed an overall sensitivity of 80% (76% for pT1 stages) when only the eight most rearranged microsatellites were considered. In conclusion, the noninvasive feature combined with the rapidity of this fluorescent and highly sensitive technique for the detection of early stages provides us with a useful help for the diagnosis of bladder cancer.

INTRODUCTION

Bladder cancer is the fourth cancer in men and the eighth in women both in terms of incidence and mortality (1–3). The main risk of these tumors is a high frequency of recurrence and progression depending on their initial stage and grade (4–6).

Diagnosis of bladder cancer at an early stage appears to be one of the main factors for patients’ survival. Cytoscopy is the “gold standard” method for diagnosis and follow-up but is still invasive for the patients. Cytology is a common noninvasive procedure for diagnosis but can miss up to 50% of tumors, especially those of low grade and low stage (5, 7). Therefore, there is a need for a diagnosis method less invasive than cytoscopy and more efficient than cytology, which could also be used for the follow-up of bladder tumors.

Genomic rearrangements are very often observed in tumors, and their accumulation is a sign of cancer progression. In bladder TCC, several studies have shown recurrent loss of heterozygosity at chromosomes 3, 4, 8, 9, 11, 13, 17, and 18 involving tumor suppressor genes such as p53 and p16 (8–16). Furthermore, chromosome 9 alterations appeared to occur early in bladder carcinogenesis (15). Recently, microsatellite analysis was shown to detect AI and genomic instability in primary tumors (17–21). Genomic or microsatellite instability describes accumulation of modifications in a number of repeats attributable to failure of the DNA mismatch repair mechanism (22–24). AI refers to partial or complete loss of one of the two alleles (previously known as loss of heterozygosity) or alternatively, amplification of one allele compared with the other (25, 26).

Recent studies have detected identical microsatellite alterations in bladder tumor and corresponding urine sediment from the same patient, demonstrating the ability to identify clonal population of tumor-derived cells in urine sediment (17, 27). In our blinded comparative study with cystoscopy and pathology diagnosis, we aimed to determine whether microsatellite analysis could be a valuable marker of lower urinary tract cancers. To detect early cancer, such analysis requires high sensitivity and specificity. Using a nonslot in and semiautomated technique, our work focused on improving the detection of tumors in the urinary tract by the characterization of microsatellite rearrangements in urine sediment.

MATERIALS AND METHODS

Patients. Urine samples and peripheral blood lymphocytes were collected from patients undergoing follow-up cystoscopy or endoscopic transurethral resection. In our blinded study performed from September 1996 to June 1998, 183 patients were included. This population comprised 136 men and 47 women (sex ratio, 2.9) of 17–88 years old (average 62). In this study, 103 bladder cancers were analyzed in addition to 47 cases of other malignancies, 7 cases of benign inflammatory urothelial diseases, and 26 patients without malignant disease as controls. In the case of suspicious bladder lesions detected by cystoscopy, the chips obtained by transurethral resection of the bladder were analyzed by the pathologist. The numbers of bladder cancer patients stratified by stage and grade are shown in Table 1. At the time of analysis, the biologist knew neither the clinical diagnosis nor the results of pathology.

Urine and Blood DNA Extraction. Up to 40 ml of urine were collected mainly through an endoscope or a catheter. In every case, 5 ml of peripheral blood were collected on EDTA. Urine sediment cells recovered after Ficoll centrifugation were washed twice with Hanks solution and then lysed at 37°C in 200 μl of buffer [8 μM urea, 2% SDS, 10 mM EDTA, 0.3 μM NaCl, 10 mM Tris (pH 8)]. After overnight digestion at 37°C with proteinase K (200 μg/ml), followed by two phenol chloroform (1:1) treatments, precipitated DNA was dissolved in 200 μl of Tris/EDTA buffer [20 mM Tris-HCl (pH 7.6), 1 mM EDTA]. For blood DNA extraction, RBCs were disrupted in hypotonic buffer TKM1 [20 mM Tris-HCl (pH 7.6), 10 mM KCl, 10 mM MgCl2, 2 mM EDTA] in the presence of NP40 (2.5%). After centrifugation at 2200 rpm for 10 min, pellets of leukocyte nuclei were washed once with TKM1 and then incubated overnight at 37°C in lysis buffer [20 mM Tris-HCl (pH 7.6), 5 mM EDTA, 1% Sarkosyl, and 40 μg/ml proteinase K]. DNA extraction was finally performed as described above.

Microsatellite Analysis. Extracted DNA from each sample was amplified by PCR using previously described primers for 17 polymorphic microsatellite markers localized on chromosomes 4, 6, 8, 9, 11, 13, 14, 16, 17, and 20 (Table 2 and Refs. 27 and 28). PCR amplification (Omnigen Hybaid Thermocycler) was carried out in a 25-μl final volume by combining 100 ng of template, 0.6 units of Taq polymerase in PCR buffer [1.5 mM MgCl2, 50 mM KCl, 20 mM Tris-HCl (pH 8.4; Life Technologies, Inc.), 80 μM of each deoxynucleotide triphosphate, and 0.16 μM of each primer]. The PCR protocol consisted of 35 cycles of 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C followed by a final 5-min extension at 72°C. One primer of each pair was 5′ Cy5-labeled. After

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3 The abbreviations used are: TCC, transitional cell carcinoma; AI, allelic imbalance; GDB, genome database; RCC, renal cell carcinoma; CV, coefficient of variation; UICC, Union Internationale Contre le Cancer.

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ASSAY REPRODUCIBILITY AND CUT-OFF DETERMINATION FOR AL.

As previously shown, it is possible, using a limited number of amplified microsatellites, to detect exfoliated bladder tumor cells in urine sediment (17). The AI, defined by modification of the allele ratio in urine DNA compared with blood-paired DNA, depends on the percentage of tumor cells that appeared highly variable in urine pellet. We strove to increase sensitivity by detecting the smallest fraction of tumor cells and thereby the smallest significant allele ratio alteration by using a semiautomated fluorescent PCR sequencing analyzer.

Intra-assay electrophoresis reproducibility was performed after quantification of the signal obtained for nine simultaneous electrophoresis of the same PCR product. As an example, for the microsatellite D16S310, the CV of the allele ratio was 2.5% for blood DNA and 3% for urine DNA with a mean allele ratio of 0.89 and 0.87, respectively. Interassay PCR reproducibility was evaluated by measuring the CV of the allele ratio for one microsatellite fragment amplified in several independent PCRs. As shown in Fig. 1, the CV calculated from peak height measured for the microsatellite D9S162 was 3.8% with a mean allele ratio of 0.72 (n = 7). By analyzing peak height variations of another microsatellite D9S747, the CV was 4.1%. These results suggested that the use of raw data and the measurement of the peak height present a good reproducibility and enabled us to next determine the variability of the allele ratio between paired urine sediment and blood DNAs. The intensity of AI was calculated as a percentage (29): AI% = absolute value ((\(B_b/B_a\)) - ((\(U_b/U_a\)) × 100/((\(B_b/B_a\)) + (\(U_b/U_a\)))) in which \(B_a\) and \(B_b\) represent the height of the two alleles in the blood and \(U_a\) and \(U_b\) in the urine. In our systematic study, the presence of an AI was confirmed by at least two independent PCRs. As expected from the interassay PCR results described above, we obtained excellent reproducibility both with strong and weak AI (Fig. 2).

We determined the cutoff value of a significant AI by analyzing urine and blood DNAs of 26 control patients. When the cutoff value for each microsatellite was calculated at 2 SD, we observed a variation of these cutoffs ranging from 7% for D9S162 (n = 17) to 14% for D9S747 (n = 18; Table 2). At 3 SD, all of the normal subjects had a normal molecular analysis, but at 2 SD, 3 of 26 control patients showed one significant AI leading to a specificity of 88%. However, despite this lower specificity, we chose cutoffs at 2 SD because it results in a better sensitivity. Thus, for the molecular detection of tumor cells, we considered indeed that it is of interest to provide the urologist with the highest sensitivity.

To evidence the linearity of tumor cells detection, we performed amplification of two highly rearranged microsatellites on serial dilution of urine DNA with blood-paired DNA (Fig. 3). A significant AI value was still observed with a 1/4 dilution but not with a 1/10 dilution. Assuming that the percentage of AI corresponds to the percentage of tumor cells in the urine sample, our experiments show that it is possible to detect an AI when urine sediment cells comprise at least 20% of tumor cells.

- **PCR**, amplified fragments were analyzed on an ALF Sequencer (Amersham-Pharmacia). This technique allows a quantitative evaluation of the allele ratio, measuring the peak height of both alleles, because it uses a unique labeling that permits access to the raw data. Thus, it is possible to use the baseline to get full sensitivity above the background.

### RESULTS

**Assay Reproducibility and Cutoff Determination for AI.** As previously shown, it is possible, using a limited number of amplified microsatellites, to detect exfoliated bladder tumor cells in urine sediment (17). The AI, defined by modification of the allele ratio in urine DNA compared with blood-paired DNA, depends on the percentage of tumor cells that appeared highly variable in urine pellet. We strove to increase sensitivity by detecting the smallest fraction of tumor cells and thereby the smallest significant allele ratio alteration by using a semiautomated fluorescent PCR sequencing analyzer.

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**Fig. 1.** Reproducible allelic profile of independent PCRs. Seven separated PCRs were performed simultaneously from the same DNA microsatellite (D9S162) and loaded on the same gel. An allelic ratio is indicated for each analysis. Horizontal axis, the length of the fragments as determined by size markers migrated on the same gel.
Results of Allelotyping and Correlation with Tumor Stage and Histological Grade. The analysis of 17 polymorphic microsatellites was performed on paired urine sediment and control blood DNA samples (Table 3). Except for the two markers IFNA and D20S48 that show only 47% and 27% of heterozygosity, respectively, the informativity of the other markers we analyzed ranged from 95 to 60%, confirming the ~70% values listed in the GDB. Among the 183 patients at initial presentation, 129 urine specimens (70%) showed molecular anomalies with cutoff values at 2 SD. AI at two or more loci was observed in 101 of 129 urine specimens, whereas 28 urine specimens showed AI at only one locus. No alteration was observed in 54 of 183 urine samples. Unlike previous studies, we never ob-

A) Patient n°1

![DNA analysis](image1)

B) Patient n°2

![DNA analysis](image2)

C) Patient n°3

![DNA analysis](image3)

Fig. 2. Reproducibility of the microsatellite analysis performed on paired blood and urine samples. Two independent PCRs (I and II) of selected microsatellites were performed on paired blood and urine DNAs isolated from three patients. A, for patient 1, a strong AI of 82% was observed for microsatellite D9S747 and was confirmed by a second PCR showing an AI of 85%. B, for patient 2, a weak AI of 18% observed at locus D16S310 was confirmed in a second analysis with an AI of 20%. C, for patient 3, the locus D16S310 was not altered in two independent PCRs.

<table>
<thead>
<tr>
<th>ID</th>
<th>Htz. nb.</th>
<th>AI %</th>
<th>Htz %</th>
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<td>32</td>
<td>63</td>
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</table>

Table 3 Percentage of AI at each locus in 103 bladder cancers

*ID, GDB identification; Htz. nb., number of informative analyzed loci; AI %, percentage of informative analyzed patients presenting an AI at the locus; Htz %, percentage of analyzed patients presenting an heterozygous locus.

Fig. 3. Example of serial dilution of urine DNA with blood-paired DNA. PCR and electrophoresis were performed as described in “Material and Methods.” When an AI of 90% was observed in urine DNA (U) as compared with blood-paired DNA (B), the 1/4 dilution (25U/75B) still presented a significant AI of 23%, in contrast to the 1/10 dilution, which lead to no significant alteration of the allele ratio.
served the presence of an additional peak corresponding to microsatellite instability (17, 30).

The code was then broken, and clinical, pathological, and molecular data were compared. In all cases, the presence of bladder TCC detected by cystoscopy was confirmed by histopathological examination of tumor specimens. Among the 103 bladder TCCs, AIs were detected in urine specimens of 87 patients, resulting in 84% sensitivity at diagnosis. Among these 87 bladder TCCs with molecular anomalies, 71 urine samples showed AI at at least two loci. AI at only one locus was observed in 16 of 87 patients. Three of these 16 patients presented weak AI values comprised between the 2-SD and 3-SD cutoffs, yielding 82% sensitivity at the 3-SD cutoff (Fig. 4A).

According to clinical stages and histological grades (TNM UICC 1997 Classification), urine specimens showed AI in 52 of 64 (81%) early pTa to 7 of 7 (100%) pT2-pT4 stages (Fig. 4A). Taking into account the grade, the frequencies of AI increased from 79% (31 of 39) in grade I to 96% (23 of 24) in grade III bladder tumors (Fig. 4B). The two secondary bladder cancers (primary were ovarian and vaginal malignancies) showed AI at two and three loci, respectively.

We next analyzed the performance of each microsatellite to detect bladder cancers. All microsatellites on chromosome 9, in addition to ACTBP2 on chromosome 6q and THO on chromosome 11, were frequently altered, ranging from 34 to 47% of analyzed informative patients. Despite their high heterozygosity, four microsatellites were altered in <15% of analyzed cases (Table 3). From our data, taking into account only the five most frequently rearranged microsatellites, D9S162, IFNA, D9S171, D9S747, and ACTBP2, the overall sensitivity of the molecular test was still 74% (76/103) as detailed in Table 4. The sensitivity calculated for each tumor grade was 74% for grade I to 92% for grade III. These results suggested that at least nine microsatellites could be removed from our panel without significant decrease of the sensitivity.

### DISCUSSION

**Sensitivity of Urine Sediment Molecular Analysis in Bladder Cancer Detection.** Several studies have shown the capacity of the microsatellite approach to reveal a urinary tract cancer through the detection of tumor cells in urine sediment (17, 28). Nevertheless, until now, no comprehensive systematic study was published to analyze by a fluorescent semiautomated method the feasibility of this molecular analysis.
ysis in a significant general population of a Urology department. In our study, we have shown that the use of fluorescent primers allowed a highly reproducible quantitative measurement within a wide range of allele signal intensity. Consequently, we were able to determine for each microsatellite the cutoff value for a significant rearrangement.

In our blinded study, we searched for the presence of molecular anomalies in a population (183 patients) large enough to allow stratification by tumor stage and grade. In contrast to previous studies (17, 27, 30) but in agreement with other groups working with nonisotopic PCR, we did not detect any microsatellite instability (31). At the 2-SD cutoff, 3 of 26 normal controls did show one significant AI. However, the possibility that these three patients (50, 55, and 76 years old, respectively) with nondetected malignant disease could present any occult urinary tract cancer cannot be completely excluded.

Similarly, the urine samples of five of seven patients with benign urothelial disease showed no altered microsatellite. The two other patients suffering from glandular cystitis and inflammatory bladder lesions presented two and one significant AI, respectively. Both could be suspicious for cancer lesion because we never observed any alterations in histological normal tissue (data not shown). It is reasonable to propose that these patients would benefit from careful follow-up. Previous studies reported similar observations (27, 30).

Our results strengthen the robustness of the microsatellite analysis because our sensitivity (84%) is in agreement with those described by several authors working with smaller populations of around 20 patients (27–31). Incomplete sensitivity could derive from two sources: first, failure of the molecular markers to detect all cases; and second, inability of small tumors to exfoliate a sufficient number of cells. In this blinded study, our cohort included patients carrying RCC and upper tract TCC as controls for cancer-bearing patients. Twenty-nine of 39 RCCs and 8 of 8 TCCs were detected, confirming recent observations (32). However, no yet-defined combination of multiple modified markers can specifically identify cellular types or tumor localization. Therefore, molecular analysis should only be interpreted by the urologist with complete clinical and histological data.

Microsatellite Analysis Is Effective in Detecting Early Bladder Cancers. We observed a similar intensity of the AI in superficial tumor and in high-stage or -grade tumors, suggesting that most of these cancers exfoliate similarly. Interestingly, 81% (52 of 64) of pT2-staged tumors, which represent the major part of our population (64 pT2 of 103 bladder cancers), were accurately detected. Considering low grade, sensitivity was up to 79%. These results appear to be more efficient than those previously described by Shigyo et al. (31) using fluorescent PCR, but with markers located only on chromosome 9. Thus, our results strongly argue in favor of using the described semiautomated molecular technique with a 2-SD cutoff. This represents significant help for both diagnosis and follow-up because patients with pT2a tumor could frequently relapse and therefore would benefit of early diagnosis. Other biochemical assays, measuring bladder tumor-associated antigen (BTA); nuclear matrix protein 22 (NMP22); telomerase activity, and fibrin/fibrinogen degradation products (FDP), have been recently developed (33–40). These tests show variable sensitivities depending on the reports, but they are generally less sensitive for diagnosis of early bladder cancer (stage pT2a grade 1), suggesting that they would be less efficient in a routine procedure than microsatellite analysis.

Optimizing the Microsatellite Anomalies Detection. In this study, we also noticed that 16 bladder tumors were not diagnosed. This lack of detection could be attributable to a limited amount of tumor cell in the collected urines. Preliminary results showed that separation of cells through Ficoll and Tris-saline buffer wash were of some help in the case of hematuria and urates. Most of the analyzed samples were obtained from patients who underwent transurethral resection of bladder; thus, to set up a noninvasive test, we checked the possibility to analyze voided urine. Preliminary assays showed that first micturition of the morning would be the most enriched sample with tumor cells in comparison with day micturition or bladder wash.

The present use of 17 different microsatellite markers could be considered as irksome and expensive. However, the use of a panel of eight microsatellites resulted only in a slight decrease of the test sensitivity from 84 to 80%. Four of these eight microsatellites were localized on chromosome 9 according to previous studies showing that chromosome 9 is frequently altered in early bladder cancer (Refs. 11 and 15 and references therein) and in agreement with alleleotyping results of Shigyo et al. (31). One microsatellite was localized on chromosome 6q13. 6q has been shown to be frequently altered in hematopoietic tumors (41) as well as in ovary carcinoma and kidney cancer (42, 43), suggesting that this microsatellite would be widely used to detect cancer cells. In agreement with previous studies (44, 45), the THO marker, located on chromosome 11, is also very frequently altered in half of our population, but often associated with chromosome 9 alteration (data not shown). In contrast, MJD52 (chromosome 14q) appeared rarely associated with AI at other loci and thus was strongly informative for the presence of tumor cells. Interestingly, TP53, located in the first intron of the p53 gene, was also frequently rearranged (32%) but less than previously described, either by immunohistochemistry or by mutation scanning (12, 46, 47). This discrepancy could be explained by differences in the cohorts analyzed.

Nevertheless, this panel of eight microsatellites should be modified to enhance the sensitivity by targeting loci that have now been shown to be frequently altered in all cancers, especially in those of bladder, kidney, and prostate (15, 48–50). This includes markers on 3p [Von Hippel Lindau gene, 51, 52 and TGFβ receptors (53)], 5q (APC gene; Refs. 54 and 55), and 10q (PTEN/MMAC1 gene; Refs. 56 and 57). Furthermore, such analysis could benefit of the use of quantitative PCR (58, 59) allowing the precise targeting of genes involved in cancerogenesis and invasiveness of urinary tract cancers.

To conclude, alleleotyping of urine sediment appears to be a reproducible and sensitive test for diagnosis of bladder tumors. It can be considered as a complementary tool to cystoscopy and pathology. Because this test can also be efficient for the detection of tumor recurrence, the next step would be to evaluate its sensitivity versus endoscopy and cytology in routine patient follow-up. Furthermore, it would be interesting to determine whether this test could be used in the screening of a selected high-risk population as well as in the diagnosis of kidney cancer.

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