Molecular Serological Detection of DNA Alterations in Transitional Cell Carcinoma Is Highly Sensitive and Stage Independent

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ABSTRACT

Purpose: To evaluate the efficacy of fluorescent microsatellite analysis (MSA) for the serological diagnosis of transitional cell carcinoma (TCC) of the urinary tract analyzing free tumor DNA in the serum of cancer patients.

Experimental Design: We applied fluorescent MSA to detect serum–DNA alterations in patients suffering from bladder and upper urinary tract TCC and prospectively collected fresh tumor, peripheral blood, and serum specimens from 61 consecutive patients to obtain the corresponding DNA. Fluorescent MSA was performed with a total of 17 polymorphic markers from the chromosomal regions 5q, 8p, 9p, 9q, 13q, 14q, 17p, 17q, and 20q in the 61 cancer patients, as well as in 20 healthy controls.

Results: Molecular serological analysis led to tumor-specific diagnosis of TCC in 80.3% (49 of 61) of cases. Four healthy controls displayed serum–DNA alterations rendering a specificity of 80%. The highest frequency of serum–DNA alterations was detected for chromosomal region 8p with 36%. Chromosomes 5q, 9p, and 20q showed serum–DNA alterations in 18 to 21%. The identification of serum–DNA alterations was not statistically associated with underlying local tumor stage (P = 0.29) but was more frequent in high-grade tumors (P = 0.08).

Conclusions: MSA offers a highly sensitive method for serological diagnosis of TCC. To optimize specificity, simultaneous analysis of tumor DNA is advised to rule out artifacts resembling allelic imbalance in MSA of serum DNA.

INTRODUCTION

Transitional cell carcinoma (TCC) accounts for ~3% of all newly diagnosed malignancies in Western countries. Within the TCCs, >90% of the tumors derive from the bladder, whereas the remainder arise from the upper urinary tract, namely the renal pelvis and the ureter. To date, a reliable serological tumor marker is not available. In contrast to most other tumors, TCCs offer the opportunity of noninvasive diagnosis via urinary cytology or other urine bound cytological tests. Urine cytological tests can be applied for the primary diagnosis of all TCCs and during follow-up for initially superficial cancers, which were treated by transurethral resection, preserving the bladder. In case of primarily invasive bladder cancers, radical cystectomy has to be performed. Local and distal recurrences of TCC can then only be diagnosed morphologically by various imaging modalities, such as computed tomography and magnetic resonance imaging scan. A reliable serological marker would ideally detect recurrence at an earlier stage with a low tumor burden and raise the effect of systemic cytoreductive therapy. In recent studies, molecular techniques have proved applicable for the detection of smallest amounts of free circulating tumor DNA in serum and plasma of cancer patients (1, 2). In one of the first studies investigating free serum–DNA alterations in transitional cell bladder cancer using the microsatellite analysis (MSA), we achieved a sensitivity for tumor-specific serological diagnosis of 84.5% in 39 bladder cancer patients (3). For this study, the specificity was estimated at 100% after applying the method on 10 healthy controls. Investigating free serum–DNA alterations in 53 renal cell carcinomas (RCC) with the same molecular method only rendered a 85% specificity in 20 healthy controls with a sensitivity of 74–87%, depending on the number of microsatellite markers incorporated in the investigation (4). This study was the first not to testify a 100% specificity for MSA of free circulating serum DNA in cancer patients. In this context, we now extended our study on TCC of the bladder and upper urinary tract investigating a total of 61 patients and 20 healthy controls using MSA with 17 microsatellite markers for chromosomes 5q, 8p, 9p, 9q, 13q, 14q, 17p, 17q, and 20q. The larger cohort size of patients and controls now served as a representative basis for estimating sensitivity and specificity of this molecular method for serological diagnosis of TCC.

MATERIALS AND METHODS

Tumor and Blood Sampling. Since 1999, preoperative peripheral blood samples (10 ml of EDTA and 10 ml of full blood samples) were prospectively collected from 61 consecutive patients treated for TCC (58 bladder cancers and 3 renal pelvis cancers). A representative fresh tumor biopsy was obtained intraoperatively either by transurethral resection or resection from the cystectomy and nephrectomy specimens. Particular attention was paid resecting a “clean” tumor sample not contaminated by healthy urothelial tissue. All samples were immediately shock frozen in liquid nitrogen and stored at −80°C before further processing. The full blood serum specimen was centrifuged for 10 min at 3000 x g to obtain the serum from the supernatant, and the isolated serum was then stored as...
above. Additionally, 10 ml of EDTA blood and 10 ml of full blood samples were collected from 20 healthy individuals (10 men and 10 women) as a control group. The pathohistological diagnosis of the main tumor specimen was undertaken by the Department of Pathology at the Philipps-University Marburg Medical School according to the Unio Internationale Contra Cancrum classification of 1997. Informed consent was obtained from the patients.

**DNA Isolation.** A 5–10-mm piece of fresh frozen tumor tissue was used for tumor–DNA isolation as published previously (3). By the technique applied, the contamination of the tumor DNA with healthy tissue DNA was reduced to a minimum. To obtain the corresponding normal DNA, the same method was applied with peripheral blood lymphocytes from the 10 ml of EDTA blood samples. DNA extraction from the serum samples was carried out with the Qiamp Midi-Kit (Qiagen, Hilden, Germany) according to the protocols for blood and body fluids supplied by the manufacturer. Serum DNA extraction was performed with 2–4 ml of clear serum supernatant obtained after centrifugation of 10 ml of full blood samples at 3000 × g for 10 min. Serum–DNA concentrations were measured photometrically.

**MSA and PCR Conditions.** For the identification of tissue and serum alterations, 17 highly polymorphic markers for the chromosomal regions 5q, 8p, 9p, 9q, and 20q (D5S1720, D5S476, D8S261, D8S560, D9S171, D9S925, D9S15, D13S153, D14S750, D14S61, D14S267, D17S799, D17S1306, D20S486, D20S607, D20S481, and D20S480) were used in the PCR-based MSA. DNA sequences for the microsatellite markers were obtained from the genome database and are shown in Ref. 3. Fifty to 100 ng of normal, tumor, and serum DNA were used as templates in 10 μl of PCR reactions as published previously (3). Fragment analysis was performed on an automated DNA laser sequencer (ALFexpressII; Amersham Pharmacia Biotech, Freiburg, Germany). Results were computed using the Fragment Manager (FM 1.02; Amersham Pharmacia Biotech) software. Allelic imbalance (AI) in heterozygous PCR products was described as loss of heterozygosity (LOH) or deletion, when loss of genetic information was known to occur for this chromosomal region, but AI could also describe differences in allele intensity caused by genetic gain from, e.g., duplication, when known for the specific site. AI caused by either loss or gain was summarized as alteration of the genetic locus.

The sensitivity of each microsatellite marker in detecting tumor-specific alterations in serum was evaluated independently and in combination with the other markers. The sensitivity was determined by measuring the overall rate of identified alterations in serum, as well as measuring the proportion of identified alterations as a fraction of the rate of alterations observed in the tumor. Serum–DNA alterations were only accepted if they were also detectable in the tumor DNA. The specificity of the method for detecting serum–DNA alterations was investigated by applying the analysis on 20 healthy controls with all 17 markers.

**Statistical Analysis.** To identify possible associations between detected serum–DNA alterations and either tumor stage and/or grade, Student’s t test and the Mann-Whitney test were used. A P ≤ 0.05 was regarded as significant.

**RESULTS**

**Histopathological Staging and Grading of Tumors.** The distribution of the 61 primary TCCs (58 bladder cancers and 3 renal pelvis cancers) investigated according to the Tumor-Node-Metastasis system (Unio Internationale Contra Cancrum 1997) and nuclear grading is shown in Table 1.

**MSA of Tumor DNA.** Results for the frequencies of tumor–DNA alterations at the nine chromosomal regions investigated with the 17 polymorphic microsatellite markers are shown in Fig. 1. The highest incidence of alterations was observed for chromosomes 8p in 57% and 20q in 54% of cases.

**MSA of Serum DNA.** Genomic DNA was detectable in each investigated serum sample. Taking all 17 microsatellite markers together, the method yielded a sensitivity of 80.3% (49 of 61) in identifying tumor-specific serum–DNA alterations, permitting the serological diagnosis of TCC with a specificity of 80% (see below). 8p-LOH at loci D8S261 and D8S560 was observed with the highest combined incidence of 36% (22 of 61). Chromosomes 5q, 9p, and 20q showed serum–DNA alterations in 18–21%. The frequency of alterations at the other investigated chromosomal regions is shown in Fig. 1. By performing the assay with only the six markers with the highest sensitivity (D8S261, D8S560, D9S171, D9S925, D9S15, and D20S486) for chromosomes 8p, 9p, 9q, and 20q, the method accomplished a serum tumor diagnosis in 57% (35 of 61). An AI in the serum DNA was only regarded as an alteration if the identical alteration was also present in the tumor DNA. Rare cases of AI only in the serum DNA were interpreted as artifacts without diagnostic relevance. Adhering to this methodological approach, artifacts are ruled out. Nevertheless, discordant AI in the serum may also represent tumor DNA with a different clonal origin from a heterogeneous tumor having gained access to the vasculature. The identification of serum–DNA alterations was not statistically associated with underlying tumor stage (P > 0.05) but was more frequent in high-grade tumors (P = 0.08;

<table>
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<th>G2</th>
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<th>Total</th>
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<td>13</td>
<td>—</td>
<td>26</td>
</tr>
<tr>
<td>pTis</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
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<td>pT1</td>
<td>2</td>
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<td>—</td>
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<td>12</td>
</tr>
<tr>
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<td>—</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>pT3b</td>
<td>—</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>pT4a</td>
<td>15</td>
<td>28</td>
<td>15</td>
<td>58</td>
</tr>
</tbody>
</table>

The 3 primary TCC renal pelvis tumors were histologically classified as pT2, pT3, G3, and pT3, G3.


**Table 1** Distribution of 58 primary bladder tumors according to the TNM system (UICC 1997)*

* TNM, Tumor-Node-Metastasis; UICC, Unio Internationale Contra Cancrum; TCC, transitional cell carcinoma.
In 4 of 20 controls (20%), we identified a total of seven PCR artifacts resembling an AI. In relation to the total number of PCRs performed (n = 340), the rate of artifacts is 2.1%. Nonetheless, the specificity of the method remains to be 80%. This specificity is in accordance to the observation of discordant tumor unspecific AI identified at a rate of 0–10% depending on the microsatellite marker used (Table 2). The average DNA concentration of all serum specimens was 169 ng/ml and slightly higher in those cases with an alteration (179 ng/ml).

DISCUSSION

During follow-up for TCC of the bladder and the upper urinary tract after radical surgery for invasive disease, clinicians have to rely on imaging studies to morphologically detect distant tumor spread or local recurrence, because currently, there are no tumor makers available. An ideal serological tumor marker could identify occult micrometastatic disease before it becomes morphologically apparent and would therefore raise the chance of systemic cytoreductive therapy attributable to a lower tumor load.

In this study, we applied MSA with a panel of 17 fluorescently labeled markers from the chromosomal regions 5q, 8p, 9p, 9q, 13q, 14q, 17p, 17q, and 20q to detect tumor-specific alterations in circulating serum DNA from patients with TCC of the bladder and upper urinary tract. We had recently published data on the evaluation of 39 bladder cancer patients and 10 healthy controls achieving a sensitivity of 84.5% (33 of 39) and a specificity of 100% with the same set of microsatellite markers (3). Applying the MSA on 53 patients with RCC and 20 healthy controls, the method in our hands yielded a sensitivity of 74–87%, whereas the specificity was calculated at 85% (4). With these results, we were prompted to extend our investigation on 61 patients with TCC and also 20 healthy controls. In an almost doubled cohort size, we were able to confirm our recent data regarding sensitivity for tumor-specific serological diagnosis with a value of 80.3% (49 of 61), but we can no longer approve the method to be 100% specific. In the underlying study, the specificity was calculated at 80%. Limiting the investigation to the six most sensitive markers (D8S261, D8S560, D9S171, D9S925, D9S15, and D20S486) in the panel of 17 markers tested, the method already achieved a sensitivity of 57% (35 of 61) for tumor-specific serum diagnosis of the TCC disease.

Fig. 1 Frequency of alterations identified at the nine chromosomal regions investigated applying 17 microsatellite markers for tumor and serum samples. The highest incidence of tumor–DNA alterations was observed for chromosomal regions 8p, 9p, and 20q (n = 61 is the 100% value for each column; alteration = loss of heterozygosity or allelic imbalance caused by either loss or gain of genetic material in one allele).

Recent studies demonstrated molecular techniques, especially MSA, to be applicable for the detection of tumor-specific alterations, even in the smallest amounts of free circulating genomic serum or plasma DNA in a variety of primary tumors, e.g., small and non-small cell lung cancer, head and neck

Fig. 2 Frequency of serum–DNA alterations observed stratified for tumor nuclear grade (three groups) and local tumor stages (two groups, i.e., superficial and muscle invasive). Serological tumor diagnosis was not statistically associated with grade or local tumor stage (P > 0.05); however, there was a trend toward higher sensitivity of the method in high-grade tumors.

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cancers, RCC, colorectal, ovarian, and breast cancers (1, 2, 5–11).

In contrast to literature, we can no longer attest the method of MSA for serological detection of tumor–DNA alterations a 100% specificity. The observed specificity of 80% with the 20% possibility of false positive PCR artifacts has to date only been described in similar fashion for our own investigations with RCC patients, and the issue has been addressed previously by Coulet et al. (4, 7). Because of the low serum DNA concentrations of ~100 ng/ml, the highly sensitive PCR-based MSA with laser product detection can mimic AI, resulting in PCR artifacts resembling DNA alterations on rare occasions. We ruled out artifacts with the simultaneous assay of the primary tumors and only regarded an AI as tumor specific if the serum–DNA alteration matched the alteration in the tumor DNA (Fig. 3). Our observed specificity of 80% is in agreement with the identification of discordant serum DNA AI seen in 0–10% of cases depending on the microsatellite marker used, although we cannot rule out the discordant serum–DNA alterations to represent tumor DNA from another clonal origin within the bladder cancer (Table 2). Comparing the observed specificity of 80% and the finding of unspecific serum–DNA alterations in only 0–10% of the present study with the results from Utting et al. (12) giving a rate of 73% for unspecific serum–DNA AI representing PCR artifacts, one has to assume a less sufficient methodology in that study. This assumption may further be supported by the fact that these authors only extracted enough serum DNA from the 15 healthy controls in 9.5%, whereas we found sufficient amounts of serum DNA in all of our 20 healthy controls.

In the underlying investigation, the sensitivity for diagnosing tumor-specific alterations in serum of bladder cancer patients is higher than reported by others, which may be related to the use of a high number of markers from various chromosomal regions. Utting et al. state a sensitivity of 27–50% using six markers from chromosomes 4, 9, and 17 in 36 bladder cancer patients, and Goessl et al. achieved a sensitivity of 63%, applying four microsatellite primers from chromosome 3p in 40 patients with RCC (2, 12). In our cohort size of 61 patients with TCC and the large panel of different microsatellite markers, we could confirm our previous results for the sensitivity levels >80% for the test in bladder cancer and RCC (3, 4). In contrast to most other investigators, we applied a wide variety of markers from different chromosomal regions known to be involved in the carcinogenesis of TCC, accomplishing a sensitivity of 80.3% (49 of 61) in the microsatellite-based serological detection of tumor-specific alterations. Hereby, we identified LOH between 8p22 and 8p12 to be the most frequent alterations in the serum of bladder cancer patients with 36% (22 of 61). Serum DNA furthermore mainly displayed alterations of the chromosome arms 5q, 9p, and 20q with an incidence of 18–21%. The simultaneous analysis of the corresponding tumor DNA showed chromosome 8p loss and 20q AI to be most frequent with an incidence of 0–10% of the cases.

### Table 2

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Marker name</th>
<th>Tumor (%)</th>
<th>Serum (%)a</th>
</tr>
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<tr>
<td>5q</td>
<td>D5S476</td>
<td>24</td>
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</tr>
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<td>5q</td>
<td>D5S1270</td>
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<tr>
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<tr>
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<td>D8S560</td>
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</tr>
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<td>D20S480</td>
<td>29</td>
<td>2 (0)</td>
</tr>
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</table>

a Values given in brackets = percentage of additional unspecific allelic imbalance (AI) identified.

![Fig. 3](clincancerres.aacrjournals.org) Curves obtained from fluorescent microsatellite analysis of tumors TCC87 (pT2, G3), TCC127 (pTa,G2), TCC130 (pTa, G2), TCC154 (pTa,G2), and TCC156 (pT1,G2) with corresponding normal DNA (N; blood lymphocytes), serum DNA (S), and tumor DNA (T) at locus D9S171 for chromosome 9p. A clear loss of heterozygosity at D9S171 is evident in all tumors (T), whereas the corresponding serum DNA (S) for tumors TCC87, TCC130, TCC154, and TCC156 only display allelic imbalance caused by “contamination” with unaltered normal DNA. Serum DNA for tumor TCC127 exhibits no allelic imbalance for the investigated locus. The clear loss of one allele in tumor DNA TCC127, TCC130, and TCC156 proves pure tumor DNA samples without “contamination” from normal DNA.
incidence of 57 (35 of 61) and 54% (33 of 61), respectively. LOH at 9p in the tumor DNA was observed in 51% (31 of 61). Tumor allelic loss at chromosomes 5q, 8p, 9p, and 20q had the highest penetrance into the serum with 35 (9p) to 63% (8p) (22 of 35; Fig. 1 and Table 2). By restricting the analysis to the six markers with the highest incidence of alterations in the tumor (D8S261, D8S560, D9S171, D9S925, D9S15, and D20S486), the method still yielded a sensitivity of 57% (35 of 61) for serum diagnosis. This subset of markers rendered a comparatively high sensitivity as alterations at the corresponding chromosomal sites were observed at the highest incidence within the DNA of the primary tumors. The results of the allelotyping of the 61 primary TCCs of the bladder and upper urinary tract are in confirmation with literature, where TCC is known to mainly exhibit a high incidence of alterations at chromosomes 8p and 9 (13, 14). Comparing the sensitivity of 57% for serological diagnosis of the six best markers out of the set of 17 markers tested in this study with the tumor-specific sensitivity of 27% achieved by Utting et al. (12) with their six markers from chromosomes 4, 9, and 17 documents the superiority of our marker selection.

In a previous investigation, we identified 5q-LOH as frequent progression-associated alterations in bladder cancer (15). Allelotyping the tumors in the present study led to the identification of 5q-LOH with an incidence of 46% (28 of 61), which almost matches the incidence of 54% achieved with >20 markers previously (15). Nevertheless, only 19% (11 of 61) of the serum–DNA samples also had 5q alterations. We recently suggested the different rates of alterations detected in the tumor as compared with alterations in serum to most probably be associated with the fragmentation of serum DNA and had argued that alterations in the fragmented serum DNA may therefore only be detected if the amplified microsatellite is small with <200 bp. Our present extended study can no longer support this assumption because now the markers D5S1720 and D8S560 (8p22) with significantly differing product sizes of 220 and 143 bp, respectively, demonstrated the highest sensitivities for serum–DNA alterations with 16 and 29.5%, respectively, and the highest penetrance rates of serum–DNA alterations as compared with alterations in the tumor DNA with 42 and 64%, respectively (Table 2).

The observation that 5q and 8p alterations were detected at the highest rate in the serum may also be the result of the subpopulation of cells with 5q and 8p LOH within a heterogeneous tumor also having the best access to the vasculature. Especially fast and solid growing tumors harboring progression-associated alterations at 5q and 8p on the other hand require a sufficient blood supply via a high density of vessels.

In addition to the chromosomal regions 5q, 8p, and 9p, a high rate of alterations in the tumor tissue was identified for chromosome 14q (LOH) and 20q (amplification) with 39 and 54%, respectively. However, serum–DNA alterations for these two regions had an incidence of only ~20% (Table 2). Confirming our results published previously, we again calculated no statistical association (P > 0.05) between local tumor stage and nuclear grade for serological TCC diagnosis in MSA, although a trend is recognizable for a higher incidence of serum–DNA alterations in higher grade tumors (Fig. 2). Other recent investigations applying molecular techniques for serum– and plasma–DNA analysis also found no association with tumor grading and disease stage (1, 2, 6, 7, 9, 11). On the other hand, several studies identified a connection between plasma–DNA detection and advanced disease and/or high grade in various malignant tumors, thus predicting poorer prognosis (4, 10, 16, 17). An association is plausible, however, considering that poorly differentiated tumors are characterized by a high mitotic index with a high genetic instability, potentially elevating free tumor–DNA levels in the serum. The missing statistical association with primary tumor stage may be interpreted as an advantage of the method of molecular serological tumor detection, because it possibly allows the identification of early stage tumors with a high potential of progression. Breaking down the present cohort in groups by differentiation (three groups), as well as by stage of disease [superficial (pTa to pT1) and invasive (pT2 to pT3b)], did result in detection levels increasing with more advanced and aggressive disease. Detection levels ranged from 53 to 88% in serum as shown in Fig. 2.

Leon et al. and Shapiro et al. (18, 19) identified significantly elevated amounts of free DNA in cancer patients compared with healthy patients or patients with benign diseases. Nevertheless, the amount of free circulating DNA within the group of cancer patients was independent of tumor size or stage, being only markedly elevated in patients with metastatic disease. Regarding the underlying data, we postulate tumors with a high genetic instability displaying numerous alterations also to be associated with higher amounts of free circulating DNA as a result of a higher proliferation rate.

In conclusion, MSA offers high sensitivity levels of >80% for the stage-independent detection of TCC-specific serum–DNA alterations. Out of a panel of 17 markers, we could identify 6 microsatellite markers for chromosomes 8p, 9, and 20q, which offered the highest sensitivity in diagnosing TCC-specific alterations in serum of cancer patients. Thus, MSA may be a valuable noninvasive tool in the follow-up of patients after radical surgery to early detect occult local or micrometastatic recurrence. To prove this assumption, additional investigations applying this test on patients during follow-up are warranted.

REFERENCES


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