Differential Mucin MUC7 Gene Expression in Invasive Bladder Carcinoma in Contrast to Uniform MUC1 and MUC2 Gene Expression in Both Normal Urothelium and Bladder Carcinoma

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Abstract

Mucins (MUCs) are high molecular weight membrane glycoproteins. The gene expression of MUCs (MUC1-MUC8) may change characteristically during malignant transformation of epithelial tissues. Total RNA was isolated from the four bladder cancer cell lines RT4, 647V, HT1376, and 486P (pathological gradings between G1 and G4) and 17 samples of transitional cell carcinomas, as well as 16 samples of normal human urothelium of the bladder from surgically removed specimens. The RNA samples were studied with MUC1-, MUC2- and MUC7-specific nested reverse transcription-PCR. Gene expression of MUC1 and MUC2 was found positive in all normal, as well as in malignant, tissue samples and in the tumor cell lines. In contrast, gene expression of MUC7 was only detected in bladder cancer cell lines and samples of invasive transitional cell carcinomas, but neither in superficial, noninvasive bladder tumors nor in normal bladder urothelium. Only one of the samples of normal urothelium obtained from 16 different tumor-bearing bladders was positive for MUC7 gene expression. These results suggest a differential MUC7 gene expression with the onset of malignant transformation of the bladder urothelium.

Introduction

MUCs are high molecular weight glycoproteins that are synthesized by glandular epithelial cells of the gastrointestinal, respiratory, and urogenital tracts. Thus far, nine human MUC genes have been identified and designated MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC6, MUC7, and MUC8 in the international nomenclature (1). A number of functions have been proposed including protection, lubrication, and formation of a selective barrier of the epithelium (2). In the bladder, MUCs provide a physical barrier between urothelial cells and urine. The MUC layer plays a role in protecting the urothelium from surgically removed specimens. The RNA samples were studied with MUC1-, MUC2- and MUC7-specific nested reverse transcription-PCRs. Gene expression of MUC1 and MUC2 was found positive in all normal, as well as in malignant, tissue samples and in the tumor cell lines. In contrast, gene expression of MUC7 was only detected in bladder cancer cell lines and samples of invasive transitional cell carcinomas, but neither in superficial, noninvasive bladder tumors nor normal bladder urothelium. Only one of the samples of normal urothelium obtained from 16 different tumor-bearing bladders was positive for MUC7 gene expression. These results suggest a differential MUC7 gene expression with the onset of malignant transformation of the bladder urothelium.

Materials and Methods

Cell Lines. Four cell lines of TCCs of the bladder (RT4 with the pathological grade G1, 647V with grade G2, HT1376 with grade G3, and 486P with grade G4) were investigated (12). The colorectal carcinoma cell line WIDR served as a positive control for the expression of MUC1, MUC2, and MUC7. RT4, 467V, HT1376, 486P, and WIDR were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were cultured as a monolayer in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS, 2 mmol/liter glutamine, and 1 mmol/liter pyruvate. HT-1376 cells were grown in Eagle’s MEM (Biochrom) with the same additives. Cultures were maintained at a temperature of 37°C in a humidified 5% CO2 atmosphere. All cell lines were routinely tested for mycoplasma contamination using a PCR mycoplasma detection kit (TaKaRa; Biomedical Europe S. A., Genevilliers, France), and contaminated cultures were discarded.

Tissues. Four samples of superficial, noninvasive tumors and one sample with severe urothelial dysplasia were obtained by transurethral resection of the bladder. Eleven samples of invasive bladder tumors and two samples of carcinoma in situ, as well as 16 samples of normal urothelial tissue, were obtained from cystectomy specimens. All samples were immediately immersed in liquid nitrogen within 10 min after removal from the patients. A representative sample was taken from each tumor for histopathological assessment, and an adjacent piece was frozen in liquid nitrogen and stored at −80°C for subsequent RNA extraction. Tumors were graded according to criteria as recommended by the WHO and staged according to the TNM classification. The pathology of the tumors was confirmed by the consensus of two pathologists.

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GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TCC, transitional cell carcinoma; MUC5B, MUC6, MUC7, and MUC8 in the international nomenclature (1). A number of functions have been proposed including protection, lubrication, and formation of a selective barrier of the epithelium (2). In the bladder, MUCs provide a physical barrier between urothelial cells and urine. The MUC layer plays a role in protecting the urothelial cell layer, whereas in TCC MUC1 stained to membranous and/or cytoplasmic cells of all urothelial cell layers, depending on tumor grade and stage (5). MUC2 was not detected immunohistochemically in normal urothelium, but was found in 40% of cases of TCC (6). In summary, only MUC1 and MUC2 have been detected in normal urothelium or in bladder carcinoma by immunohistochemical techniques, but no comprehensive studies have analyzed their mRNA expression yet. To our knowledge thus far, no reports have focused on the investigation of any other MUC expression in human urothelium and TCC of the bladder. We have screened a set of gastrointestinal and other tumor cell lines for the expression of MUCs. Colorectal and bladder cancer cell lines exhibited positive results for MUC7 in this screening. The structure and chromosomal localization of the human salivary MUC gene MUC7 was first described by Bobek et al. (7) in 1993 (8). Since then, MUC7 gene expression has also been detected in the pancreas, in submucosal glands of the bronchus, and in cholangiocarcinomas (9–11). On the basis of these findings, the aim of this study was to investigate gene expression of three MUCs (MUC1, MUC2, and MUC7) by a nested RT-PCR in cell lines of TCC, benign, and malignant tissues of urothelial origin.
ogists. The histopathological tumor stage of the superficial, noninvasive blad-
der tumors ranged between pTaG1 and pTaG2. All invasive bladder tumors
were classified as TCCs. The histopathological tumor stage of the invasive
bladder carcinomas ranged between pT1 G3 and pT4 G3 (see Table 2).

Isolation of Total RNA. Total RNA was isolated from tumor cell lines and
tissue samples (100–500 mg) based on the guanidinium-thiocyanate-pheno-
chloroform single-step isolation method (14). The RNA from tumor cell lines
was extracted using RNA-Clean (AGS-Chemie, Heidelberg, Germany), and
frozen tissue specimens were extracted for total RNA by RNAzol (WAK-
CHEMIE MEDICAL GmbH, Bad Homburg, Germany). To optimize the yield
of RNA, 5 μg of glycogen (Boehringer Mannheim, Mannheim, Germany)
were added to the aqueous phase before precipitation with isopropanol. The
RNA preparations were dissolved in 40 μL of RNase-free water. RNA recovery
and purity were controlled by absorption measurement at 260 nm and 280 nm
(Gene Quant II; Pharmacia Biotech, Freiburg, Germany), respectively. Sam-
ple of 2 μg of the extracted total RNA were separated in 1% agarose gel
(Small DNA agarose; Biozym, Hessisch Oldendorf, Germany). The integrity
of RNA was determined, and samples with evidence of rRNA degradation
were discarded.

RT-PCR. The total RNA (2 μg) of each sample was dissolved in a volume
of 10 μL, denatured for 10 min at 70°C and then quickly chilled on ice. The
cDNA was synthesized in a total volume of 20 μL containing 4 μL of 5 × first-
strand buffer, 2 mm DTT, 200 units of SuperScript II (all purchased from Life
Technologies, Inc., Egggenstein, Germany), 20 units of RNase inhibitor, 5 μM
random hexamers, and 1 mM deoxynucleotide triphosphate mix (all purchased
from Perkin-Elmer Applied Biosystems GmbH, Weiterstadt, Germany). The
reaction mix was incubated for 10 min at 24°C and 60 min at 42°C. Reverse
transcriptase was then inactivated for 3 min at 94°C. The MUCl-, MUC2-, and
MUC7-specific PCR were performed as nested PCR. Primers were synthesized
by MWG-Biotech (Ebersberg, Germany).

MUC1, MUC2, and MUC7 Nested RT-PCR. MUCl-A sense, 5'-
TCATTCCCCAGCCAC CATCCT- MUCIB antisense, 5'-AGTTCTTTCG-
GGCAGCTGGAC- MUCIC sense, 5'-GGCAGCCTAGCACCAACAGA CT-
MUCI-D antisense, 5'-TGACAGACAGCAAGCAC TTG. External MUCl
RT-PCR resulted in a 595-bp fragment; the internal PCR yielded a 539-bp
product.

MUC2-A sense, 5'-CTGATCGGTTGTTGAGGGA AGA; MUC2-B antisense,
5'-CAGCGTGA CGGAAAGGAGAC; MUC2-C sense, 5'-GAGAT-
CAAGCCCCCTTGAGGAGA; MUC2-D antisense, 5'-CAAAGCTCTTTGGT-
TACACCTG. External MUC2 RT-PCR resulted in a 317-bp fragment; the
internal PCR yielded a 259-bp product.

MUC7-A sense, 5'-CAGGGCTTGA GCTGAGGAGGAA; MUC7-B antisense,
5'-ATGGAGCTTGG-GCGGAGTAGA C; MUC7-C antisense, 5'-GTATA-
GGCAGATAGGAGG- GAAA; MUC7-D antisense, 5'-GCCACATGATAGGATGAGG-
MUC7-D antisense. External MUC7 RT-PCR resulted in a 439-bp fragment; the
internal PCR yielded a 348-bp product.

For each specific MUCl, MUC2, or MUC7 nested RT-PCR, 20 μL of the
cDNA synthesis mix were used in a final volume of 50 μL. The reaction mix
contained 3 μL of 10 × tricine buffer III (15), 200 μL deoxynucleotide
triphosphate mixture, 1.5 μM MUCl-A sense, 1.5 μM MUC2-B antisense primer,
and 1 unit of Taq-DNA polymerase (Life Technologies, Inc.). The cycling
protocol for the MUCl, MUC2, and MUC7 nested RT-PCRs (lower band
product of 180 bp was amplified from extractions of the four different
MUCl-specific PCR products through all investigated samples (data not shown). The analyzed
sequence of one of the MUCl-specific nested RT-PCR products
(derived from cell line HT1376) of 539 bp showed 100% identity with
the published MUCl mRNA sequence (GenBank accession No.
J05581).

RT-PCR. A GAPDH-specific RT-PCR producing a 359-bp frag-
ment demonstrated the integrity of isolated RNA and success of the
cDNA synthesis procedure for all MUC nested RT-PCRs (lower band
of Fig. 1, A–C).

RT-PCR of MUC1. Products of 539 bp amplified by a MUC1
nested RT-PCR were detected in all isolates from the four bladder
carcinoma cell lines RT4, 647V, HT1376, and 486P with pathological
gradings between G1 and G4 (Fig. 1A, Lanes 18–21). The colorectal
cell line WIDR serving as a MUC1-positive control also showed a
nested RT-PCR product of 539 bp (Fig. 1A, Lane 1). MUC1 gene
expression could be detected in all tissue samples of bladder carci-
nomas with tumor stages ranging from pTaG1 (two of four selected
samples in Fig. 1A, Lanes 9 and 10) to pT4G3 (Fig. 1A, Lane 17), as
well as in two samples with carcinoma in situ of the bladder (Fig. 1A,
Lanes 11 and 12). All isolates of normal urothelium were also found
positive for MUC1 gene expression (6 of 16 selected samples in Fig.
1A, Lanes 2–7). The corresponding negative controls (amplification
without reverse transcription) showed no MUC1 gene expression
throughout all investigated samples (data not shown). The analyzed
sequence of one of the MUC1-specific nested RT-PCR products
(derived from cell line HT1376) of 539 bp showed 100% identity with
the published MUC1 mRNA sequence (GenBank accession No.
J05581).

RT-PCR of MUC2. The expected MUC2-specific nested RT-PCR
product of 180 bp was amplified from extractions of the four different
bladder cancer cell lines and of the control cell line WIDR (Fig. 1B,
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Lane 1). MUC2 gene expression was detected in all tissue samples of bladder carcinomas including two carcinomas in situ (selected samples in Fig. 1B, Lanes 9–20) as well as in all samples of normal urothelium (selected samples in Fig. 1B, Lanes 2–7) and in urothelial dysplasia (Fig. 1B, Lane 8). Sequencing of one of the 180-bp fragments of the MUC2-specific nested RT-PCR (derived from cell line HT1376) proved 100% similarity with known sequences from the GenBank nucleotide database (GenBank accession Nos. L21998, M94132, and M86523). The majority of samples also displayed the 317-bp long external product on completion of the MUC2-specific nested RT-PCR (Fig. 1B, Lanes 6–10 and 12–21). Correspondence of the 317-bp fragments, as seen in Fig. 1B, with the expected MUC2 external product was also confirmed by automatic sequencing of one sample (derived from cell line HT1376). Furthermore, an additional amplified fragment of 796 bp was found in 13 extracted tissue samples (Fig. 1B, Lanes 2–5, 7, 9–11, 13–15, 17, 19) This fragment was identified as a genomic DNA contamination because it also appeared as a single fragment in the otherwise negative controls that were run without reverse transcription (data not shown). Performing a PCR with DNA from a commercially available genomic library (Promega, Madison, WI) with the MUC2-C sense and MUC2-D antisense primers confirmed once more the genomic origin of the detected fragment by yielding the same 796-bp fragment (data not shown). This fragment was identified by automatic sequencing as having included a novel intron of 616 bp (EMBL accession No. AJ007575), located between positions 1098 and 1099 of GenBank accession No. M94132, between positions 13710 and 13711 of GenBank accession No. L21998, and between positions 209 and 210 of GenBank accession No. M86523, respectively. Therefore, it was concluded that intron spanning primers were established for the MUC2-specific nested RT-PCR applied in this study. The corresponding negative control (amplification without reverse transcription) showed no MUC2 gene expression throughout all investigated samples (data not shown).

**RT-PCR of MUC7.** RT-PCR products of 348 bp amplified by a MUC7 nested RT-PCR were detected in all isolates from the four different bladder cancer cell lines and in the control cell line WIDR. Furthermore, all tissue samples of invasive TCCs, including two samples of carcinoma in situ of the urinary bladder, showed a MUC7 gene expression. In contrast to these samples and to the findings with MUC1 and MUC2, no MUC7 amplification was detected in the superficial, noninvasive bladder tumors (Fig. 1C, Lanes 9 and 10) or in the sample with severe urothelial dysplasia (Fig. 1C, Lane 8). Except for one sample of normal urothelium that was obtained from a tumor-bearing bladder (Fig. 1C, Lane 2), all 15 samples of normal urothelium were repeatedly found negative for MUC7 gene expression (5 of 15 selected samples in Fig. 1C, Lanes 3–7). The fragment length and analyzed sequence of one MUC7-specific nested RT-PCR product of 348 bp was in accordance with the known MUC7 cDNA nucleotide sequence (GenBank accession No. L13283). The corresponding negative control (amplification without reverse transcription) showed no MUC7 gene expression in all cases (data not shown).

Table 2 gives an overview of the MUC7 gene expression in all tissue samples of the bladder in regard to the tumor stage.

**Discussion**

During recent years, a number of biochemical studies on the structure and the organ specificity of several MUC proteins have been published. Due to extensive research, it is now possible to identify nine different MUCs of the MUC family (1). MUC1 is the best characterized MUC and is expressed on the apical surface of most polarized epithelial cells (2). MUC2 production and secretion was first demonstrated in endoderm-derived epithelial cells, mainly in the

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**Fig. 1. MUC1-, MUC2-, and MUC7-specific nested RT-PCR of normal urothelium of the bladder, bladder tumor, and bladder carcinoma cell lines. A, MUC1-specific nested RT-PCR (internal PCR product, 539 bp). B, MUC2-specific nested RT-PCR (MUC2 intron product, 796 bp; external PCR product, 317 bp; internal PCR product, 180 bp). C, MUC7-specific nested RT-PCR (internal PCR product, 348 bp). Samples were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. Representative data from a panel of 20 specimens are presented. The colon carcinoma cell line WIDR served as positive control for MUC1, MUC2, and MUC7 gene expression (Lane 1), normal urothelial tissues of the urinary bladder (Lanes 2–7), severe urothelial dysplasia (Lane 8), superficial, noninvasive bladder tumors (Lanes 9 and 10), carcinoma in situ (Lanes 11 and 12), invasive bladder carcinoma with T1G3 (Lane 13), T2G3 (Lane 14), T3G2 (Lane 15), T3G3 (Lane 16), T4G3 (Lane 17), and bladder carcinoma cell lines with RT4 (Lane 18), 647V (Lane 19), HT1376 (Lane 20), and 468P (Lane 21). M, molecular weight marker. The corresponding control reaction was performed as GAPDH RT-PCR with a 359-bp product and is shown in the lower band. Data of corresponding negative control (amplification without reverse transcription) are not shown.

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**Table 2.** MUC7 gene expression in tissue samples of the bladder with different TNM stages assessed by MUC7-specific nested RT-PCR

<table>
<thead>
<tr>
<th>Histology of the bladder</th>
<th>UICC classification (TNM stage)</th>
<th>No. of samples with MUC7 gene expression</th>
<th>total no. of samples</th>
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<tr>
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<td>Stage 0a</td>
<td>0/4</td>
<td></td>
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<tr>
<td>Invasive carcinoma</td>
<td>Stage I</td>
<td>1/1</td>
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<tr>
<td></td>
<td>Stage II</td>
<td>2/2</td>
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<tr>
<td></td>
<td>Stage III</td>
<td>6/6</td>
<td></td>
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<tr>
<td></td>
<td>Stage IV</td>
<td>2/2</td>
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a UICC, Union Internationale contre le Cancer.
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gastrointestinal and respiratory tracts (16). MUC7 has been cloned and sequenced from a salivary cDNA library (7). We investigated the three MUCs (MUC1, MUC2, and MUC7) that are among the few MUCs that have been fully sequenced at the cDNA level (1). MUC sequences like MUC5B and MUC6 consist mainly of VNTRs, which makes them inaccessible for specific PCR detection (17). Because MUC1, MUC2, and MUC7 have distinct mRNA sequences apart from expression of MUC7, a MUC7 gene expression might point to a premalignant genetic transformation. This sample was classified as normal urothelial tissue, the up-regulation of MUC7 GENE EXPRESSION IN INVASIVE BLADDER CANCER

TCCs were probably missed by immunohistochemical staining methods because the antibody might not bind to the glycosylated mature form of MUC2. This shortcoming in studying MUC expression by immunohistochemistry is avoided by applying a RT-PCR technique that detects MUC gene expression independent of cellular localization or posttranslational processing.

The investigation of MUC7 gene expression in normal and maligantly transformed urothelium has not been described thus far. This work demonstrates, for the first time, the differential gene expression of MUC7 detected by a nested RT-PCR in invasive bladder carcinomas and in TCC cell lines. Particularly, MUC7 gene expression was found positive in tissue samples of carcinoma in situ of the bladder, which is a preinvasive morphological alteration of epithelial cells commonly preceding invasive tumor growth. These findings indicate a high specificity of MUC7 gene expression also with respect to preinvasive TCC of the bladder. In contrast to these results, no MUC7 gene expression was detected in superficial, noninvasive bladder tumors. Except for one tissue sample, all 15 samples of normal urothelium of the bladder were found negative for MUC7 gene expression. The one positive sample of normal urothelium was obtained from a tumor-bearing bladder. Although histological examination classified this sample as normal urothelial tissue, the up-regulation of MUC7 gene expression might point to a premalignant genetic transformation of the bladder urothelium that was not detectable by histopathological criteria. Our results confirm that neoplastic transformation of the urothelium is accompanied by switching on the gene expression of MUC7. The analysis of MUC7 gene expression by a nested RT-PCR technique may be of value in the early diagnosis of neoplastic transformation of the bladder urothelium, therefore, adding a sensitive molecular diagnostic parameter to the disease of TCC of the urinary bladder. These data encourage additional studies investigating the detection of MUC7-positive cells in voided urine samples by RT-PCR technique and immunocytochemistry.

Cancer-related dysregulation of MUC gene expression has been studied extensively. The functions of MUCs in tumor cells may include protection from hostile environments, protect the tumor cells from the immune system by acting as a blocking agent, and steric hindrance for cell surface antigens that are involved in immune recognition. Therefore, tumors that express higher amounts of MUCs may have a better chance of survival and a higher tendency to metastasize once they have reached the blood and lymphatic system. Consistent with these theories, it was previously shown that the up-regulation of MUC1 expression in breast cancer is associated with tumor invasion and progressive disease (19). Taken together, it may well be possible that MUC quantity and alterations in their composition may be influential on the biological behavior of a tumor cell and, consequently, on the clinical outcome.

An interesting clinical aspect of MUC expression involves the interaction of BCG as an immunotherapeutic agent for superficial bladder carcinoma and MUC-expressing urothelial cells. Badalament et al. (20) found in animal experiments that attachment of the BCG cells to the urothelium, which is a prerequisite for effective therapy, depends on the state of glycosylation of the urothelial MUC layer. The authors hypothesize that removal of the urothelial MUC layer may be associated with increased BCG adherence to the bladder surface and, therefore, enhancement of the antitumoral effect. It is concluded that altered MUC expression in neoplastic changes of the bladder urothelium might influence the effectiveness of this established form of therapy.

In conclusion, this study revealed a differential MUC gene expression for MUC7 in preinvasive and invasive bladder carcinomas in contrast to superficial, noninvasive bladder tumors and normal urothelium. These results might indicate a high sensitivity of MUC7 gene expression in premalignant transformation of the bladder urothelium. The clinical implication of the differential MUC7 gene expression in TCC and its role as a potential tumor marker warrant additional investigations and studies on a larger scale of patients.

References


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