Detection of Circulating Cancer Cells by Reverse Transcription-Polymerase Chain Reaction for Uroplakin II in Peripheral Blood of Patients with Urothelial Cancer

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INTRODUCTION

Approximately 50% of patients with muscle-invasive cancers of the urinary tract already have distant metastases (1). In addition, roughly 5% of patients with well-differentiated and moderately differentiated superficial papillary urothelial cancers may unexpectedly manifest distant metastases after eradication of the primary tumors (1). Vascular or lymphatic infiltration presented in the surgical specimen is an important predictor of systemic spread. Even thorough histopathological examination, however, sometimes misses such findings. On the other hand, it is still controversial as to the clinical usefulness of molecular markers, including aberration of p53 for the prediction of metastasis in urothelial cancers (2, 3).

Attempts to find cancer cells in the circulatory blood, bone marrow, or lymph nodes using the present PCR technique have been made in various types of malignant tumors, including melanoma (4, 5), colon (6, 7), breast (8), and prostate (9–11) cancers. Carcinoembryonic antigen and epidermal growth factor receptor are tactile molecular targets for the detection of circulating cancer cells in patients with colon and breast cancers, respectively (7, 8). Prostate-specific antigen and prostate-specific membrane antigen are not specific to malignant neoplasms of the prostate, but detection of prostate cancer cells in the circulatory blood has effectively been realized by the RT-PCR3 method owing to their high specificity for the prostatic epithelia (9–11). On the other hand, few attempts at the molecular detection of urothelial cancer cells in the blood or lymph nodes have been made because neither urothelial cancer specific nor urothelium-specific molecules or genetic changes were available.

In 1990, genes that encode urothelium-specific transmembrane proteins, uroplakins (UPs), were cloned. We established a nested RT-PCR assay for the detection of UP-II-positive cells in the blood of urothelial cancer patients (14, 15). In our preliminary examination, UP-II-positive cells were found in three of three patients with metastatic urothelial cancers (15). The present series of investigations using a larger number of patients and multiple blood sampling have indicated a promising role for UP II mRNA detection using the circulatory blood of patients with urothelial cancer as a new staging marker.

ABSTRACT

Few attempts have been made at the molecular detection of urothelial cancer cells in the blood or lymph nodes mainly because of an absence of good candidate molecular or genetic changes specific to urothelial cancer or urothelium. In 1990, however, genes that encode urothelium-specific transmembrane proteins, uroplakins (UPs), were cloned. We have established a method of detecting circulating cancer cells in peripheral blood of patients with transitional cell carcinoma by nested reverse transcription-PCR assay for UP II. UP II mRNA-positive cells were detected in 3 (10.3%) of 29 patients with superficial cancers (pTa–1 N0 M0), 4 (28.6%) of 14 patients with muscularly invasive cancers (pT2–4 N0 M0), 2 (40.0%) of 5 loco-regional node-positive patients (pN1–2 M0), and 6 (75.0%) of 8 patients with distant metastases. Positive rates, therefore, increased with tumor extension (P = 0.0033, Kruskal-Wallis test). Furthermore, sequential blood sampling was performed in three patients with metastases during and after systemic chemotherapy, and UP-II-positive cells were found to have disappeared in two patients who responded well to the systemic chemotherapy. These results suggest that our nested reverse transcription-PCR assay for UP II is highly specific and might be used as a tumor marker for molecular staging of urothelial cancers, although the sensitivity is not so optimal.

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3 The abbreviations used are: RT-PCR, reverse transcription-PCR; UP, uroplakin; TCC, transitional cell carcinoma.
PATIENTS AND METHODS

Patients and Blood Samples. The first sample set consisted of peripheral blood obtained from 56 urothelial cancer patients who were hospitalized and treated at Kyoto University Hospital during the period of January 1, 1999, to December 31, 1999. All 14 patients with locally invasive cancers (pT2–4 N0 M0) and 13 patients with metastases (5 had loco-regional lymph node metastases without distant metastases and 8 had distant metastases) treated during the above period were enrolled in this study. In addition, 29 randomly selected patients with superficial urothelial cancers (pTa–1 N0 M0) treated endoscopically were also investigated. The histopathological diagnosis was TCC in all cases. Clinicopathological profiles of the 43 patients with localized disease are summarized in Table 1. Blood sampling was performed before any treatment for the first sample set.

The second sample set consisting of peripheral blood from 10 healthy volunteers and 10 patients with untreated renal cell carcinomas (6 patients with metastasis and 4 patients without metastasis) was prepared for the present study as a control. In addition, sequential blood sampling over the treatment course was performed to yield a third sample set for three patients with nodal or distant metastases that were treated with systemic chemotherapy.

Grading and Staging of Primary Tumors, and Estimation of Metastatic Tumor Volumes. Grade and stage of primary tumors were classified according to the WHO criteria (16) and TNM classification (17), respectively. The volume of metastatic tumor was estimated from an imaging study.

Tumor Specimens. Tumor tissue samples of 23 TCCs (7 were grade G1, 8 were G2, and 8 were G3), 1 bladder squamous cell carcinoma, 1 renal cell carcinoma, and 1 melanoma were obtained for the present study.
transilluminator. We have used the result of the dilution experiment to ethidium bromide solution for 30 min, and visualized on a UV transilluminator.

bp were separated on a 2% agarose gel, stained in 0.5 M 4M, and the second antisense primer, 5'-TCTCTGGTC-3'. The first primer pair produces a PCR product of 268 bp in size, and the second primer pair produces a PCR product 400 bp in length and larger than that of RT-PCR. The 25-M deoxynucleotide triphosphates, 25 pmol of each primer, 0.2 units of AmpliTaq polymerase.

The PCR products of several blood samples as well as HT 1197 cells that were positive for UP II expression were sequenced with ABI prism 330 (Perkin-Elmer, Norwalk, CT). All were confirmed to be identical to the UP II cDNA sequence. PCR for the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was performed and used as an internal control. A negative control was included in each step of the RT-PCR by adding water in place of reverse transcriptase and AmpliTaq polymerase.

The nested RT-PCR was performed twice for each total RNA sample. A third nested RT-PCR was performed only when the results were inconclusive.

Sensitivity Testing of the Nested RT-PCR Assay. HT1197 cells (1 × 10^6) were suspended in 1 ml of PBS and serially diluted from 10 to 10^6 times. One ml of each diluted sample was added to 5 ml of peripheral blood from a healthy volunteer and subjected to the nested RT-PCR assay described above.

Ethical Issues. Written informed consent was obtained from all of the patients as well as the 10 healthy volunteers who participated in this study before blood sampling.

RESULTS

UP II mRNA Expression in Tumor Tissues. The 17 primary and 6 metastatic TCCs were all positive for UP II mRNA expression by both the first round of RT-PCR and the nested PCR. On the other hand, UP II-specific products were not detected even by the nested PCR in a bladder squamous cell carcinoma and in three renal cell carcinomas (Fig. 1).

Sensitivity of the Nested RT-PCR for UP II mRNA. The UP-II-positive control cell line, HT 1197, was sequentially attenuated and added to peripheral blood of a healthy volunteer negative for UP II mRNA expression and examined for UP II mRNA expression. UP II mRNA could be detected even in a sample in which the cell suspension was diluted up to 10^5 times (Fig. 2). The limit of this assay therefore was calculated to be two cancer cells in 1 ml of blood. We defined this band intensity as a standard for positivity throughout the study.

Detection of UP II mRNA in the Peripheral Blood of Patients and Controls. Peripheral blood from 56 urothelial cancer patients with localized tumors (n = 43), loco-regional lymph node metastases (n = 5), or distant metastases (n = 8) was analyzed for the presence of UP II mRNA (Fig. 1). In 4
The relation between positivity on positive expression of UP II by the nested RT-PCR. From 10 healthy volunteers and 10 renal cancers. None showed multiple blood UP-II-positive cells in metastatic patients.

Influence of Systemic Chemotherapy on Detection of UP-II-positive Cells in Metastatic Patients. Multiple blood sampling was done for three patients (sample set 3) who underwent systemic combination chemotherapy consisting of cisplatin, epidoxorubicin, and methotrexate for their metastatic disease (Table 3). UP-II-positive cells were detected in the blood collected before chemotherapy in two patients (patients 8 and 11), whereas UP II was negative in patient 53. These three patients were treated with three to four cycles of chemotherapy. Patients 8 and 11 responded to chemotherapy well, and their UP-II-positive cells disappeared from the first cycle through to the final cycle of chemotherapy. In case 53, rather conflicting results were obtained for UP II RT-PCR. UP-II-positive cells could not be detected before chemotherapy, although the metastatic tumor burden was very large. UP-II-positive cells, however, were once detected just after the first cycle of chemotherapy, although 90% of lymph node metastases and liver metastases were eradicated by that time. This patient has thus far undergone four cycles of chemotherapy and now harbors only small metastatic foci in the liver and the lumbar vertebral bones. RT-PCR for UP II in the blood was consistently negative after two cycles of the chemotherapy.

**DISCUSSION**

In this study, circulating UP II mRNA-positive cells were detected in patients with urothelial cancers of varying stages, and the detection rates were increased with stage. Circulating UP II mRNA-positive cells were detected in 28.6% of patients with invasive tumors (pT2–4N0M0) and 40.0% of patients with regional lymph-node metastases (pN1–2M0). This data are particularly important in that a tumor marker would have a significant impact on the clinical management of such a disease status.
i.e., whether to conduct adjuvant chemotherapy after radical surgery. Li et al. (18) found circulating UP-II-positive cells in 3 of 10 metastatic tumor patients and in 0 of 50 nonmetastatic patients. The difference in detection rates between the series of Li et al. (18) and ours may be attributable to a difference in the sensitivity of the assay. We used nested PCR as described previously, whereas Li et al. (18) used a single round of PCR. Li et al. (18) preferred a single round of PCR because they were afraid of pseudo-positive results in the nested PCR assay. In fact, 10.3% of patients with superficial urothelial cancers in this study showed positive results for UP II expression in the blood. Occult metastases rarely exist in this subset of patients, but 5–15% of stage T4 to T1 patients will have metastases in the future (1). All of the three superficial cancer patients positive for UP II harbored five or more tumor lesions in the bladder. Pathological understaging is apt to occur in patients with such multiple lesions in the bladder. It is therefore possible that the positive UP II RT-PCR in the superficial cancer patients resulted from microscopically infiltrating cancer cells rather than from illegitimate expression. We consider this nested PCR protocol to have succeeded in improving sensitivity without compromising specificity because we obtained negative results in all healthy volunteers and in all 10 patients with renal cell carcinomas in duplicated assays.

Approximately 30% of patients with musculocutaneous invasive cancers without apparent metastases showed the presence of circulating cancer cells in this study. This result is interesting in that ≥50% of this subset of patients will later have metastatic diseases even if they undergo radical surgery (1). Longer follow-up for these patients will clarify whether the positive results obtained in the present analysis are related to the occult existence of metastasis.

A similar approach for the detection of circulating cancer cells has been adopted in urothelial cancer by Fuji et al. (19) using cytokeratin 20 as a molecular marker. Cytokeratin 20 is an epithelial-specific antigen expressed in gastric and intestinal epithelium, urothelium, and Merkel cells, whether normal or malignant (20, 21). The results in gastric cancer and colorectal cancer have already been reported (21–23). The reported detection rates in urothelial cancer are compatible with our values. Cytokeratin 20, however, is expressed in tissues other than urothelium and urothelial cancers. UPS are therefore considered to be more suitable and reliable markers for urothelial cancers in that the expression of UPS is highly specific for urothelial cells. One squamous cell carcinoma of the urinary tract did not express UP II mRNA. The only concern that we have is the reduction in UP expression in poorly differentiated TCCs because UPS are assumed to be closely involved in the differentiation of urothelium (12, 13).

The monitoring of UP-II-positive cells in the blood during the clinical course of various treatments, including surgery, radiation, and chemotherapy, may provide information about disease status. The disappearance of UP-II-positive cells in the blood of patients who show a favorable response to chemotherapy may indicate that the metastatic disease is consolidated and less active. On the other hand, the persistence of UP-II-positive cells may suggest incomplete eradication of cancer cells even if the patient clinically shows no residual tumor.

In conclusion, UP-II-positive cells were detected in the peripheral blood of patients with TCC of the urinary tract. The detection rates changed with disease status. These results suggest that our nested RT-PCR assay for UP II is highly specific and might be used as a tumor marker for molecular staging of urothelial cancers, although the sensitivity is not so optimal. Studies of a larger scale and long-term follow-up are warranted to clarify the clinical usefulness of this assay.

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