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Under-representation of Bladder Transitional Cell Tumour 9q, 11p and 14q LOH in Urine and Impact on Molecular Diagnosis

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Abstract. *Background: To investigate whether the recently reported evidence of differences in the overall loss of heterozygosity (LOH) frequency between urine and tumour tissue in patients with transitional cell tumours (TCT) of the urinary bladder involved specific chromosomal sites, and their impact in diagnosis. Materials and Methods: Blood, tissue and urine specimens were obtained from 55 patients and 25 controls. Sixteen microsatellites were PCR-amplified and blindly analyzed for LOH through a laser-based capillary electrophoresis system. Results: Significant frequence differences between tumour tissue and urine sediment LOH were found in 9q and 11p in non-invasive disease and 14q in invasive disease. There was no significant difference for all the other chromosomal arms analyzed. Conclusion: The contribution in the urine sediment of cells belonging to tumours of the same histological classification differs according to the specific genetic alterations these cells carry. Furthermore, the location regarding these differences could indicate regions involved in tumour exfoliation or apoptosis.*

Transitional cell tumours (TCT) of the bladder are the most common malignancies of the urinary tract. Non-invasive tumours have the propensity for recurrence and a 15% risk of progression to invasive disease (1). Close patient follow-up for tumour recurrence is necessary and is performed routinely by cystoscopy and cytology. Cystoscopy offers direct vision of the bladder and the possibility of endoscopic biopsy. However, cystoscopy is invasive and expensive. Cytology, on the other hand, is non-invasive and less expensive, but also less sensitive than cystoscopy for low grade tumours (2, 3). The ideal alternative to the above diagnostic methods should be non-invasive, inexpensive, highly specific and sensitive. One potential biomarker candidate is microsatellite alterations in the urine sediments (MAUS) of patients (4, 5). Early studies reported an almost complete concordance between the loss of heterozygosity (LOH) of microsatellite markers in the urine sediment and the tumour tissue (4, 1), while disparities have been found in recent studies (6, 7). However, these disparities have been reported as overall percentage differences between the alterations (LOH) in the urine sediment and tumour tissue and not to the specific genetic alterations in the tumour tissue.

There is growing evidence of specific microsatellite alterations in tumour tissue providing prognostic value (8-10). It is therefore essential to know to what extent these alterations are represented in the urinary sediment, thus affecting both the sensitivity and specificity of diagnostic procedures based on MAUS. Does the previously mentioned difference in percentage of microsatellite alterations between the urine and the tumour equally regard all alterations independently of chromosomal site, or is there a difference between them? In order to answer this question, the prevalence of specific chromosomal changes were assessed using 16 markers, in paired specimens of tumour tissue and urine sediment of 55 patients with histologically confirmed bladder TCT.

Materials and Methods

Patients. Paired blood, urine and tissue specimens were obtained from 72 patients suffering from bladder tumours and from 25 control patients at Herlev University Hospital of Copenhagen, Denmark. Histopathological classification was disclosed only after the microsatellite analysis had been completed. It followed that data from 17 patients could not be included in the study either because of co-presence of prostatic cancer or total resection of

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tumours 1-2 weeks before sample collection. Therefore, the data from 55 patients with bladder tumours (male/female ratio: 1.75, age range: 55-91 years and median: 70.96 years) and 25 controls (with benign prostatic hyperplasia (BPH) and with interstitial cystitis (IC)) (male/female ratio: 24/1, age range: 40-96 years and median: 67.08 years). Six of the BPH patients presented inflammation in their urine samples. Thirty-five tumour biopsies were obtained from transurethral resections (TUR) and 20 from cystectomies. All 55 patients had TCT as assessed by routine histology. The tumours were graded according to Bergquist *et al*. (11) and staged according to the tumour-node-metastasis (TNM) system (Table I). (According to the WHO grading (12), all grade I and most of grade II belong to the neoplasms of low malignant potential. Whereas, few grade II and the remaining grade III and IV belong to the malignant lesions).

All control specimens were obtained from TUR. There were no bladder tumours present in the controls.

The Copenhagen Ethical Committee, Denmark, approved the study.

Blood treatment. Three millilitres of venous blood were drawn in tubes containing EDTA the morning prior to surgery and were centrifuged at 2,860 xg. The leukocyte fractions were collected and used for DNA extraction.

Urine treatment. Two hundred millilitres of voided urine were collected and divided into 50 ml tubes on the morning of surgery. The tubes were centrifuged at 4,211 xg for 10 min and the supernatants discarded. The remaining sediment was diluted up to 50 ml with PBS (pH: 7.2-7.4). The centrifugation and discarding of the supernatant were repeated before DNA extraction of the urine sediment.

Tissue treatment. Biopsies were divided into small pieces using a new sterile blade for each.

DNA extraction. DNA extractions from the leukocyte fractions, the urine sediments and the tissue samples were done in separate sessions with QIAamp® DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), using the manufacturer's protocols.

The DNA concentrations were determined spectrophotometrically at 260 nm.

Primers. Sixteen highly polymorphic dinucleotide (CA)n microsatellites, (D3S1285, D4S1540, D4S394, D6S419, D8S259, D8S513, D9S157, D9S167, D11S907, D11S929, D13S175, D14S288, D14S51, D17S654, D17S261 and D17S786), located on chromosomal arms 3p, 4p, 4q, 6q, 8p, 9p, 9q, 11p, 13q, 14q and 17p were amplified by PCR. The primer sequences and the amplified fragment lengths were retrieved from the human genome database (http://www.gdb.org/). The 5' end of the forward primer of each primer pair was labelled by a fluorescent dye (HEX, TET, or 6-FAM).

PCR. The leukocyte, urine and tissue DNA amplifications were performed in a 25 μl reaction volume containing 7.5 pmol of each primer, 1.25 units of AmpliTaq® DNA polymerase (Applied Biosystems, Foster City, CA, USA), 0.2 mM of each dNTP, 2.5 μl of 10x buffer with 1.5-3.0 mM of $MgCl_2$ and 70-90 ng of DNA according to the primer pair. The PCR was carried out in a GeneAmp® PCR System 9700 (Applied Biosystems) using the following programme: an initial denaturation step of 4 min at 95˚C, followed by 25 cycles of denaturation-annealing-extension (30 sec at 94˚C, 30 sec at 60˚C and 30 sec at 72˚C) and a final extension step of 7 min at 72˚C (5, 13).

Table I. *Classification of tumours according to grade (gr.) and stage (pT).*

	gr. I	gr. II	gr. III	gr. IV	Total
pTa	1	20	1	0	22
pT1	0	3	7	3	13
pT2	0	0	9	2	11
pT3	0	0	2	3	5
pT4	0	0	3	1	4
Total	1	23	22	9	55

Microsatellite analysis. One microlitre of each PCR product was mixed with 12 μl of H_2O and 0.5 μl of internal lane standard GENESCAN-500. Each sample mix was analyzed in a laser-based, capillary electrophoresis system, ABI PRISM™ 310 Genetic Analyzer, and the data were processed by GeneScan™ software (Applied Biosystems).

Loss of heterozygosity (LOH) assessment. LOH was scored for every heterozygote microsatellite, where a difference greater than 20% was observed between the allelic ratio of the urine or tissue and the reference (leukocyte) DNA (14).

Statistics. All significance tests were performed by use of Fisher's exact test and all values of $p \leq 0.05$ were considered significant.

Results

Overall prevalence of LOH in urine sediment and tumour tissue with relation to tumour grade and stage. The prevalence of LOH was 49% (27/55) in the urine sediment and 78% (43/55) in the tumour tissue of patients with bladder tumours and did not relate to tumour grade or stage. There were some rare cases of LOH in controls (12% and 8% in urine and tissue, respectively), all of which were borderline LOH (the difference between the allelic ratio of the urine or tissue and the reference DNA was >20% and ≤30%). Borderline LOH and inflammation were not related.

Concordance between LOH in urine sediment and tumour tissue. LOH was observed in both the urine and tissue of 23 patients of whom 22 (96%) shared at least one common LOH. In total 87 markers showed a LOH in the urine sediment of which 69% (60/87) matched the tissue LOH.

A total of 142 markers with LOH were seen in tumour tissue samples.

Chromosome-specific LOH in urine sediment and tumour tissue related to tumour grade and stage. The presence of LOH at 13q in urine sediment related to a high tumour stage ($p=0.04$), but we failed to demonstrate this relation in tumour tissue. No other chromosome-specific LOH relations were found between urine sediment and tumour tissue grade and stage.

LOH at 6q in tumour tissue was associated with high grade ($p=0.01$) and stage ($p=0.02$), loss at 14q was associated with high grade ($p=0.05$), while loss at 9p was associated with low grade ($p=0.03$) and stage ($p=0.05$). The remaining chromosome-specific LOH in tumour tissue did not relate to tumour grade or stage.

Comparison between chromosome-specific LOH in urine sediment and tumour tissue. The prevalence of LOH in 9q, 11p and 14q was significantly higher in tumour tissue than in urine sediment ($p=0.01$, $p=0.02$ and $p=0.02$, respectively). No significant differences were found between the prevalence of urine and tissue LOH in the remaining chromosomes.

Classification according to tumour grade and stage showed that only patients with low grade transitional cell tumours had a significantly higher prevalence of LOH in 9q in tissue than in urine ($p=0.05$). Similarly, the higher prevalence of LOH in 11p was only significant in low grade ($p=0.03$) and stage ($p=0.05$) disease. In contrast, the higher prevalence of LOH in 14q was only significant in high grade ($p=0.02$) and stage ($p=0.03$) disease.

Neither multiple, nor small (≤2 cm) tumours showed any correlation with 9q, 11p or 14q tumour LOH under-representation in urine.

Discussion

Previous studies have investigated the diagnostic value of urine and tissue microsatellite alterations in several chromosomal arms (4, 1, 5, 13). In the present study, for the first time to our knowledge, the differences between urine and tissue chromosome-specific alterations were studied. We analyzed for LOH 16 microsatellites on 11 chromosomal arms of 55 patients with TCT and 25 controls. Most interestingly, we found that the prevalence of LOH in 9q, 11p and 14q was significantly lower in urine sediment than in tumour tissue in low grade, non-invasive and in invasive transitional cell tumours, respectively.

The above disparities were probably not caused by difference in the access of tumour cells to the urinary tract, as they were both observed in non-invasive and invasive disease. Moreover, analysis revealed that neither could they be explained by the presence but not sampling of other tumours in case of multiple tumours, nor by the presence of tumours of reduced size.

The results of the present study are important for two reasons. First, they indicate that the contribution of tumour cells in the urine sediment differs according to which microsatellite alterations these cells harbour. This could suggest a difference in the physiological behaviour of cell clones belonging to tumours of the same histological classification. The reduced shedding/exfoliation may be the result of a drop in tumour senescence, and/or apoptosis caused by a loss of tumour suppressor genes in the regions surrounding our 9q, 11p and 14q markers. The presence of a tumour suppressor gene (TSG) candidate has been long postulated in 9q22.3, near the location of our LOH marker. This region contains the locus for Gorlin Syndrome and Multiple Self-Healing Squamous Epithelioma (15, 16). Similarly, 11p13 is a region containing a putative TSG (17).

Secondly, urine analysis of markers lying in the above regions could be insufficient if not complemented by the correspondent analysis in tissues, thus reducing the advantage of MAUS, being non-invasiveness.

Furthermore, we found that the method had a specificity of 88% for urine LOH and 92% for tissue LOH. Such specificities have been previously observed in both urine (18) as well as tissue LOH (19). It would be of clinical relevance to investigate whether these alterations are early predictive markers.

We found no association between the overall prevalence of LOH in urine sediment and tumour tissue and tumour grade and stage in accordance with most previous studies (7, 20-22), and in contrast to a single report (23).

Additionally, we found that a total of 69% of the urine markers showing LOH were tumour-specific, contrary to the earliest studies reporting a 100% tumour specificity of urine LOH (4, 1), but in agreement with more recent studies showing various degrees of discrepancies between urine and tissue LOH (6, 7, 22-24).

The association between chromosome-specific LOH found in urine or tissue LOH and tumour grade or stage also accords with most previous studies (25-28, 16, 29-31), with the exception of a single report (32).

In conclusion, transitional tumour cells of the bladder carrying LOH in 9q, 11p and/or 14q are significantly under-represented in the urine sediment compared to tumour cells having LOH on other chromosomal arms. This could indicate regions involved in tumour exfoliation or apoptosis. The drop in the LOH frequency observed in 9q, 11p and 14q of urine sediment should be taken into account when performing MAUS without concomitant tumour collection, as this may lead to a reduced diagnostic and/or prognostic sensitivity.

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