Expression of Uroplakin Ib and Uroplakin III Genes in Tissues and Peripheral Blood of Patients with Transitional Cell Carcinoma

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Uroplakins (UPs), urothelium-specific transmembrane proteins, are present only in urothelial cells. We have determined the nucleotide sequences of human UP-Ib and UP-III and synthesized specific primer pairs. The two UP genes were expressed in both cancerous and noncancerous urothelia taken from all patients examined by reverse transcription-polymerase chain reaction (RT-PCR). These genes were also detected in the peripheral blood of 3 patients with metastatic transitional cell carcinoma (TCC), but not in that from 9 patients with non-metastatic TCC or 3 healthy volunteers. The sensitivity of our assay was sufficient to detect one cancer cell in 5 ml of peripheral blood. Detection of UP gene-expression in blood by RT-PCR may provide helpful information for the diagnosis and management of TCC.

Key words: Uroplakin Ib — Uroplakin III — Transitional cell carcinoma — Reverse transcription-polymerase chain reaction

Uroplakins (UPs), including UP-Ia, UP-Ib, UP-II and UP-III, have previously been described only as urothelial-specific transmembrane proteins.1–3 UPs are believed to stabilize the luminal surface of the epithelium and to prevent a rupture of the surface during bladder distention.4,5 In an immunohistochemical study, UPs were shown to be detectable in most transitional cell carcinomas (TCC), irrespective of grade or stage, and were never detected in other cancers.6 Expression of UPs is a specific marker for urothelium, so UPs are good candidates as tumor markers specific for TCC. However, only UP-II gene expression has been confirmed in human TCC tissues,7 as the UP-Ib and UP-III genes have not yet been characterized sufficiently.

In this study we determined the nucleotide sequences of human UP-Ib and UP-III and investigated the expression of the human UP-Ib and UP-III genes in TCC using reverse transcription-polymerase chain reaction (RT-PCR). We also investigated the expression of these genes in the peripheral blood of patients with non-metastatic and metastatic TCC.

First, to obtain the nucleotide sequences of human UP-Ib and UP-III, we synthesized the UP-Ib primers, UP-IbS1 (5′-GGCCTTGGGGGAATCCTGAAG-3′, nucleotides 41 to 61 of bovine UP-Ib 5′-untranslated region) and UP-IbA2 (5′-CCTACAGTCTATAACATGTGCA-3′, nucleotides 1047 to 1070 of bovine UP-Ib 3′-untranslated region), and the UP-III primers, UP-IIS1 (5′-GTCGCACGTCTTCTCCCCAGGCCG-3′, nucleotides −20 to −1 of bovine UP-III 5′-untranslated region) and UPIIIA2 (5′-CCTGTCCAGGGACGCCCCC-3′, nucleotides 812 to 831 of bovine UP-III 3′-untranslated region), according to the reported sequences of bovine UP-Ib9 and UP-III10 respectively. Specimens analyzed in this study were obtained by transurethral resection and cut in half. One sample was frozen immediately and stored at −80°C until RNA extraction. The other was fixed with neutralized buffered formalin for routine histopathologic examination. Total cellular RNA was extracted from normal bladder tissue by the acid guanidium thiocyanate-chloroform method previously described,10 and first-strand cDNA was synthesized from 5 μg of total RNA using 20 units of RAV-2 reverse transcriptase (Takara, Otsu) and random nonamers. Portions (1 μl) of the cDNA were amplified by PCR as described previously.11 The reaction mixture (50 μl) consisted of 25 mM Tris-HCl (pH 9.0) containing 50 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol, 200 μM dNTPs, 1.25 units of Ampli-Taq DNA polymerase (Takara) and 200 ng each of primers. Amplification was performed with 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 1 min). The double-stranded DNA fragment generated by PCR was prepared by membrane filtration with Suprec-02 (Takara) and directly sequenced. The nucleotide sequences of these fragments were determined and the corresponding amino acid sequences were deduced (Fig. 1, A and B). The
deduced amino acid sequences of human UP-Ib and UP-III were 93% and 85% identical with those of bovine UP-Ib and UP-III, respectively.8,9) Secondly, in order to examine the expression of human UP-Ib and UP-III genes in the tissues and peripheral blood of patients with TCC, we synthesized two human UP-Ib-specific primer pairs; the HUPIb first primer pair (HUPIbS3 5′-ATGGCCAAAGACAACTCAACT-3′, nucleotides 1 to 21 of human UP-Ib and HUPIbA4 5′-ATAT-TCAATTCTGTCCTCCAGTAG-3′, nucleotides 759 to 780 of human UP-Ib) and the second primer pair (HUPIbS5 5′-CAACGAGACTTTTTCACACCC-3′, nucleotides 334 to 354 of human UP-Ib and HUPIbA6 5′-CCCCCGGAGGTTGGCCATCA-3′, nucleotides 677 to 696 of human UP-Ib) according to the nucleotide sequence of the human UP-Ib gene (Fig. 1A). The HUPIb first and second primer pairs amplified a 780 base pair (bp) fragment and a 363 bp fragment, respectively. We also synthesized two human UP-III-specific primer pairs; the HUPIII first primer pair (HUPIIIA4 5′-ATAT-TCAATTCTGTCCTCCAGTAG-3′, nucleotides 840 to 858 of human UP-III) and the second primer pair (HUPIIIA6 5′-CCCCCGGAGGTTGGCCATCA-3′, nucleotides 641 to 661 of human UP-III) according to the nucleotide sequence of the human UP-III gene (Fig. 1B). The HUPIII first and second primer pairs amplified 783 bp and 392 bp fragments, respectively. A human β-actin (β-actin) specific primer pair, 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCCTAATGTCACGCAGATTTC-3′, was used as a control primer pair as described previously.12) We analyzed the expression of human UP-Ib and UP-III genes in normal transitional cells from 3 patients with noncancerous diseases and 12 primary TCCs, including 3 of grade 1, 7 of grade 2 and 2 of grade 3, of the urinary bladder by RT-PCR with the respective second primer pairs. The histologic diagnosis and clinical stage were determined according to the General Rule for Clinical and Pathological Studies on Bladder Cancer in Japan.13) The human UP-Ib and UP-III genes were both amplified in all 3 normal urothelium samples and all 12 bladder cancers (Fig. 2). These results indicated that the human UP-Ib and UP-III genes are highly conserved, and are expressed not only in normal transitional cells, but also in TCC.

We assessed the feasibility of detecting UP-Ib and UP-III gene-expressing cancer cells by nested RT-PCR using HT1197,14) a TCC cell line. HT1197 cells were serially
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diluted from 10^6 to 1 cell in 5 ml of peripheral blood. From blood samples, cells were isolated by a density separation method using Leucoprep (Becton & Dickinson, Franklin Lakes, NJ), washed once in phosphate-buffered saline and stored at −80°C until RNA extraction. Total RNA was isolated from the diluents and used for nested RT-PCR using the respective UP first and second primer pairs to determine the sensitivity in detecting UP-Ib and UP-III gene-expressing cancer cells in blood. In the peripheral blood of three healthy volunteers, neither the UP-Ib nor the UP-III gene was detected. Expression of both genes could be detected in one cancer cell in 5 ml of normal blood. Expression of β-actin was used as a control.

Finally, expression of the human UP-Ib and UP-III genes in the peripheral blood of patients with TCC was analyzed by nested RT-PCR using the respective first and second primer pairs, as described above. Clinicopathologic features of the peripheral blood of the additional 12 patients examined are summarized in Table I. Expression of both genes was detected in the peripheral blood of all three patients with metastatic TCC, but not in any of the 9 non-metastatic TCC cases or in 3 healthy volunteers (Fig. 4). These results indicate that circulating TCC cells can be detected by nested RT-PCR using the human UP-specific primer pairs.

Recently, PCR-based methods for analyzing tumor-associated gene expression have been reported to be highly sensitive in the detection of circulating tumor cells in blood.15–17) The UP-Ib and UP-III genes were also indicated to be TCC-specific when expressed in the peripheral blood in this study (Fig. 4). Until now, no tumor marker specific for TCC in serum has been known. Expression of UPs is a marker for urothelium, suggesting that UPs are candidates for TCC-specific serum tumor markers. We have now produced recombinant human UP proteins in Escherichia coli, BL21 competent cells (Novagen, Madison, WI) using a pET32a expression vector system (Novagen) and are raising monoclonal antibodies against

| Table I. Clinicopathologic Data of Patients with TCC of Urinary Bladder Whose Peripheral Blood Was Used for Nested RT-PCR Analysis |
|---|
| No. | Sex | Age | Grade | TNM | UP-Ib | UP-III |
|---|
| 1 | 60 | F | G1 | TaN0M0 | − | − |
| 2 | 63 | M | G3 | TisN0M0 | − | − |
| 3 | 30 | M | G1 | T1aN0M0 | − | − |
| 4 | 65 | M | G2 | T1bN0M0 | − | − |
| 5 | 73 | M | G2 | T1bN0M0 | − | − |
| 6 | 61 | M | G2 | T2N0M0 | − | − |
| 7 | 56 | M | G2 | T2N0M0 | − | − |
| 8 | 79 | F | G3 | T3bN0M0 | − | − |
| 9 | 63 | M | G3 | T3bN0M0 | − | − |
| 10 | 56 | F | G2 | T2N2M1 | + | + |
| 11 | 52 | F | G2 | T2N3M1 | + | + |
| 12 | 68 | M | G2 | T1bN2M1 | + | + |

UPIb, UPIII: expression of UPIb and UPIII detected (+) or not (−) by nested RT-PCR.
the UPs to investigate these proteins in serum samples from patients with TCC.

In summary, we determined the nucleotide sequences of human UP-Ib and UP-III and synthesized human UP-Ib and UP-III specific primers. Expression of human UP-Ib and UP-III genes in both normal urothelium and TCC arising in the urinary bladder was detected in peripheral blood samples of patients with metastatic TCC. We could detect one cancer cell in 5 ml of peripheral blood with our assay system. Detection of UP-Ib and UP-III genes by RT-PCR may provide useful information in the diagnosis and management of TCC. To our knowledge, this is the first report of the detection by RT-PCR of circulating cancer cells in patients with TCC.

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