Jpn. J. Cancer Res. **90**, 753–757, July 1999

Detection of Disseminated Urothelial Cancer Cells in Peripheral Venous Blood by a Cytokeratin 20-specific Nested Reverse Transcriptase-Polymerase Chain Reaction

Yasuhisa Fujii,[1] Yukio Kageyama, Satoru Kawakami, Kazunori Kihara and Hiroyuki Oshima

Department of Urology, Tokyo Medical and Dental University School of Medicine, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519

More than half of all patients with invasive urothelial cancer subsequently develop metastatic disease even after radical resection of the primary cancer. In these patients, neoplastic cells may be disseminated prior to or during the operation. A nested reverse transcriptase-polymerase chain reaction (nested RT-PCR) assay which amplifies cytokeratin (CK) 20 transcripts was used to detect cancer cells in the peripheral blood of urothelial cancer patients. This assay was able to detect 10 bladder cancer cell line cells in a sample of ten million peripheral-blood mononuclear (PBMN) cells. CK 20-specific signals were detected in 9 (22.5%) of 40 PBMN cell samples prepared from 40 urothelial cancer patients in relation to the tumor stage, including 0/13 patients with a superficial tumor, 4/21 (19%) with a regionally invasive tumor and 5/6 (83%) with a metastatic tumor (P=0.0002 in χ^2 test). No signals were detected in any of 25 healthy donor PBMN cell samples. The present results indicate that the CK 20 RT-PCR assay is applicable for detection of urothelial cancer cells in the peripheral blood. The assay also confirms that hematogenic dissemination occurs in invasive urothelial cancers but rarely in superficial ones.

Key words: Bladder neoplasm — Hematogenous dissemination — Circulating carcinoma cells — Cytokeratin 20 — Polymerase chain reaction

While the presence of tumor cells in the circulation does not indicate that metastasis has occurred, it is the first crucial step in the cascade of hematogenic metastasis. Sensitive detection of such cells may have important therapeutic and prognostic implications. Reverse transcriptase-polymerase chain reaction (RT-PCR) has been applied to amplify mRNA sequences unique to breast cells, prostatic cells or other malignant cells in the circulation for early detection of micrometastases.[1, 2] However, few studies have reported the detection of transitional cell carcinoma (TCC) cells in blood or bone marrow by RT-PCR since mRNAs specific to urothelial cells have not been known. Very recently uroplakin Ib and III mRNA has been proposed as a specific marker for detection of TCC cells in the peripheral blood.[3]

Cytokeratins (CKs) are intermediate-sized filaments that are characteristically present in epithelial cells and tumors, but have also been detected in many normal and neoplastic non-epithelial cells using immunohistochemical and RT-PCR techniques. In earlier studies using RT-PCR, CK 8 and CK 18 expression was found in normal blood and bone marrow,[4] and CK 19 mRNA was detected in normal blood.[5, 6] CK 20 expression, on the other hand, is almost entirely confined to gastric and intestinal epithelium, urothelium and Merkel cells, whether normal or malignant.[7, 8] The CK 20 RT-PCR assay has enabled detection of gastrointestinal cancer cells in bone marrow and venous blood.[5, 9–12] Here we describe the detection of TCC cells in the peripheral blood by the CK 20 RT-PCR technique.

PATIENTS, MATERIALS, AND METHODS

Bladder cancer cell lines Three bladder cancer cell lines JTC 30, JTC 32,[13] and T24[14] were used in the study. JTC 30 and JTC 32 were established from well-differentiated and anaplastic TCC of the human bladder, respectively. JTC 30 and JTC 32 were cultured in DM 170 medium (Kyokuto Pharmaceutical Industrial Co., Tokyo) containing 10% fetal calf serum (FCS), and T24 was cultured in α minimum essential medium (MEM) (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS.

Patients The study population consisted of 40 patients having histologically diagnosed TCC of various stages; 13 patients with superficial TCC (Ta,T1,Tis N0 M0 in TNM classification[15]), 21 with invasive and regional TCC (T2-T4 N0 M0), and 6 with metastatic TCC (Tx N1-N3 Mx or Tx Nx M1) (Table I). Twenty-five healthy adults served as a control group. Written informed consent was obtained from all of them.

Blood samples Fourteen milliliter aliquots of peripheral blood were drawn from 40 patients and 25 healthy donors,

[1] To whom correspondence and requests for reprints should be addressed.
E-mail: y-fujii.uro@med.tmd.ac.jp

Table I. Correlation of CK 20-specific RT-PCR Results with the Patient Profile

Site[a)]	Stage	No. of cases	CK 20 signal	
			positive	negative
UUT	superficial	1	0	1
	regionally invasive	6	1	5
	metastatic	5	4	1
Bladder	superficial	11	0	11
	regionally invasive	15	3	12
	metastatic	1	1	0
Urethra	superficial	1	0	1
Total		40	9	31

a) UUT, upper urinary tract.

Table II. Cycling Protocol of PCR for CK 20 and β-Actin

Step	Denaturation (s/°C)	Annealing and extension (s/°C)	Cycles
1	40/94	120/74	1
2	40/94	115/69	1
3	40/94	110/68	1
4	40/94	105/67	1
5	40/94	100/66	1
6	40/94	95/65	1
7	40/94	90/64	1
8	40/94	85/63	1
9	40/94	80/62	1
10–30	40/94	60/61 and 90/72	21

and diluted with 14 ml of balanced salt solution. The mononuclear cell fraction was isolated as an intermediate layer by density centrifugation in the presence of Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) for 30 min at 400*g*.[16)]

RNA extraction Total RNA from cell lines and peripheral-blood mononuclear (PBMN) cell preparations was extracted using a commercially available RNA extraction kit in accordance with the recommendations of the manufacturer (Isogen; Nippon Gene, Toyama). Each RNA preparation was dissolved in 20 μl of RNase-free water. The amount and purity of RNA prepared from each sample was determined from the absorption at 260 nm and the absorption ratio of 260 to 280 nm (Gene Quant II; Pharmacia Biotech AB).

RT-PCR cDNA was synthesized with a reverse transcription kit (Life Technologies, Inc.), using 4 μg of total RNA, 2 μl of random hexamers (50 ng/μl) and 1 μl (200 units) of SuperScript II in a total volume of 20 μl.

The CK 20-specific nested RT-PCR assay was carried out as previously described,[9)] with PCR primers synthesized by Amersham Pharmacia Biotech, Tokyo. CK 20-A sense within exon 1, 5′-GCGTTTATGGGGGTGCTGGAG; CK 20-B antisense within exon 5, 5′-AAGGCTCTGGGAGGTGCGTCTC; CK 20-C sense in exon 1, 5′-CGGCGGGGACCTGTTTGT; CK 20-D antisense in exon 4, 5′-CAGTGTTGCCCAGATGCTTGTG. The first CK 20-specific PCR was performed in a final volume of 50 μl. The reaction mixture contained 18 μl of cDNA, 10× PCR buffer, 200 μM deoxynucleotide triphosphate (dNTP) mix, 0.5 μM CK 20-A and 0.5 μM CK 20-B primers, and 5 units of *Taq* DNA polymerase (TaKaRa Biomedicals, Otsu). The cycling protocol for PCR is listed in Table II.

The second RT-PCR was performed in the same manner as in Table II, but used 1 μl of the first PCR reaction mixture, and the primers CK 20-C and CK 20-D in a final volume of 50 μl. The internal (second) PCR yielded a 485-bp product.

To monitor cDNA synthesis, RT-PCR for β-actin was simultaneously performed with 1 μl of cDNA, and β-actin sense, 5′-CCAAGGCCAACCGCGAGAAGATGAC and antisense, 5′-AGGGTACATGGTGGTGCCGCCAGAC primers (0.5 μM each) in a volume of 50 μl. The product length of β-actin PCR was 587 bp.

One microgram of total RNA prepared from the bladder cancer cell line JTC 30 was used as a positive control. Contamination was routinely checked by RT-PCR assay of RNA-free samples (water control). All RT-PCR products were separated by electrophoresis in a 1.5% agarose gel in Tris-acetate-EDTA (TAE)-buffer and visualized by ethidium-bromide staining. The molecular weights were determined by using DNA molecular-weight marker 4 (Nippon Gene).

Nucleotide-sequence determination The CK 20 RT-PCR product (485 bp) from positive controls and randomly chosen patients was sequenced using the ABI PRISM 377 DNA sequencer (Perkin Elmer, Foster, CA) as specified by the manufacturer. DNA sequences were aligned and analyzed using an Apple Macintosh computer.

Statistical analysis The association between the CK 20 mRNA detection rate and tumor stages was examined by χ^2 test.

RESULTS

Sensitivity and specificity of CK 20 RT-PCR In all three bladder cancer cell line samples, the CK 20-specific nested RT-PCR amplified 485-bp product was distinctly visible as a single band. No CK 20-specific signals were detected in PBMN cells of the 25 healthy donors.

To determine the sensitivity of the assay, JTC 30 cells were mixed with 10×10^6 PBMN cells obtained from a healthy donor as indicated in Fig. 1. RNA extracted from each sample was processed by CK 20-specific RT-PCR.

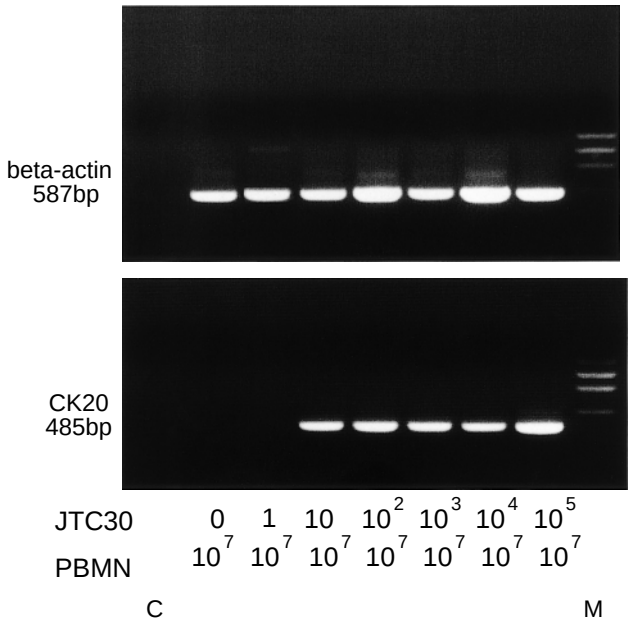

Fig. 1. CK 20-specific nested RT-PCR amplification product [485 bp] of JTC 30 bladder cancer cells. Cells were mixed with 10×10^6 PBMN cells of a healthy donor as indicated. The control test was performed as RT-PCR for β-actin and is shown in the upper band. Ten JTC 30 cells were detected. C, water negative control; M, molecular weight marker.

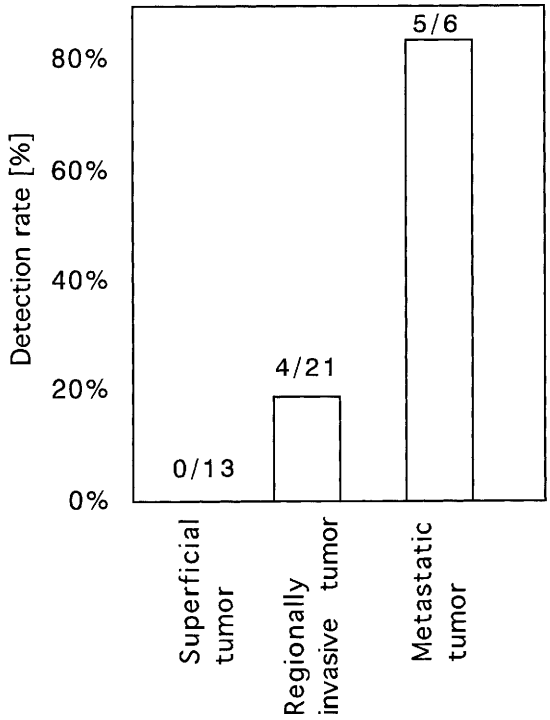

Fig. 3. Correlation of CK 20-specific RT-PCR results with stages of disease in urothelial cancer. The number of positive cases and the total number of cases, respectively, are shown above the columns.

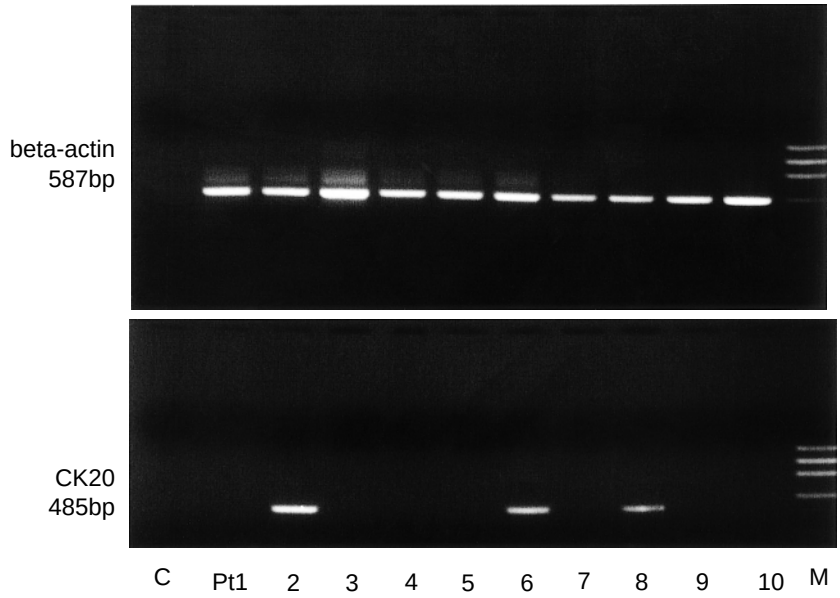

Fig. 2. CK 20-specific nested RT-PCR amplification product [485 bp] from PBMN cell samples obtained from patients with urothelial cancer. Ten arbitrarily chosen specimens (1–10) demonstrated. Patients 2, 6 and 8 show a CK 20-specific product; the others were negative. C, water negative control; M, molecular weight marker.

The results are shown in Fig. 1. Ten JTC 30 cells were found in a sample of 10×10^6 PBMN cells.

Detection of CK 20 mRNA in patients' blood samples

Among urothelial cancer patients, 9/40 (22.5%) exhibited a CK 20-specific signal in the PBMN cell fractions. A representative electrophoresis of CK 20 RT-PCR products is presented in Fig. 2. As shown in Fig. 3, the CK 20-specific signal was detected in 4 of 21 (19%) patients with regionally invasive tumors and in 5 of 6 (83%) with metastatic lesions. A CK 20-specific signal was undetectable in any of 13 with superficial tumors ($\chi^2=16.796$, $P=0.0002$ in χ^2 test). The site (upper urinary tract, bladder or urethra) of urothelial cancer seems to be unrelated to the RT-PCR results (Table I).

Sequencing of the final PCR products revealed that the amplified products were consistently identical with the CK 20 cDNA sequence.

DISCUSSION

The present study addresses the applicability of CK 20-specific nested RT-PCR assay to the detection of urothelial TCC cells. All three bladder cancer cell line cells tested positive for CK 20 mRNA. The system is able to detect 10 JTC 30 cells per 10×10^6 PBMN cells. In other words, approximately 4 pg of total RNA of JTC 30 cells mixed with 4 μg of total RNA of PBMN cells is detectable. This supports the previous result that the CK-20 RT-PCR assay detected 0.1 pg of unmixed total RNA of the pancreatic tumor cell line A818-4 and 0.5 pg of total RNA of colorectal tumor cell line HT29.[9] None of the blood samples from the 25 healthy individuals exhibited the CK 20-specific signal.

The CK 20 signal was detected in PBMN cell fractions of some invasive urothelial cancer patients. The presence of circulating tumor cells does not necessarily predict subsequent metastatic disease. Most circulating tumor cells are destroyed rapidly,[17] and only a small number of cells may implant. In addition, the metastatic potential of individual tumor cells may vary. Therefore, the prognostic value of circulating tumor cells in urothelial cancer patients is still uncertain. It has been reported, however, that the survival rate of gastrointestinal cancer patients with CK 20 mRNA-positive bone marrow was significantly lower than that of negative patients.[10] Further clinical observation will reveal the prognostic value of detection of circulating urothelial tumor cells by CK 20 RT-PCR amplification.

No CK 20-specific signal was observed in patients with superficial urothelial cancers. Superficial bladder cancer is generally believed not to have metastatic potential and can be managed by organ-sparing treatment including transurethral resection of the tumor. The current result supports this modern urological policy.

In conclusion, the CK 20 RT-PCR assay is highly sensitive and specific, and therefore capable of detecting urothelial TCC cells circulating in the peripheral blood.

ACKNOWLEDGMENTS

This study was supported in part by Grant-in-Aid 9771205 for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.

(Received April 2, 1999/Revised May 10, 1999/Accepted May 13, 1999)

REFERENCES

1) Johnson, P. W., Burchill, S. A. and Selby, P. J. The molecular detection of circulating tumour cells. *Br. J. Cancer*, **72**, 268–276 (1995).
2) Raj, G. V., Moreno, J. G. and Gomella, L. G. Utilization of polymerase chain reaction technology in the detection of solid tumors. *Cancer*, **82**, 1419–1442 (1998).
3) Yuasa, T., Yoshiki, T., Tanaka, T., Kim, C. J., Isono, T. and Okada, Y. Expression of uroplakin Ib and uroplakin III genes in tissues and peripheral blood of patients with transitional cell carcinoma. *Jpn. J. Cancer Res.*, **89**, 879–882 (1998).
4) Traweek, S. T., Liu, J. and Battifora, H. Keratin gene expression in non-epithelial tissues. Detection with polymerase chain reaction. *Am. J. Pathol.*, **142**, 1111–1118 (1993).
5) Burchill, S. A., Bradbury, M. F., Pittman, K., Southgate, J., Smith, B. and Selby, P. Detection of epithelial cancer cells in peripheral blood by reverse transcriptase-polymerase chain reaction. *Br. J. Cancer*, **71**, 278–281 (1995).
6) Krismann, M., Todt, B., Schroder, J., Gareis, D., Muller, K. M., Seeber, S. and Schutte, J. Low specificity of cytokeratin 19 reverse transcriptase-polymerase chain reaction analyses for detection of hematogenous lung cancer dissemination. *J. Clin. Oncol.*, **13**, 2769–2775 (1995).
7) Moll, R., Lowe, A., Laufer, J. and Franke, W. W. Cytokeratin 20 in human carcinomas. A new histodiagnostic marker detected by monoclonal antibodies. *Am. J. Pathol.*, **140**, 427–447 (1992).
8) Moll, R., Zimbelmann, R., Goldschmidt, M. D., Keith, M., Laufer, J., Kasper, M., Koch, P. J. and Franke, W. W. The human gene encoding cytokeratin 20 and its expression during fetal development and in gastrointestinal carcinomas. *Differentiation*, **53**, 75–93 (1993).
9) Soeth, E., Roder, C., Juhl, H., Kruger, U., Kremer, B. and Kalthoff, H. The detection of disseminated tumor cells in bone marrow from colorectal-cancer patients by a cytokera-

tin-20-specific nested reverse-transcriptase-polymerase-chain reaction is related to the stage of disease. *Int. J. Cancer*, **69**, 278–282 (1996).
10) Soeth, E., Vogel, I., Roder, C., Juhl, H., Marxsen, J., Kruger, U., Henne-Bruns, D., Kremer, B. and Kalthoff, H. Comparative analysis of bone marrow and venous blood isolates from gastrointestinal cancer patients for the detection of disseminated tumor cells using reverse transcription PCR. *Cancer Res.*, **57**, 3106–3110 (1997).
11) Funaki, N. O., Tanaka, J., Ohshio, G., Onodera, H., Maetani, S. and Imamura, M. Cytokeratin 20 mRNA in peripheral venous blood of colorectal carcinoma patients. *Br. J. Cancer*, **77**, 1327–1332 (1998).
12) Weitz, J., Kienle, P., Lacroix, J., Willeke, F., Benner, A., Lehnert, T., Herfarth, C. and von Knebel Doeberitz, M. Dissemination of tumor cells in patients undergoing surgery for colorectal cancer. *Clin. Cancer Res.*, **4**, 343–348 (1998).
13) Kakuya, T., Yamada, T., Yokokawa, M. and Ueda, T. Establishment of cell strains from human urothelial carcinoma and their morphological characterization. *In Vitro*, **19**, 591–599 (1983).
14) Bubenik, J., Baresova, M., Viklicky, V., Jakoubkova, J., Sainerova, H. and Donner, J. Established cell line of urinary bladder carcinoma (T24) containing tumour-specific antigen. *Int. J. Cancer*, **11**, 765–773 (1973).
15) Sobin, L. H. and Wittekind, C. H. "UICC TNM Classification of Malignant Tumours," 5th Ed., pp. 183–190 (1997). Wiley-Liss, New York.
16) Boyum, A. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.*, **21** (Suppl. 97), 77–89 (1968).
17) Fidler, I. J. Metastasis: quantificative analysis of distribution and fate of tumor cell emboli labeled with [^{125}I]iodo-2′deoxyuridine. *J. Natl. Cancer Inst.*, **45**, 773–782 (1970).