

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

Takeshi Yuasa,¹ Tatsuhiro Yoshiki,^{1,3} Tsutomu Tanaka,¹ Chol Jang Kim,¹ Takahiro Isono² and Yusaku Okada¹

¹Department of Urology and ²Central Research Laboratory, Shiga University of Medical Science, Seta, Otsu 520-2192

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 urothelium-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'-GGCTTGGGGGAAATCCTGAAG-3', nucleotides 41 to 61 of bovine UP-Ib 5'-untranslated region) and UP-

IbA2 (5'-CCTACAGTCTATAACAATGTTGCA-3', nucleotides 1047 to 1070 of bovine UP-Ib 3'-untranslated region), and the UP-III primers, UP-III S1 (5'-GTCCG-CAGCTTCTCCCAGGCG-3', nucleotides -20 to -1 of bovine UP-III 5'-untranslated region) and UP-III A2 (5'-CCTGTCCAGGGACGGCCCCC-3', nucleotides 812 to 831 of bovine UP-III 3'-untranslated region), according to the reported sequences of bovine *UP-Ib*⁸ and *UP-III*,⁹ 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

³ To whom reprint requests and all correspondence should be addressed.

⁴ The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB010637 and AB015234.

A

```

ATGCCAAGACAACCTCACTGTTGCTGCTCCAGGGCCTGCTGATTTTGGAAATGTG 60
M A K D N S T V R C F Q G L L I F G N V
ATTATTGGTTGTTGGCGCATTGCCCTGACTCGGAGTGCATCTCTTTGTATCTGACCAA 120
I I G C C G I A L T A E C I F F V S D Q
CACAGCCTTACCCTACTGCTGAAGCCACCCGACACGATGACATCTATGGGGCTGCCTGG 180
H S L Y P L L E A T D N D D I Y G A A W
ATCGGCATATTTTGGGCATCTGCTCTCTGCTGCTGTTCTAGGCATTGTAGGCATC 240
I G I F V G I C L F C L S V L G I V G I
ATGAAGTCCAGCAGAAAATTTCTCTGGCCTATTTCTATTCTGATGTTTATAGTATATGCC 300
M K S S R K I L L A Y F I L M F I V Y A
TTTGAAGTGGCATCTTTATCACAGCAGCAACACACAGAGACTTTTTCACACCCCACTC 360
F E V A S C I T A A T Q R D F F T P N L
TTCCTGAAGCAGATGCTAGAGAGGTACCAAAAACAACAGCCCTCCAAACGATGATGACCA 420
F L K Q M L E R Y Q N N S P P N D D D Q
TGAAAAACAATGGAGTACCACAAAACCTGGGACAGGCTCATGCTCCAGGACAATTGCTGT 480
W K N N G V T K T W D R L M L Q D N C C
GGCCTAAATGGTCCATCAGACTGGCAAAAATACACATCTGCCCTCCGACTGAGAAATAT 540
G V N G P S D W Q K Y T S A F R T E N N
GATGCTACTATCCCTGGCCTGCTCAATGCTGTGTTATGAACAATCTTAAAGAACCTCTC 600
D A D Y P W P R Q C C V M N N L K E F L
AACCTGGAGGCTTGAATACTAGGCGTGCCTGGTTTTTATCACAATCAGGGCTGCTATGAA 660
N L E A C K L G V P G F Y H N Q G C Y E
CTGATCTCTGGTCCAATGAACCCGACCGCTGGGGGTTGCCTGGTTTGGATTGCGCATT 720
L I S G P M N R H A A W G V A W F G F A I
CTCTGCTGACTTTTGGGTTCTCCTGGTACCATGTTCTACTGAGCAGAAATGAATAT 780
L C W T F W V L L G T M F Y W S R I E Y
TAA
*
    
```

B

```

ATGCCCTCCGCTCTGGGCGCTGCTGGCCCTCGGCTGCTGCGGTTGCGCTCGGCTGTGAAC 60
M P P L W A L L A L G C L R F G S A V N
CTGCAGCCCAACTGGCCAGTGTGACTTTGCCCCAACAAACCCACACTTACCCTGTG 120
L Q P Q L A S V T F A T N N P T L T T V
GCCTTGGAAAAGCCTCTCTGCATGTTGACAGCAAGAGGCCCTCACTGGCACCACGAG 180
A L E K P L C M F D S K E A L T G T H E
GTCTACCTGTATGCTCCTGCTGACTCAGCCATTTCCAGGAATGCTCAGTGAAGACAGC 240
V Y L Y V L V D S A I S R N A S V Q D S
ACCAACACCCCACTGGGCTCAACGTTCCACAAACAGAGGGTGGGAGGACAGTCCCTAC 300
T N T P L G S T P L Q T E G G R T G P Y
AAAGCTGTGGCCTTTGACCTGATCCCTGACGAGTACCTGCCCCAGCTGGATGCCATTGG 360
K A V A F D L I P C S D L P S L D A I G
GATGTGTCCAAGGCTCACAGATCCTGAATGCCTACCTGGTCCAGGTGGGTGCCAACGG 420
D V S K A S Q I L N A Y L V R V G A N G
ACCTCCCTGTGGATCCCAACTTCCAGGCGCTCTGTAAACCCACCCCTGTCCGACGCCAG 480
T C L W D P N F Q G L C N P P L S A A T
GAGTACAGGTTCAAGTATGCTCCTGCTCAATATGCTCCCGGGCTTGTAGAGGACCCAG 540
E Y R F K Y V L V N M S T G L V E D Q T
CTGTGGTCAGACCCCATCCGACCAACAGCTCACCCATCACTGACGATCCGACCCCTGG 600
L W S D P I R T N Q L T P Y S T I D T W
CCAGGCGGGGAGCGGAGGCGATGATGCTCATCACTTCCATCCTGGGCTCCCTGCCCTTC 660
P G R R S G G M I V I T S I L G S L P F
TTTCTACTGTGGGTTTGTGCTGGCGCCATTGCCCTCAGCCCTCGTGGCCATGGGGATTCT 720
F L L V G F A G A I A L S L V A M G S S
GATGGGAAACGACTCAGCAGTCCCAATCACTCAGGAGGCTGTTCACCAAGTCCGCTGG 780
D G E T T H D S Q I T Q E A V F K S L G
GCCTGGAGCTTCTTACAGTCCGTAACCGGGGCGCCACTGACAGGCGCTOAGGTC 840
A S E S S Y T S V N R G P P L D R A E V
TATTCCAGCAAGCTCCAAGACTGA
Y S S K L Q D *
    
```

Fig. 1. Human *UP-Ib* (A) and *UP-III* (B) nucleotide sequences and the deduced amino acid sequences.

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'-ATGGCCAAAGACAACCTCAACT-3', nucleotides 1 to 21 of human *UP-Ib* and HUPIbA4 5'-ATAT-TCAATTCTGCTCCAGTAG-3', nucleotides 759 to 780 of human *UP-Ib*) and the HUPIb second primer pair (HUPIbS5 5'-CAACGAGACTTTTTTCACACCC-3', nucleotides 334 to 354 of human *UP-Ib* and HUPIbA6 5'-CCCCAGGCGTGTGCGTTCA-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 (HUPIIIS3 5'-GCCAGTGT-GACTTTTCGCCACC-3', nucleotides 76 to 96 of human *UP-III* and HUPIIIA4 5'-TTGGAGCTTGCTGGAATAC-3', nucleotides 840 to 858 of human *UP-III*) and the HUPIII second primer pair (HUPIIIS5 5'-ACAAACA-GAGGGTGGGAGGACAG-3', nucleotides 270 to 292 of

human *UP-III* and HUPIIIA6 5'-AGAAGGGCAGG-GAGCCCAGG-3', nucleotides 641 to 661 of human *UP-III*) according to the nucleotide sequence of the human *UP-III* (Fig. 1B). The HUPIII first and second primer pairs amplified 783 bp and 392 bp fragments, respectively. A human *beta-actin* (β -actin) specific primer pair, 5'-GTGGGGCGCCCCAGGCACCA-3' and 5'-CTCCTTA-ATGTCACGCACGATTTTC-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



Fig. 2. RT-PCR analysis for expression of the human *UP-Ib* and *UP-III* genes in normal urothelium and TCC. Expression of human *UP-Ib* and *UP-III* genes was analyzed in 3 normal transitional cell samples (Normal) and 12 TCC samples (TCC: No. 1–3, grade 1; No. 4–10, grade 2; and No. 11, 12, grade 3) by RT-PCR. Expression of *β-actin* was used as a control.

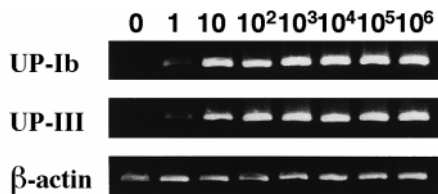


Fig. 3. Sensitivity of detection of *UP-Ib*- and *UP-III*-expressing cancer cells using HT1197 cells in blood by nested RT-PCR. Each lane includes 0, 1, 10, 10², 10³, 10⁴, 10⁵, or 10⁶ cancer cells in 5 ml of normal blood. Expression of *β-actin* was used as a control.

diluted from 10⁶ 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 blood (Fig. 3), indicating that our assay system is highly sensitive.

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.

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	UPIb	UPIII
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.



Fig. 4. RT-PCR analysis for expression of human *UP-Ib* and *UP-III* genes in peripheral blood of healthy volunteers and patients with TCC. The expression of *UP-Ib* and *UP-III* genes was analyzed in peripheral blood of 3 healthy volunteers (Normal) and 12 patients with TCC (TCC) by nested RT-PCR. Numbers correspond to those in Table I. Expression of *β-actin* was used as a control.

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

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.

We wish to thank Mr. Masafumi Suzuki of the Central Research Laboratory, Shiga University of Medical Sciences, for his technical assistance. This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

(Received June 8, 1998/Revised July 8, 1998/Accepted July 16, 1998)

REFERENCES

- 1) Yu, J., Manabe, M., Wu, X.-R., Xu, C., Surya, B. and Sun, T.-T. Uroplakin I: a 27-kD protein associated with the asymmetric unit membrane of mammalian urothelium. *J. Cell Biol.*, **111**, 1207–1216 (1990).
- 2) Wu, X.-R., Manabe, M., Yu, J. and Sun, T.-T. Large scale purification and immunolocalization of bovine uroplakins I, II, and III. *J. Biol. Chem.*, **265**, 19170–19179 (1990).
- 3) Wu, X.-R., Medina, J.-J. and Sun, T.-T. Selective interactions of UPIa and UPIb, two members of the transmembrane 4 superfamily, with distinct single transmembrane-domained proteins in differentiated urothelial cells. *J. Biol. Chem.*, **270**, 29752–29759 (1995).
- 4) Minsky, B. D. and Chlapowski, F. J. Morphometric analysis of the translocation of transitional epithelial cells during the expansion-contraction cycles of mammalian urinary bladder. *J. Cell Biol.*, **77**, 685–697 (1978).
- 5) Sariaks, S. N. and Chlapowski, F. J. Effect of ATP inhibitors on the translocation of luminal membrane between cytoplasm and cell surface of transitional epithelial cells during the expansion-contraction cycles of the rat urinary bladder. *Cell Tissue Res.*, **246**, 107–117 (1986).
- 6) Moll, R., Wu, X.-R., Lin, J.-H. and Sun, T.-T. Uroplakins, specific membrane proteins of urothelial umbrella cells, as histological markers of metastatic transitional cell carcinomas. *Am. J. Pathol.*, **147**, 1383–1397 (1995).
- 7) Wu, R.-L., Osman, I., Wu, X.-R., Lu, M.-L., Zhang, Z.-F., Liang, F.-X., Hamza, R., Scher, H., Cordon-Cardo, C. and Sun, T.-T. Uroplakin II gene is expressed in transitional cell carcinoma but not in bilharzial bladder squamous cell carcinoma: alternative pathways of bladder epithelial differentiation and tumor formation. *Cancer Res.*, **58**, 1291–1297 (1998).
- 8) Yu, J., Lin, J.-H., Wu, X.-R. and Sun, T.-T. Uroplakins Ia and Ib, two major differentiation products of bladder epithelium, belong to a family of four transmembrane domain (4TM) proteins. *J. Cell Biol.*, **125**, 171–182 (1994).
- 9) Wu, X.-R. and Sun, T.-T. Molecular cloning of a 47 kDa tissue-specific and differentiation-dependent urothelial cell surface glycoprotein. *J. Cell Sci.*, **106**, 31–43 (1993).
- 10) Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159 (1987).
- 11) Isono, T., Isegawa, Y. and Seto, A. Sequence and diversity of variable gene segments coding for rabbit T-cell receptor beta chains. *Immunogenetics*, **39**, 243–248 (1994).
- 12) Tachibana, O., Nakazawa, H., Lampe, J., Watanabe, K., Kleihues, P. and Ohgaki, H. Expression of Fas/APO-1 during the progression of astrocytomas. *Cancer Res.*, **55**, 5528–5530 (1995).
- 13) The Japanese Urological Association, Japanese Pathological Society and Japan Radiological Society. “General Rule for Clinical and Pathological Studies on Bladder Cancer,” 2nd Ed. (1993). Kanehara Co., Tokyo.
- 14) Rasheed, S., Gardner, M. B., Rongey, R. W., Nelson-Rees, W. A. and Arnstein, P. Human bladder carcinoma: characterization of two new tumor cell lines and search for tumor viruses. *J. Natl. Cancer Inst.*, **58**, 881–890 (1977).
- 15) Smith, B., Selby, P., Southgate, J., Pittman, K., Bradley, C. and Blair, G. E. Detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction. *Lancet*, **338**, 1227–1229 (1991).
- 16) Moreno, J. G., Croce, C. M., Fischer, R., Monne, M., Vihko, P., Mulholland, S. G. and Gomella, L. G. Detection of hematogenous micrometastasis in patients with prostate cancer. *Cancer Res.*, **52**, 6110–6112 (1992).
- 17) Matsumura, M., Niwa, Y., Hikida, Y., Okano, K., Kato, N., Shina, S., Shiratori, Y. and Omata, M. Sensitive assay for detection of hepatocellular carcinoma associated gene transcription (alpha fetoprotein mRNA) in blood. *Biochem. Biophys. Res. Commun.*, **207**, 813–818 (1995).