Circulating Tumor Cells and Aberrant Methylation as Tumor Markers in Patients with Esophageal Cancer

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Abstract. *Background: This study was designed to compare the detection rates of conventional tumor markers with two molecular diagnostic approaches on blood samples from patients with esophageal squamous cell cancer. Materials and Methods: Preoperative blood samples were obtained from 44 esophageal cancer patients and were subjected to CEA-specific reverse transcriptase-polymerase chain reaction (RT-PCR) assay and methylation-specific polymerase chain reaction (MSP) assay for p16, E-cadherin and RARβ genes. Results: Circulating tumor cells were detected in 12 patients (27%); 14 patients (32%) had aberrant methylation in the promoter region of at least one gene (6, 4 and 4 patients, for p16, E-cadherin and RARβ, respectively). No abnormality was detected by either assay in control plasmas. Altogether, 23 patients (53%) had a positive result in either molecular assay. There was no correlation between either assay result and those of conventional serum markers. Conclusion: The RT-PCR and MSP assays can serve as complementary markers for screening and monitoring esophageal cancer patients.*

Patients with esophageal cancer have no typical disease-related symptoms at an early-stage; accordingly, most patients with esophageal cancer are found with advanced disease that is often incurable, with direct invasion into adjacent organs and distant metastases (1). Moreover, esophageal cancer frequently develops recurrent disease after an apparently curative operation and is subsequently associated with extremely poor survival rates (2). In order to achieve a true cure in esophageal cancer, the primary disease must be detected at an early stage and recurrent disease must be minimal, or clinically occult, at the initiation of adjuvant therapies, such as chemo- and/or radiation therapy.

Among the various approaches for screening and monitoring cancer patients, serum tumor markers have been of the most convenient and useful non-invasive diagnostic tools. However, for esophageal cancer, the detection of conventional tumor markers, such as squamous cell carcinoma (SCC) antigen and cyfra, lacks sufficient sensitivity and specificity to enable early diagnosis (3, 4). Several approaches using molecular techniques have been used to detect various primary and recurrent cancers. One method detects a small number of circulating tumor cells by a reverse transcription-polymerase chain reaction (RT-PCR) (5-8). The other method detects circulating tumor-related nucleic acids in the plasma/serum of patients with various cancers (9, 10).

Several studies have reported an increase of circulating cell-free DNA fragments in various malignancies (11, 12) and have identified tumor-specific DNA alterations in the plasma/serum of cancer patients. These alterations include genetic mutations in *K-ras* and *p53*, microsatellite instability, loss of heterozygosity and also aberrant methylation of tumor-related genes (9-13). Among these, aberrant methylation, which can be detected by a methylation-specific polymerase chain reaction (MSP), is one of the most promising targets providing sufficient sensitivity for the screening and monitoring of cancer patients.

This study was designed to investigate the diagnostic value of circulating tumor cells and plasma aberrant methylation for *p16, E-cadherin*, and retinoic acid receptor beta *(RARβ)* genes, which are related to esophageal cancer, in patients with esophageal squamous cell cancer. The results of both assays were compared with conventional tumor markers.

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Materials and Methods

Sample collection. Forty-four consecutive esophageal squamous cell cancer patients who underwent radical esophagectomy were enrolled in this study, as were 12 healthy volunteers. A 10 ml peripheral blood sample was collected from each patient. Immediately after collection, 5 ml the sample was subjected to an RNA extraction procedure. Nucleated cells were isolated on Ficoll-Isopaque (Pharmacia, Freiburg, Germany). The total RNA was extracted using the RNeasy mini kit according to the manufacturer's instructions (QIAgen, Hilden, Germany). The remaining 5 ml was subjected to an 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) for preventing the contamination of cellular nucleic acids. Cell-free genomic DNA was isolated from 400 μl of plasma samples using the QIAamp blood mini kit (Qiagen, Hilden, Germany).

Resected specimens from each patient were fixed in buffered formalin and embedded in paraffin for pathological examination using standard methods. Macroscopic and microscopic classification of tumors was based on the TMN staging system (14).

Reverse transcription-polymerase chain reaction (RT-PCR) assay. Quantitative RT-PCR was carried out with a Quantitect Probe RT-PCR kit (Qiagen) on a LighyCycler System (Roche Diagnostics, Mannheim, Germany). Primers and probe sequences for CEA and have been described elsewhere (15). The β-actin gene was also amplified as a quality control. The standard reaction volume was 20 μl and contained 10 μl of Probe Buffer, 0.2 μl of RT Mix, 1 μl of each primer and probe (20 μM) and template mRNA equivalent to 50 μl plasma samples. Samples were incubated for 5 min at 50˚C and for 15 min at 95˚C, after which amplification was carried out with 45 cycles under the following conditions: 95˚C for 1 sec, 60˚C for 60 sec. Patients were considered to harbor circulating tumor cells if CEA mRNA was detected no later than after 40 PCR cycles. This threshold was defined in preliminary experiments by the analysis of samples from patients without cancer who never had CEA mRNA detected below this threshold.

Methylation-specific polymerase chain reaction (MSP). 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 PCR as follows. Sense and antisense primer sequences for the methylated and unmethylated promoter regions have been described elsewhere (9, 16). PCR was performed using specific primers in 25 μl of a mixture containing 1 x PCR buffer, 0.5 μM concentration of each primer, 0.5 mM dNTP, 1 unit of Taq polymerase, and the modified DNA as template. PCR conditions were as follows: initial denaturation at 95˚C for 10 min, then denaturation using 40 cycles of 95˚C for 30, 30 sec annealing at 66˚C for *p16*, or 57˚C for *E-cadherin* and *RARβ*, and 72˚C for 30 s extension. Final extension was for 10 min at 72˚C The PCR product was electrophoresed on a 2% agarose gel together with a size marker, stained with ethidium bromide and visualized under ultraviolet illumination. Control methylation-positive DNA and -negative DNA were amplified performed for each reaction

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

Results

Forty-four preoperative patients with esophageal cancer included 1 stage 0, 5 stage I, 18 stage II, 12 stage III and 6 stage IV; the remaining 2 patients had no residual tumor after chemoradiotherapy. Circulating tumor cells were detected in 12 patients (27%). No CEA mRNA amplification was detected before 40 PCR cycles in the plasma from volunteers who served as controls. No significant association was found between detection using RT-PCR and various clinicopathological features in this study (Table I).

Aberrant methylation of p16, *E-cadherin* and *RARβ* genes was demonstrated in 6 (14%), 4 (9%) and 4 patients (9%), respectively. Representative MSP results are shown in Figure 1. Altogether, 14 patients (32%) had aberrant methylation of the promoter region of at least one gene in their plasma samples. Unmethylated promoter regions of these genes were detected by MSP in all patients, reflecting the presence of normal DNA derived from non-neoplastic cells. No aberrant methylation was detected in the plasma from volunteers who served as controls. Comparison of the assay results with the pathological findings of the primary tumors suggested that the patients with plasma aberrant methylation were relatively young and somewhat more likely to have poorly-differentiated carcinoma. However, we found no statistical association between the results and tumor size, lymph node metastasis, or disease-stage (Table I).

Altogether, 23 patients (53%) were detected positively using RT-PCR and/or MSP assays. By contrast, 14 patients (32%) exhibited elevations of serum SCC and cyfra tumor markers. The number of positive detections using the molecular approaches tended to be higher than that using serum markers, although the difference was not significant ($p=0.05$). There was no correlation between the conventional tumor markers and the other assay results in this study (Table II).

Discussion

Serum tumor markers have proven valuable and convenient for the early detection and subsequent management of various primary and recurrent cancer. However, due to their low sensitivity, of approximately 30%, the markers, such as SCC and cyfra have little practical use in the early detection of patients with esophageal squamous cell cancer (3, 4).

In recent years, several molecular approaches have been assessed for their ability to detect various primary and recurrent cancers at an early stage (5-13). One is the RT-PCR assay, which is a highly sensitive method capable of detecting a small number of circulating tumor cells in the peripheral blood of patients (5-8). The existence of circulating tumor cells has been proven to be a powerful

Table I. *Relationship between RT-PCR and MSP results and clinicopathological features.*

	RT-PCR			MSP		
	positive	negative	*p*-value	positive	negative	*p*-value
Age (years)	63	66	0.45	61	67	0.04
Gender						
Male	10	27		13	24	
Female	2	5	0.93	1	6	0.27
Depth						
T0 ~ T2	3	15		7	11	
T3 ~ T4	9	17	0.19	7	19	0.4
Size (mm)	45	46	0.88	39	49	0.2
Histology						
*Diff.	9	19		6	22	
*Undiff.	3	11	0.47	8	6	0.02
Lymphatic invasion						
negative	6	17		7	16	
positive	6	13	0.7	7	12	0.66
Venous invasion						
negative	5	16		8	13	
positive	7	14	0.49	6	15	0.51
Lymph node metastasis						
negative	6	18		7	17	
positive	6	14	0.71	7	13	0.68
Chemoradiotherapy						
none	6	13		4	14	
*CRT	6	19	0.58	10	16	0.26
Stage						
0, I, II	7	19		10	10	
III, IV	5	13	0.95	4	14	0.08

*Diff.: differentiated squamous cell carcinoma; Undiff.: undifferentiated squamous cell carcinoma; CRT: chemoradiotherapy.

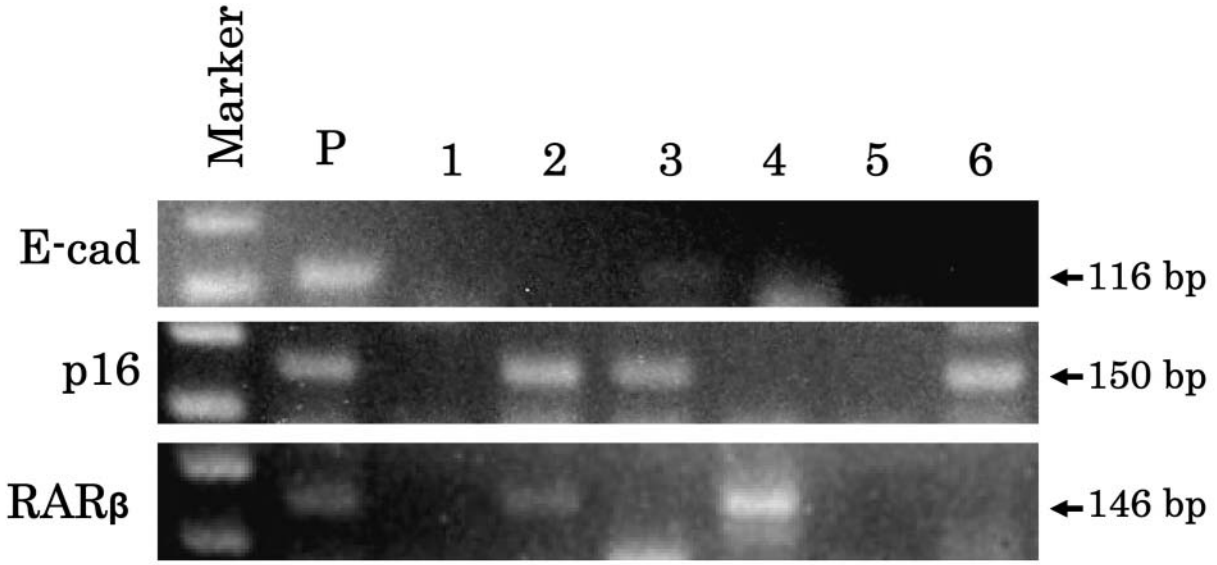

Figure 1. *Representative result of methylation-specific polymerase chain reaction (MSP) analysis. MSP analysis of each gene promoter was performed by amplifying methylated promoter sequences using specific primers. Methylated E-cadherin (E-cad) PCR products were detected from case 3, p16 products were from cases 8, 9 and 12, and RARβ products were from cases 14 and 16. P: positive control for methylation.*

Table II. *Relationship between RT-PCR and MSP results and conventional tumor markers.*

	RT-PCR			MSP		
	positive	negative	*p*-value	positive	negative	*p*-value
SCC and/or Cyfra						
positive	2	12		7	7	
negative	10	20	0.17	7	23	0.08

prognostic factor in various types of cancer. However it has long been known that circulating cell-free DNA fragments are increased in the plasma/serum of patients with various types of cancer (11, 12). Recently, aberrant methylation in the promoter region was reported to correlate with the down-regulation or silencing of several tumor suppressor genes, and the MSP assay is a sensitive method which detects aberrant methylation of tumor-related genes in the plasma of patients with cancer (9, 10). Much interest, therefore, has been focused on the clinical application of circulating nucleic acids.

To our knowledge, this is the first comprehensive analysis comparing the diagnostic value of detection of circulating tumor cells using a RT-PCR assay with that of a tumor-related aberrant methylation assay in patients with esophageal squamous cell carcinomas. We found that the positive percentages of circulating tumor cells by the RT-PCR assay was 27% was while that by the MSP assay was 32%. The positive detection rate using the RT-PCR assay in the present study is lower than those of other previous reports (5-8). We tested for the presence of circulating tumor cells using CEA mRNA *via* a real-time PCR technique, using specific primers and a probe. The relatively low positive detection rate, as described above, could be explained by such differences in the PCR assays. On the other hand, the MSP assay demonstrated that the positive rate of detection of aberrant methylation in *p16*, *E-cadherin*, and *RARβ* genes is not enough for clinical use. However, the addition of analyses for other related genes might increase the cancer detection rate and provide more reliable and useful information for the screening and monitoring of esophageal cancer patients.

These molecular diagnostic strategies analyzed two different targets for the detection of cancer: (i) the RT-PCR assay for detecting circulating tumor cells among otherwise normal blood cells; and (ii) the MSP assay that utilizes enriched plasma DNA fragments which are released from tumors into the circulation by necrosis and/or apoptosis. In fact, only 3 patients (7%) had concurrent positive results by both assays; a total of 23 patients (52%) had a positive result by the RT-PCR and/or MSP assays. The overall detection rate of both assays was higher than that of conventional tumor markers ($p=0.05$). Meanwhile, the appropriate specificity of both assays was confirmed since positive results were not obtained by the RT-PCR and MSP assays using plasma from healthy volunteers.

More recently, RNA has also been found circulating in the plasma/serum of cancer patients and in healthy individuals (17, 18). The detection of tumor-related cell-free mRNA opens up a new and interesting field in the screening and monitoring of cancer patients (19, 20). This additional new target might increase the overall detection rate of cancer-specific abnormalities in the peripheral blood of patients with this aggressive disease.

These new molecular assays using peripheral blood have several potential clinical uses: (i) screening patients at high risk for esophageal cancer, (ii) detecting recurrent disease during long term follow-up after an apparently curative operation, and (iii) confirming the completeness of tumor resection or efficacy of adjuvant therapies.

Conclusion

The RT-PCR and MSP assays using peripheral blood showed a relatively low sensitivity but sufficient specificity in patients with esophageal cancer. As there was no correlation between the results of either assay and conventional tumor markers, these tools may serve as complementary diagnostic markers for the screening and monitoring of patients with esophageal cancer. This may also permit the selection of patients requiring more intensive screening or aggressive chemotherapy.

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