SPANDIDOS PUBLICATIONS

Genomic instability analysis of urine sediment versus tumor tissue in transitional cell carcinoma of the urinary bladder

JACEK TURYN[1], MARCIN MATUSZEWSKI[2] and BEATA SCHLICHTHOLZ[1]

[1]Department of Biochemistry, Medical University of Gdansk, 80-211 Gdansk;
[2]Department of Urology, Medical University of Gdansk, 80-402 Gdansk, Poland

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Abstract. Microsatellite alterations are a common feature of neoplastic cells. Our study aimed to compare the profile of microsatellite DNA alterations in tumor tissue and urine sediment at 12 selected microsatellite loci in transitional cell carcinoma of the bladder, and to determine which of the 12 markers or combination of markers has potential for the non-invasive diagnosis of bladder cancer. DNA alterations were examined using microsatellite markers on chromosomes 2p, 3p, 8p, 9p, 9q, 12q, 13q, 17p and 18q in 38 patients, including 12 with superficial Ta/T1 and 26 with muscle invasive T2-T4 bladder tumors. Microsatellite instability was a rare event in comparison with loss of heterozygosity and was related to a low rate of defects in mismatch repair genes. The sensitivity of microsatellite analysis was 75% (9/12) for Ta/T1 tumors and 69% (18/26) for T2-T4 tumors. Two tetranucleotide markers, D9S242 and D9S252, when combined, displayed microsatellite alterations in 59% (16/27) of microsatellite analysis-positive patients. DNA alterations were not detected in 21 non-tumor specimens. Twenty of 51 (39%) tumor DNA alterations were re-detected in urine sediments, and 7 alterations found in urine sediments were not found in the corresponding tumor specimens. No association was found between the DNA alterations and any of the prognostic parameters. However, the overall survival correlated with microsatellite alterations (P=0.04, log-rank test). These data suggest that markers at tetranucleotide repeats on chromosome 9q have particular diagnostic potential in bladder cancer. Moreover, microsatellite analysis is suitable for the selection of patients with a less favorable outcome.

Introduction

In Poland, bladder cancer is the fourth most common cancer in men, and is responsible for about 3% of all cancer-related deaths (1). The transitional cell carcinoma (TCC) occurs in approximately 90% of bladder cancer patients. Diagnosis of primary and recurrent bladder cancer is one of the most difficult problems in urology and cytology. Cystoscopy (endoscopic examination of the interior of the bladder) and urine cytology are considered the standard methods for diagnostic evaluation of patients with symptoms of bladder cancer. However, the sensitivity of cytology in urinary specimens is limited, since many of the non-invasive cancers (stage pT1) are missed (2). Other methods for detecting low grade TCCs of the bladder include cytogenetic studies and DNA measurements by flow cytometry (3). Analysis of microsatellite instability (MSI) and/or loss of heterozygosity (LOH) seems to be a very attractive, alternative method with a high potential in predicting the presence of bladder tumor. Microsatellite analysis has been found to have a sensitivity of 95% in detecting bladder cancers that had been confirmed by cystoscopy, compared with only 50% sensitivity for conventional cytology (4).

Several studies have reported that LOH on chromosome 9 can occur early in bladder tumorigenesis (5,6). LOH on chromosomes 3p, 4p, 8p, 13q, 17p and 18q in high stages has also been observed (7-10). Although hundreds of different microsatellite markers have been used for microsatellite alteration analysis, it is unclear how many markers and which chromosomal loci should be used to evaluate MSI and/or LOH. The frequency of MSI can vary from 0 to 100% in sporadic bladder cancer (11,12). Moreover, it is very important to distinguish lesions that have a significant effect on survival. In this study, we tested 12 different microsatellites in 38 transitional cell carcinomas of the bladder to investigate whether there are any microsatellite loci that are especially susceptible for instability. We addressed the following questions: i) is the genetic instability observed in some uroepithelial carcinomas due to mutations of MMR genes? ii) which loci have the highest sensitivity and specificity in the detection of bladder cancer? iii) what is the frequency of microsatellite alterations in urine-sediment DNA? and iv) are the changes found in tumor tissue reflected in urine sediment?

Materials and methods

Patients and tumor samples. The Department of Urology at the Medical University of Gdansk provided a total of 38 bladder tumors and blood samples from patients undergoing transurethral resection or cystectomy. The corresponding urine

Correspondence to: Dr Beata Schlichtholz, Department of Biochemistry, Medical University of Gdansk, Debinki 1, 80-211 Gdansk, Poland
E-mail: bsch@amg.gda.pl

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Table I. Microsatellite alterations and clinicopathological characteristics of 38 bladder cancer patients.

Variable	Tumor			Urine sediment		
	No. Pts. (%)	No. LOH/MSI - tumor tissue positive (%)	P-value	No. Pts. (%)	No. LOH/MSI - urine sediment positive (%)	P-value
Female	8 (21)	6 (75)	NS[a]	7 (20)	5 (71)	NS[a]
Male	30 (79)	21 (70)		28 (80)	11 (39)	
Age						
<65	15 (39)	9 (60)	NS[a]	13 (37)	4 (31)	NS[a]
≥65	23 (61)	18 (78)		22 (63)	12 (55)	
Pathological stage						
pTa/T1	12 (32)	9 (75)	NS[a]	10 (29)	6 (60)	NS[a]
pT2-pT4	26 (68)	18 (69)		25 (71)	10 (40)	
Lymph node status						
Positive	9 (24)	8 (89)		8 (23)	6 (75)	
Negative	23 (61)	16 (23)	NS[b]	22 (63)	7 (32)	NS[b]
Cannot be assessed	6 (16)	3 (50)		5 (14)	3 (60)	

NS, not significant; [a]Calculated by two-sided Fisher's exact test; [b]Calculated by Pearson's χ^2 test.

Table II. Characteristics of microsatellite markers.

Name/ locus	Chromosome	Primer sequence	Type	Size (bp)	Max het.	Reference GDB
BAT26	2p16	-TGACTACTTTTGACTTCAGCC -AACCATTCAACATTTTTAACCC	Mono	120	-	gdb:9834505 BAT-26
D2S123	2p16	-GACTTTCCACCTATGGGACT -TCAACATTGCTGGAAGTTCT	Di-	197-227	0.773	gdb:187953 AFM093xh3
D3S1611	3p24.2-3p22	-CCCCAAGGCTGCACTT -AGCTGAGACTACAGGCATTTG	Di-	Min 260	0.664	gdb:200039 AMF338xe5
D8S133	8p21.3-8q11.1	-CAGGTGGGAAAACTGAGGGA -AGCAACTGTCAACATATTGCTC	Di-	94-112	0.775	gdb:180979 D8S133CA
D9S171	9p21	-AGCTAAGTGAACCTCATCTCTGTCT -ACCCIAGCACTGATGGTATAGTCT	Di-	159-177	0.804	gdb:188218 AFM186xc3
D9S273	9p21-9q21	-AGCTGGGTTATTTAAGTCAAGTTT -AGCTGGGTGTGGTCAGG	Di-	Min 203	0.740	gdb:199715 AFM280th5
D9S252	9q13-9q22	-ACAATGAACATCCATATACCC -ACCATGATTTGTCAACTCCTA	Tetra-	Min 212	0.875	gdb:198806 UT2103
D9S242	9q32-9q33	-ACTCCAGTACAAGACTCTG -GTGAGAGTTCCTTCTGGC	Tetra-	148-208	0.833	gdb:198484 UT914
D12S81	12q21	-CTATTCCAGATGAGGGGGTC -TCAAATCATAAGGGTATCAGAAATT	Di-	146-166	0.819	gdb:187977 AFM102xg9
Rb1.20	13q14.3	-AATTAACAAGGTGTGGTGG -TGTATCGGCTAGCCTATC	Tetra-	446	0.940	gdb:171126 RB1.20
TP53	17p13.1	-ACTGCCACTCCTTGCCCCATTC -AGGGATACTATTCAGCCCGAGGTG	Di-	103-135	-	gdb:191095 TP53.PCR15
D18S34	18q12.2-18q12.3	-CAGAAAATTCTCTCTGGCTA -CTCATGTTCCTGGCAAGAAT	Di-	103-119	0.810	gdb:180546 Mfd26

were available from 35 patients. In addition, non-tumor adjacent to tumor tissue from 11 patients with bladder cancer, and non-tumor tissue and urine from 10 patients undergoing surgery for reasons unrelated to bladder cancer were obtained. The patients' mean age was 62±9 years (ranging from 46-77). Patient information is shown in Table I. Tissue collected included obvious tumor tissue that was confirmed by subsequent histopathological diagnosis at the Pathology Department of the Medical University of Gdansk. Samples were immediately frozen in liquid nitrogen and stored at -80˚C. DNA was extracted from frozen bladder tumor samples using DNA extraction kit (A&A Biotechnology, Gdynia, Poland) and stored at 4˚C.

Blood. Venous blood from each patient was collected in EDTA tubes. Leukocytes were isolated from 0.5 ml of whole blood, adding 1.5 ml TM buffer (5 mM $MgCl_2$; 20 mM Tris; pH 7.4) for 5 min of incubation at room temperature. Cells were collected by centrifugation at 700 x g for 10 min. The pellet was washed with 1 ml PBS buffer (0.8% NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 8 mM Na_2HPO_4; pH 7.4) three times to remove hemoglobine. Leukocyte DNA was isolated using DNA extraction kit (A&A Biotechnology) and stored at 4˚C.

Urine. Voided urine (50 ml) from each patient was centrifuged at 700 x g for 10 min at 4˚C. The pellet was washed with 25 ml PBS buffer (0.8% NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 8 mM Na_2HPO_4; pH 7.4) three times. DNA was isolated using DNA extraction kit (A&A Biotechnology) and stored at 4˚C.

Microsatellite analysis. A PCR-based method was used to assess the allelic loss and/or microsatellite instability occurring at loci which lie within or in close proximity to *MSH2* (2p16), *hMLH1* (3p21), *CDKN2A* (9p21), *PTCH* (9q22), *DBCCR1* (9q33), *Rb* (13q14), and *TP57* (17p13) genes, and at chromosomes 8p21, 18q12, and 12q21. The characteristic of the polymorphic loci is shown in Table II. Microsatellite alterations in matched leukocytes and tumor tissue were detected by evaluating either the allelic loss or length of poly(A) repeat (BAT26) by a poly(CA) repeat at eight loci (D2S123, D3S1611 D8S133, D9S273, D9S171, D12S81, TP53, D18S34), by a poly(CTTT) repeat (Rb1.20, D9S242), and by a poly(CTAT) repeat (D9S252). Primer sequences were taken from Genome Database and commercially synthesized (GenSet; Paris, France). PCR amplifications were performed with 100 ng of purified genomic DNA in a 25 μl reaction volume containing 0.4 μM of each primer and 200 μM dNTPs. PCRs were carried out using MJ Research PTC200 thermocycler. PCR parameters were 95˚C for 5 min, 94˚C for 30 sec, annealing temperature (46˚C for BAT26; 55˚C for D2S123, D3S1611, D9S242, Rb1.20, D18S34, D12S81; 60˚C for TP53, D8S133, D9S171, D9S273, D9S252) for 30 sec, 72˚C for 30 sec with 35 cycles, and then 5-min elongation at 72˚C. Subsequently, PCR products were analyzed by 6% polyacrylamide/8 M urea gel electrophoresis (2 h 1800 V, 47˚C) in a sequencing gel chamber (Owl Separation Systems) and by silver nitrate staining (13). The gel image was analyzed using GelDoc 2000 and LOH was scored by Quantity One software from Bio-Rad. LOH was determined from those loci that were heterozygous. The allelic ratio (R) was derived as the ratio of the peak heights

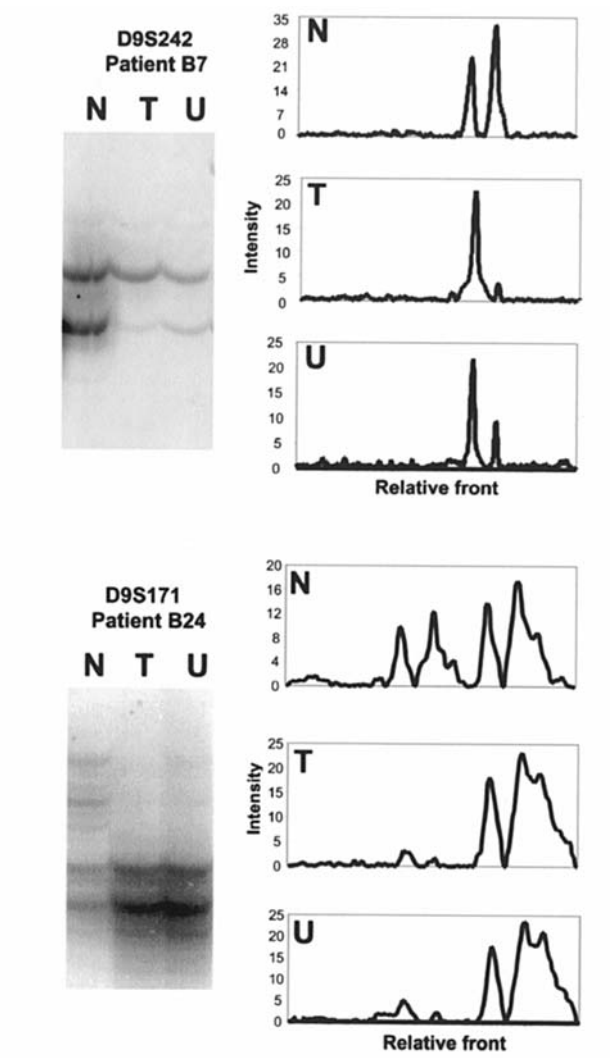

Figure 1. Detection of LOH on chromosome 9 in tumor and urine DNA from patients with bladder cancer. Two representative examples of microsatellite analysis for patient B7 and B18. Allelic profiles are shown on the left. L, normal DNA isolated from corresponding leukocytes; T, DNA from tumor tissue; U, DNA from urine sediment.

in the leukocyte-derived samples divided by the peak heights of the alleles from the tumor. If R<0.5 or >2, one of the alleles has decreased >50%, resulting in LOH (Fig. 1). Change in allele size or the appearance of a new band in tumor DNA was scored as a band shift. Samples were scored as positive when they had at least one marker with LOH or MSI. Every marker alteration (LOH or MSI) was confirmed by re-amplification of the starting material.

Statistical analysis. Based on available clinical and molecular data, the associations between the presence of microsatellite alterations in tumor or urine specimens and sex, age, stage and lymph node status were examined. Bivariate analysis using two-sided Fisher's exact test and Pearson's χ^2 test were performed where appropriate. In addition, the association between the presence of microsatellite alterations and overall survival (OS) was examined, OS defined as the time from primary therapy to death (from any cause) or last follow-up. The last follow-up evaluation was performed in November 2004. Total length of the study at the time of analysis was 56

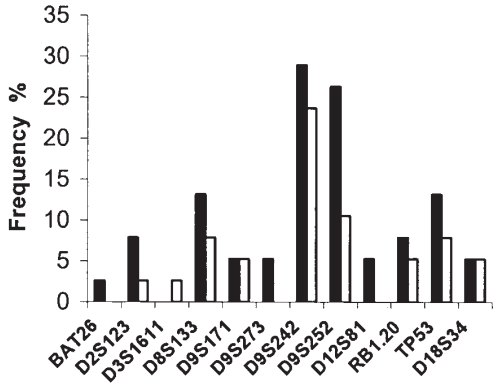

Figure 2. The frequency of microsatellite alterations at individual markers in tumor (black bars) and urine sediment DNA (white bars).

months and the median follow-up was 28 months (range, 2-56 months). In bivariate association, clinical variables were dichotomized as follows: age at diagnosis, ≤65 versus >65 years; tumor stage, Ta/T1 versus T2-T4. $P<0.05$ was considered statistically significant. The analyses were performed with the software Statistica version 6 (StatSoft, Inc. Tulsa, USA)

Results

Microsatellite analysis. We chose 12 microsatellite markers in order to study loci from different parts of the genome representing the regions of potential interest in bladder carcinoma. In our study, DNA obtained from tumor and urine sediment was compared with peripheral leukocytes for microsatellite alterations (LOH or/and MSI). The overall frequency of microsatellite alterations was 71% (27/38). Patients [39% (15/38)] had DNA alterations at >2 microsatellite loci. When stratified by stage, sensitivities were found to be 75% (9/12) for Ta/T1 stage tumors and 69% (18/26) for T2-T4 stage tumors (Table I). Moreover, microsatellite alterations found in tumor tissue were not observed in adjacent non-tumor tissue from eleven microsatellite analysis-positive patients with bladder cancer, and were not detected in both tissue and urine sediment from ten, age-matched non-tumor patients (100% specificity). The microsatellite alterations on chromosome 9 were found in 58% (7/12) of Ta/T1 tumors and in 31% (8/26) of T2-T4 tumors. Interestingly allelic losses on chromosome 9q were more frequently observed than on chromosome 9p. The frequency of alterations at individual markers ranged from 3 to 29% (Fig. 2). Two markers, D9S171 and TP53, which frequently changed in other studies (14,15), were quite stable in our group of bladder cancer patients, showing allelic loss in only 8% (3/38) and 13% (5/38) of cases, respectively. In contrast, two tetranucleotide markers, D9S242 and D9S252, were altered in 16 out of 27 (59%) microsatellite analysis-positive patients. The overall frequencies of microsatellite alterations at these two loci in tumor tissue were 26% (10/38) at D9S252 and 29% (11/38) at D9S242 (Fig. 2). These two markers were found to be equally altered in 42% (5/12) of Ta/T1 tumors. The D9S252 marker was lost or unstable in 23% (6/26) and D9S242 in 31% (8/26) of T2-T4 tumors. However, a statistically significant association between either of them and the stage was not found.

A

Sample \ Chromosome	Tumor 2	3	8	9	12	13	17	18	Urine 2	3	8	9	12	13	17	18	Stage of tumor
B1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Ta
B2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Ta
B3	-	-	-	-	-	-	-	-	-	■	-	-	-	-	-	-	T1
B4	-	-	-	■	-	-	■	-	-	-	-	■	-	-	■	-	T1
B5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T1
B6	-	-	-	■	-	-	-	-	-	-	-	-	-	-	-	-	T1
B7	-	-	-	■	-	-	-	-	-	-	-	■	-	-	-	-	T1
B8	-	-	-	■	-	-	-	-	-	-	-	■	-	-	-	-	T1
B9	-	-	-	■	-	-	-	-	-	-	-	-	-	-	-	-	T1
B10	-	-	-	-	-	-	-	-	-	-	-	■	-	-	-	-	T1
B11	-	-	-	■	-	-	-	-	-	-	-	-	-	-	-	-	T1
B12	-	-	-	-	-	-	-	-	-	-	-	■	-	-	-	-	T1
Summary:	0	0	0	7	1	0	1	0	0	1	0	5	0	0	1	0	

B

Sample \ Chromosome	Tumor 2	3	8	9	12	13	17	18	Urine 2	3	8	9	12	13	17	18	Stage of tumor
B13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T2
B14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T2
B15	-	-	-	■	-	-	-	-	-	-	-	-	-	-	-	-	T2
B16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T2
B17	-	-	■	-	■	-	■	-	-	-	-	-	-	-	■	-	T2
B18	-	-	-	■	-	-	■	-	-	-	-	■	-	-	-	-	T2
B19	-	-	-	-	-	-	■	-	-	-	-	-	-	-	-	-	T3
B20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T3
B21	■	-	-	-	-	■	-	-	■	-	-	-	-	-	-	-	T3
B22	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T3
B23	-	-	■	■	-	-	-	-	-	-	■	■	-	-	-	-	T3
B24	-	-	■	■	-	-	-	-	-	-	■	■	-	-	-	-	T3
B25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T3
B26	-	-	■	-	-	-	-	-	-	-	-	-	-	-	-	-	T3
B27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T3
B28	■	-	-	-	-	■	-	-	-	-	-	-	-	-	-	-	T3
B29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T3
B30	-	-	-	■	-	-	-	-	-	-	-	-	-	-	-	-	T3
B31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T4
B32	-	-	-	■	-	-	-	-	-	-	-	■	-	-	-	-	T4
B33	-	-	-	-	-	-	-	■	-	-	-	-	-	-	-	■	T4
B34	-	-	-	-	■	-	-	-	-	-	-	-	-	-	-	-	T4
B35	■	-	-	-	-	-	-	■	-	-	■	-	-	-	-	■	T4
B36	-	-	-	■	-	-	■	-	-	-	-	■	-	■	■	-	T4
B37	-	-	■	-	-	-	-	-	-	-	-	-	-	-	-	-	T4
B38	-	-	-	■	-	■	-	-	-	-	-	■	-	■	-	-	T4
Summary:	4	0	5	8	2	3	4	2	1	0	3	6	0	2	2	2	

Figure 3. Summary of raw data after LOH/MSI analysis of tumor and urine DNA. A, stage Ta/T1 tumors; B, stage T2-T4 tumors.

MSI was a very rare event in this group of patients. A shift in the electrophoretic mobility of the amplified fragment was observed in 8 out of 53 (15%) tumor microsatellite alterations, and was found in 6 out of 38 (16%) patients. Five of these 6 patients also showed LOH, all six MSI-positive patients had muscle-invasive tumors. The instability at the mismatch repair genes MSH2 (BAT26, D2S123) and hMLH1 (D3S1611) was found only in five cases. Thus, suggesting that another mechanism than mutation in MMR genes is responsible for genomic instability observed in transitional cell carcinoma of the bladder.

In urine sediment, microsatellite alterations were found in 46% (16/35) of bladder cancer patients (Table I). The sensitivity was 60% (6/10) for Ta/T1 tumors and 40% (10/25) for T2-T4 tumors. Twenty of fifty-one (39%) tumor DNA alterations were re-detected in urine sediments. The pattern of microsatellite alterations in tumor tissue with that found in urine sediments is shown in Fig. 3, and the frequency of alterations at selected locus in Fig. 2. In addition 7 alterations found in urine sediments were not observed in corresponding tumor specimens.

Correlation between clinicopathological parameters and survival analysis. We found no association between micro-

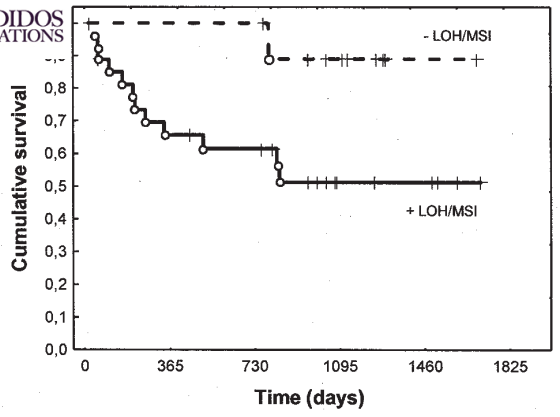

Figure 4. Cancer specific overall survival for bladder cancer stratified by the LOH/MSI status. P=0.04, log-rank test.

satellite alterations at one or more microsatellite markers and any of prognostic parameters (Table I). In addition, changes at any given microsatellite marker were not associated with any clinical parameters as the group of patients may have not been large enough. The Kaplan-Meier method and log-rank test were used to evaluate the effect of microsatellite alterations in at least one locus on overall survival for 38 patients with bladder cancer. The overall 4-year survival was 66%. There was a significant association between the shorter survival and both stage (P=0.03) and microsatellite alterations (P=0.04, Fig. 4). Tumor-specific 4-year survival was 91% for patients without DNA alterations (n=11), whereas it was 56% (n=27) for patients with DNA alterations in at least one locus.

Discussion

In the present study, we aimed to compare the sensitivity of the microsatellite analysis (LOH and/or MSI) of urine sediment with that of tumor tissue, and determine which of the 12 markers or combination of markers have the highest potential for predicting the presence of bladder cancer. Microsatellite alterations were found in 75% (9/12) of Ta/T1 stage tumors, and in 69% (18/26) of T2-T4 stage tumors. Microsatellite instability represented only 8 out of 53 (15%) tumor microsatellite alterations. Conventionally, the diagnostic criterion of an MSI tumor relies upon the number of unstable loci tested, ranging from 2 to >100, and the number of loci required to score a tumor as MSI usually ranges from 1 to 2 (16). Recently, it has been shown that the microsatellite marker, BAT 26, is sensitive to MSI, even if studied as the one and only locus (17). The likelihood of MSI in bladder cancer varies from 0 to 100% (11,12,18). Bonnal *et al* did not found any MSI in 33 TCCs and four bladder cancer cell lines using 6 microsatellite markers, including mono- and dinucleotide repeats (11). Gonzalez-Zulueta *et al* (19) determined microsatellite instability in only 3% of bladder tumors using six microsatellites in a group of 200 TCCs while Mao *et al* (4) found alterations in 40% of cases using thirteen microsatellite markers in a group of 25 TCCs. In another study, 100% instability was reported for 14 cases of bladder tumor with a set of 22 microsatellites used in the study (12). In our study we have shown that microsatellite instability is a rare event compared with loss of heterozygosity in bladder cancer, and these results are in agreement with recently published data by Saetta *et al* who found MSI in 16.6% (12/72) of bladder tumors using six microsatellite markers (20). Therefore, the disconcordance between some reports and our results may be due to differences in the number and type of microsatellite markers. Also, we expect some differences related to the studied population and macroenvironment as some markers with a high rate of instability in one study display a low rate of instability in another. Nevertheless, the high frequency of MSI found in some bladder cancer studies may suggest that the same type of genetic defects in MMR genes that are observed in hereditary non-polyposis colorectal cancer (HNPCC) can occur somatically in sporadic cancers (4,21). As inactivation of MMR genes in sporadic cancers is incompletely defined (22), in the present study BAT26, D2S123 and D3S1611 were selected to give information on MSI related to MMR defects (16). In contrast to the results of Christensen *et al* (12), who reported the occurrence of LOH at microsatellites located close to DNA mismatch genes, *hMSH2* and *hMLH1,* our study has not revealed the frequent occurrence of alternations in the structure of *hMLH1* using D3S1611 or *hMSH2* using BAT26 and D2S123 microsatellites. The low level of genetic defects in MMR could be responsible for the low frequency of MSI observed in our study. Although it cannot be ruled out that analysis of more microsatellites would reveal unstable loci. Further analysis with a broader panel of microsatellites should be performed. However, our results do not exclude involvement of the mutator genes in tumorigenesis and/or progression of TCC of the bladder. Other alterations, such as point mutations and DNA cytosine methylation, may be responsible for MMR gene inactivation (23).

In this study, a panel of 12 microsatellite markers allowed us to determine the microsatellite alterations in 71% (27/38) of tumors, which corresponds with the findings by Utting *et al* (14). In agreement with the general concept that the microsatellite alterations on chromosome 9 are the most frequent lesions in TCCs (reviewed in ref. 24), allelic losses on chromosome 9 were the most frequently observed alterations, suggesting inactivation of *CDKN2A* at 9p21, *PTCH* at 9q22 and *DBCCR1* at 9q33. Using only two tetranucleotide markers on chromosome 9q (D9S242 and D9S252) that showed alteration rates of 26 and 29%, sixteen out of 27 (59%) microsatellite analysis-positive patients were identified. A higher frequency of allelic losses found on chromosome 9q in comparison with losses on 9p is consistent with the findings by Kimura *et al* (25). Moreover, our data supports the study by Berger *et al* which showed that a 25% alteration rate per microsatellite locus represents a reasonable limit for the selection of microsatellite markers that could be used in clinical practice (26).

The high potential of microsatellite analysis for non-invasive diagnosis of bladder cancer based on urine DNA analysis has been addressed in many studies. In the classic study by Mao *et al*, microsatellite alterations matching those in tumors have been detected in the urine sediment of 95% of patients (4). Application of fluorescent microsatellite analysis allowed for the detection of tumor-specific urine DNA alterations in 27 (72%) of 39 cases (27). In contrast, in the study by Fornari *et al* (28), LOH has been found in 49%

(16/33) of T1-T4 bladder tumors. In our study, tumor-specific microsatellite alterations in exfoliated cells in urine were found in 46% (16/35) of patients. In total, 39% (20/51) of all alterations were re-detected in urine sediments, which is not satisfactory; however, it is higher than the 27% found in the study by Utting *et al* (14). These findings suggest that the failure to detect microsatellite alterations in urine sediment may be due to the presence of a mixture of cancer, normal and inflammatory cells. The sensitivity of the technique used for the analysis is also important. The comparison analysis of three different methods for the detection of microsatellite alterations demonstrated that the fluorescence method is more sensitive than autoradiography and silver-stained gels (29). In addition, there is a limit of detection and the positive cells should represent at least 10% of the total number of cells in a sample (30). In our study, a 50% reduction of signal between two peaks was regarded as LOH while, in some studies, it was reduced to 30%. Reducing the threshold for LOH scoring improves the sensitivity and substantially reduces the specificity, as has been shown by Little *et al* (31). In their study, the initial frequency of LOH in urinary DNA was 49% (18/37) and the specificity was 89% (68/76) and, after reduction from 50 to 30%, the sensitivity increased to 73% and specificity decreased to 63%. In our study, the higher frequency of microsatellite alterations found in tumors in comparison with urine sediment using 50% reduction in peak height as indicative of LOH suggests that it was not the only reason for the low frequency of DNA alterations in urine. A possible explanation for this observation might be that the urine of bladder cancer patients contains leukocytes (32). As in a study by Primdahl *et al* (33) who found the discrepancies between allelic loss in tumor and urine sediment in 3 loci, we also found 7 alterations in 6 loci in urine sediment only. However, in our study the instability in urine sediments was independent of the stage of bladder cancer patients. The presence of microsatellite alterations in urine sediment DNA but not in corresponding tumor tissue has also been reported by Mao *et al* and Berger *et al* (4,26). Considering that genomic instability may increase with progression, the above observations could result from the presence in the urine sample of a more advanced clonal population of cells that were omitted from the tumor specimen. Nevertheless, a very high specificity (100%) makes microsatellite analysis of urine sediment DNA a very good adjunct to cystoscopy and/or cytology.

It has been reported that there was no difference in patient survival when the patients were categorized by allelic loss (34). In contrast, Uchida *et al* showed that patients with multiple LOHs at both 18q21.1 and 9p21-22 had a much worse prognosis than single LOH or no LOH patients (35). In the study by Tzai *et al*, it has been found that the combined loss of heterozygosity on chromosomes 9p and 14q was associated with a worse prognosis, compared with other patterns of microsatellite alterations by univariate analysis (36). According to our univariate analysis, the microsatellite alterations in at least one locus were significantly associated with a shorter survival.

In conclusion, our data have shown that MSI in comparison with LOH is a rare event in bladder cancer and is related to a low rate of defects in MMR genes. Moreover, two markers, D9S242 and D9S252, with a high rate of alterations were especially useful in the analysis of genomic instability in bladder cancer, which suggests that the identification of markers with elevated genomic instability at tetranucleotide repeats should have a potential application for the molecular detection of bladder cancer. Moreover, a low frequency of microsatellite alteration in urine sediment and some discrepancies between alterations observed in tumor tissue and urine suggest that this type of analysis awaits validation for the non-invasive diagnosis of bladder cancer.

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