Circulating Cell-free mRNA in Plasma as a Tumor Marker for Patients with Primary and Recurrent Gastric Cancer

NOBUYUKI TANI[1], DAISUKE ICHIKAWA[1], DAITO IKOMA[1], HARUHISA TOMITA[1], SOUJIN SAI[1], HISASHI IKOMA[1], KAZUMA OKAMOTO[1], TOSHIYA OCHIAI[1], YUJI UEDA[1], EIGO OTSUJI[1], HISAKAZU YAMAGISHI[1], NORIMASA MIURA[2] and GOSHI SHIOTA[3]

[1]*Department of Surgery, Division of Digestive Surgery, Kyoto Prefectural University of Medicine, Kyoto, 602-8566;*
[2]*Department of Pathophysiological and Therapeutic Science, Division of Pharmacotherapeutics and*
[3]*Department of Genetic Medicine and Regenerative Therapeutics, Division of Molecular and Genetic Medicine, Graduate School of Medicine, Tottori University, Yonago, 683-8503, Japan*

Abstract. *Background: The diagnostic value of circulating mRNA for the early detection of primary and recurrent gastric cancer was evaluated. Patients and Methods: Circulating hTERT and MUC1 mRNA were amplified in the plasma from 52 gastric cancer patients (40 preoperative and 12 postoperative patients) and 20 healthy controls. The results were compared with those of a circulating cancer cell assay and methylation-specific polymerase chain reaction assay. Results: Cell-free mRNA of the analyzed genes was detected in 6 (15%) preoperative gastric cancer patients (hTERT: 3 and MUC1: 4 patients) and 2 follow-up patients. These mRNAs were not detected in the plasma from healthy volunteers. There was no correlation between the results of the cell-free mRNA and the other assays. Conclusion: Detection of circulating cell-free mRNA might serve as a new complementary tumor marker for gastric cancer.*

Recent molecular analyses have improved our understanding of oncogenesis and progression in cancer cells (1). Nevertheless, the diagnosis and treatment decisions for cancer patients rely largely on various conventional examinations in clinical practice. Endoscopic examination, barium study and computed tomography (CT) have been recognized as reliable and valuable methods for diagnosis including screening and follow-up in gastric cancer patients. Serum tumor markers, such as carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9, have also been used as convenient diagnostic assays. These conventional serum markers, however, lack sufficient sensitivity and specificity for early detection of cancer (2-4).

Several molecular approaches using the peripheral blood of patients with cancer have been assessed recently for their ability to detect various early stage cancers. One method uses cellular nucleic acids and can detect a small number of circulating cancer cells (5, 6). Another method uses the cell-free nucleic acids that are released from tumors into the circulation system (7-9). The latter method is further divided into two categories according to their detection targets, cell-free DNA and RNA. We have previously reported that the detection of small numbers of circulating cancer cells and plasma/serum aberrant methylation can serve as tumor markers in gastric cancer patients (6, 8). The relatively low sensitivity of each assay, however, represents a significant limitation, therefore a combination of various assays might be more informative for the early detection of cancer.

RNA has also been found circulating in the plasma/serum of cancer patients and in healthy individuals, and the detection of tumor-related cell-free mRNA opens up a new and interesting field in the screening and monitoring of cancer patients (10, 11). In this study, we set out to investigate the diagnostic value of circulating cell-free mRNA for the early detection of primary and recurrent gastric cancer, comparing the results with those of the circulating cancer cell assay and the methylation-specific polymerase chain reaction assay (MSP).

Patients and Methods

Patients and samples. Preoperative plasma samples were collected prospectively from 40 patients with gastric cancer, as well as from 20 healthy volunteers. The resected gastric cancer specimens were fixed in buffered formalin and embedded in paraffin for pathological examination using standard methods. Macroscopic

Correspondence to: Dr. Daisuke Ichikawa, Department of Surgery, Division of Digestive Surgery, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto, 602-8566, Japan. Tel: +81 75 251 5527, Fax: +81 75 251 5522, e-mail: ichikawa@koto.kpu-m.ac.jp

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and microscopic classification of tumors was based on the UICC/TMN staging system (12). Another 12 postoperative patients 1-year after curative gastrectomy were also enrolled in this study and plasma samples were collected from the patients on their periodical postoperative check-up. During regular visits to out-patients clinic, they were followed up by chest roentegenography, ultrasonography, endoscopy, CT and conventional tumor markers such as CEA and CA19-9. Informed consent was obtained from all participants for this protocol before obtaining their blood samples.

A 10 ml peripheral blood sample was collected from each participant. Immediately after collection, 5 ml of the sample were subjected to isolation of nuclear cells on Ficoll-Isopaque (Pharmacia, Freiburg, Germany) as described elsewhere, the remaining 5ml was subjected to isolation of cell-free nucleic acids by 3 spin protocol (1500 rpm for 30 min, 3000 rpm for 5 min, and 4500 rpm for 5 min) to prevent the contamination of cellular nucleic acids. Plasma samples were then stored at –80˚C until further processing. Cell-free mRNA and genomic DNA were isolated from 280 μl and 400 μl of plasma samples by the QIAamp viral RNA kit and the QIAamp blood mini kit, respectively (Qiagen, Hilden, Germany) according to the manufacturer's instruction.

Reverse transcription-polymerase chain reaction (RT-PCR) for cell-free mRNA. Quantitative RT-PCR was carried out with a Quantitect SYBR Green RT-PCR kit (Qiagen) on a LightCycler System (Roche Diagnostics, Mannheim, Germany). Sense and antisense primer sequences for *hTERT* and *MUC1* genes are described elsewhere (10, 13). Primers for the *GAPDH* gene were also used as a quality control (13). The standard reaction volume was 20 μl and contained 10 μl of SYBR Green Buffer, 0.2 μl of RT Mix, 1 μl of each primer (20 μM) and template mRNA equivalent to that from a 50 μl plasma sample. Samples were incubated for 5 min at 50˚C and for 15 min at 95˚C. Amplification was carried out for 45 cycles under the following conditions: 95˚C for 0 sec, 57˚C for 10 sec, 72˚C for 10 sec and 82˚C for 5 sec (to measure the fluorescence) for the hTERT gene. The temperature was 84˚C when measuring the MUC1 gene. Melting curve analysis was performed directly after PCR. Each run of amplification included RNA of the MKN45 cell line used as a positive control and a negative control without RNA. None of the 20 plasma samples of healthy volunteers presented any detectable mRNA of the analyzed genes in this study (Figure 1A), therefore a signal appearing within the first 40 cycles was scored as positive for the existence of the target cell-free mRNA in the plasma samples.

Comparison with circulating cancer cell assay and methylation-specific polymerase chain reaction (MSP) assay. The RT-PCR using CEA-specific primers was performed for identification of circulating cancer cells in plasma samples as described elsewhere (6). The plasma-derived DNA was treated with sodium bisulfate using the CpGenome DNA modification kit (Intergen, New York, NY, USA). The modified DNA was then subjected to methylation-specific PCR for detecting aberrant methylation of *p16*, *E-cadherin* and *retinoic acid receptor-beta* genes, as described elsewhere (14).

Statistical analyses. Statistical comparisons were performed using Fisher's exact test to examine associations between serum promoter methylation results and clinicopathological features. *P*-values <0.05 were considered to indicate significance.

Table I. *Relationship between cell-free plasma mRNA and clinicopathological features.*

	Circulating cell-free mRNA		
Factor	Positive	Negative	*P*-value
Age (mean)			
(year)	65	64	0.95
Gender			
Male	4	22	
Female	2	12	0.93
Macro			
Localized	5	29	
Diffuse	1	5	0.90
Tumor size (mean)			
(mm)	49	54	0.74
Histological type			
Diff.*	2	17	
Undiff.*	4	17	0.45
Depth of invasion (T)			
1,2	4	21	
3,4	2	13	0.82
Lymphatic invasion			
Negative	3	17	
Positive	3	17	1.00
Vascular invasion			
Negative	4	24	
Positive	2	10	0.85
Lymph node metastasis (N)			
Negative	3	18	
Positive	3	16	0.89
Stage			
I and II	4	22	
III and IV	2	12	0.93

*Diff.: Differentiated adenocarcinoma, Undiff.: Undifferentiated adenocarcinoma.

Results

Cell-free mRNA in preoperative gastric cancer patients. The 40 preoperative patients with primary tumors included 20 with TNM stage I, 6 with stage II, 8 with stage III and 6 with stage IV. Representative results of the RT-PCR assays using cell-free mRNA are shown in Figure 1. Cell-free mRNA of *hTERT* and *MUC1* genes was detected in 3 (8%) and 4 (10%) patients, respectively. Altogether, 6 (15%) patients were positive for at least one tumor-related mRNA. GAPDH mRNA was detected in all the plasma samples from cancer patients and healthy control individuals, reflecting the presence of cell-free mRNA in the circulating plasma from both neoplastic and non-neoplastic sources. On the other hand, no mRNA of *hTERT* or *MUC1* was detected in the plasma from the volunteers who served as controls in this study. No statistical association between the results of these assays and various clinicopathological factors (Table I) was found.

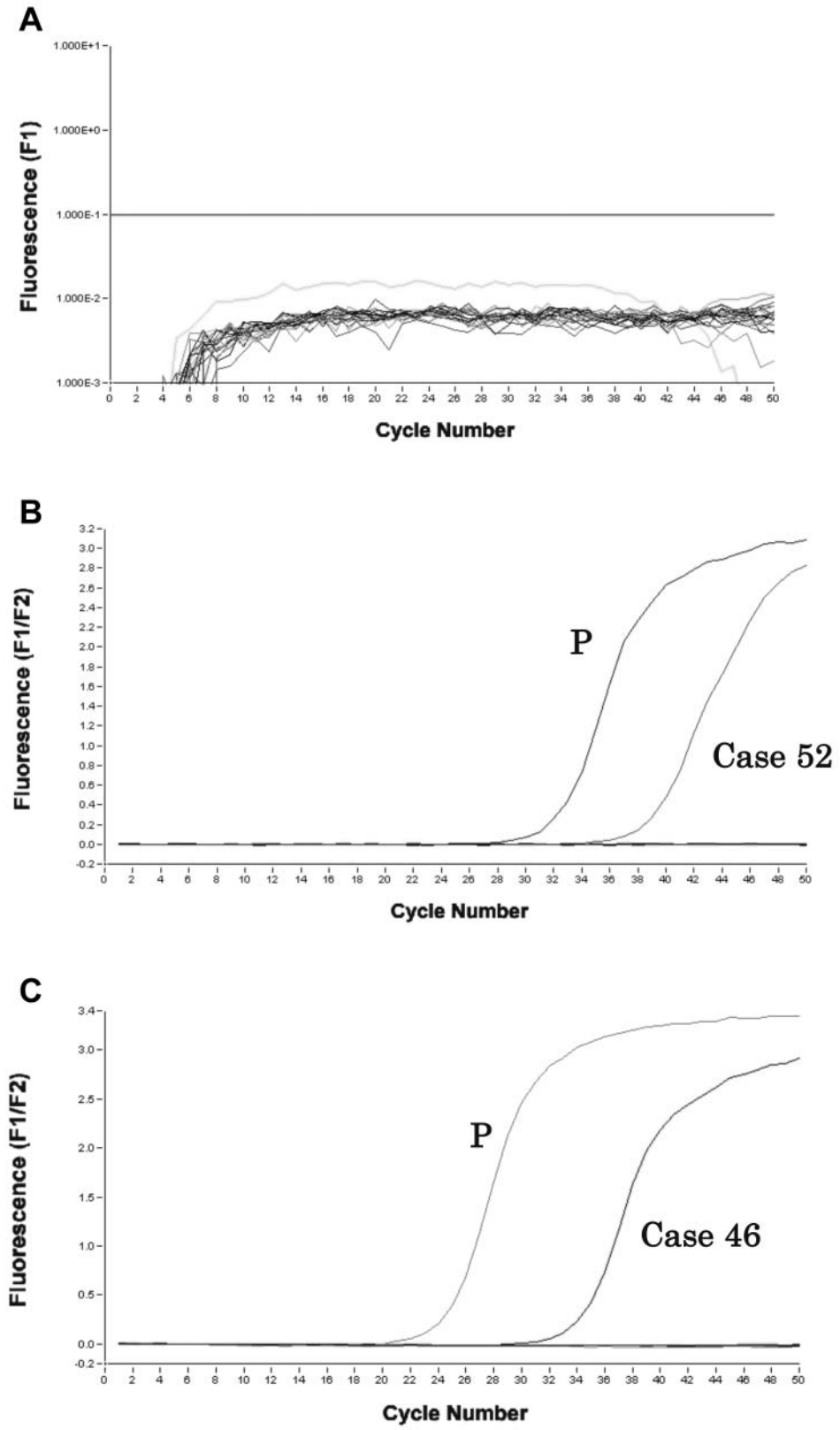

Figure 1. *Representative results of RT-PCR amplification plots. No plasma mRNA of hTERT or MUC1 was detectable in healthy individuals (A). Representative result of analysis for hTERT (B) and MUC1 (C). P: Positive control from MKN45 cancer cell line.*

Table II. *The result of circulating cell-free mRNA and clinicopathological characteristics of 12 patients in the follow-up series.*

No.	Age	Gender	Histology*	Stage	Circulating cell-free mRNA	Recurrences
1	75	M	Undiff.	III b	–	None
2	75	M	Undiff.	I b	–	None
3	57	M	Undiff.	III a	–	None
4	70	M	Diff.	III b	–	None
5	55	M	Diff.	IV	–	None
6	53	M	Diff.	IV	*hTERT*	Recurrence
7	57	F	Diff.	I b	–	None
8	56	F	Undiff.	I b	–	None
9	46	F	Diff.	II	–	None
10	71	F	Undiff.	IIIa	*hTERT, cMET*	5 months later
11	61	M	Undiff.	II	–	None
12	78	M	Diff.	II	–	None

* Diff.: differentiated adenocarcinoma, Undiff.: undifferentiated adenocarcinoma.

Cell-free mRNA in patients during follow-up after gastrectomy. In the follow-up patients, 2 out of 12 patients were positive for circulating cell-free tumor-related mRNA in their plasma samples. The results and clinical details are summarized in Table II. One of the two patients demonstrating tumor-related cell-free mRNA showed definite findings of recurrence by CT at the time of analysis, the other had no definite findings of recurrence at the time of the analysis. This latter patient, however, did develop para-aortic lymph node recurrences 5 months later.

Comparison with results of the other assays. Circulating tumor cells were detected in 4 patients (2 preoperative and 2 follow-up) using the CEA-specific RT-PCR assay and aberrant methylation of at least one gene was detected in 12 patients (*p16* in 5, *E-cadherin* in 5 and *retinoic acid receptor-beta* in 2) (8 preoperative and 4 follow-up) with the MSP assay in this study. The positive rate of the cell-free mRNA assay was equivalent to that of the MSP assay, however, there was no correlation with the results of either of the others assays (Table III). On the other hand, the circulating cancer cell assay had a lower positive rate than the other two assays described above. Circulating cancer cells were not detected in 10 out of the 12 patients positive for tumor-related cell-free mRNA in the plasma.

Table III. *Correlation between results of the cell-free mRNA assay and the MSP assay.*

	MSP		
	Positive (n=12)	Negative (n=40)	*P*-value
Cell-free mRNA			
Positive (n=8)	3	5	
Negative (n=44)	9	35	0.29

Discussion

Patients with early stage gastric cancer have no typical disease-related symptoms, so gastric cancer is detected at an advanced stage in most patients, often when it is incurable with distant metastases (15). Therefore, primary and recurrent tumors must be detected when they are clinically occult and minimal in order to improve the cure rate of gastric cancer. Serum tumor markers, such as CEA and CA19-9, are convenient and valuable diagnostic assays for the screening and/or follow-up of patients with gastric cancer. However, their low sensitivity and specificity prompted us to find more reliable noninvasive screening tests for the early detection of cancer (2-4).

Many molecular approaches have been studied for the monitoring of various cancer patients (5-9, 16). Almost all recent studies are based on the polymerase chain reaction (PCR) amplification of several target molecules. One is a method for detecting circulating cancer cells in the blood of patients with malignancies (5, 6). Despite having been proven to be powerful tools, these techniques have limitations for the early detection of malignancies. The appearance of cancer cells in the circulation is thought to be a relatively late event in cancer development and the circulating cancer cells might be clusters or clumps rather than being distributed homogenously. Another approach is to detect circulating cell-free nucleic acids released from tumors into the circulation (7-9, 16). The presence of cell-free DNA fragments has been described in plasma/serum samples from cancer patients (17). Detection of tumor specific alterations in the plasma/serum, such as K-*ras* mutations and promoter hypermethylation, has been reported as diagnostically useful for cancer management

(7). However, not all cancers are currently associated with known specific DNA abnormalities and the low sensitivity and complexity of the method that involves modifying DNA with sodium bisulfate makes it less attractive for clinical applications.

Lo *et al.* have reported a detailed investigation of cell-free mRNA in the blood from cancer patients and found that such tumor-derived mRNA could be useful for the early detection and diagnosis of various cancers (18, 19). In this study, we selected two genes, *hTERT* and *MUC1* to analyze for circulating cell-free mRNA, since the over-expression of these genes has been reported to correlate with tumor progression in gastric cancer (20, 21). Although these genes are also involved in a variety of normal functions, tumor-related cell-free mRNA was not detected in the plasma samples from healthy volunteers in this study. Overexpression of the target genes in gastric cancer and their abundant release from tumors into the circulation *via* neo vessels in cancer tissues might permit the detection of the cell-free mRNA in the peripheral blood from gastric cancer patients only and not the healthy volunteers. Normal gastrointestinal epithelial cells are exfoliated and subsequently their cellular mRNA might mainly be released into the gastrointestinal tract, not into the circulation. The circulating cell-free mRNA assay, therefore, has sufficient specificity as a screening method for the diagnosis of cancer in this study. We analyzed only two genes involved in gastric carcinogenesis, so adding analyses for other related genes might increase the cancer detection rate.

The mechanism, however, accounting for this increase in plasma/serum mRNA is not well understood. The mRNA may simply be released from the primary tumors into the circulation by necrosis and apoptosis, or originates from the circulating tumor cells which are detected by the cellular RT-PCR method. Interestingly, patients with circulating tumor-derived mRNA in the plasma did not necessarily exhibit circulating cancer cells in this study. Therefore the circulating cell-free mRNAs are likely released not only from circulating tumor cells but also from tumor tissues themselves.

Conclusion

The detection of circulating cell-free mRNA might serve as a new complementary marker for gastric cancer. This assay might be helpful in detecting patients with primary gastric cancer at an early stage, as well as detecting clinically occult recurrences during the follow-up period after a gastrectomy.

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