Quantification of Circulating Plasma DNA Fragments as Tumor Markers in Patients with Esophageal Cancer

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Abstract. *The quantity and quality of circulating DNA fragments was analyzed by quantitative real-time polymerase chain reactions (qPCR) in plasma from patients with esophageal carcinomas, in order to assess their diagnostic value. Patients and Methods: Plasma was collected preoperatively from 24 patients with esophageal cancer and 21 healthy controls. qPCR was performed using two primer sets for the beta-actin gene, amplifying short and long segments. Results: The DNA concentrations in both the short and long assays of esophageal cancer patients were significantly higher than the controls ($p<0.001$). The area under the receiver-operating characteristic curve was 0.83 (short) and 0.91 (long) for esophageal cancer patients versus the controls. There was also a significant difference in DNA integrity (short/long) between esophageal cancer patients and the control group ($p=0.001$). Conclusion: qPCR assays for plasma DNA concentrations and their integrity can serve as new diagnostic markers for screening and monitoring patients with esophageal cancer.*

The surgical results of esophagectomy for esophageal carcinomas have improved in recent decades (1, 2). Most of the improvement, however, can be attributed to advances in diagnostic and postoperative management. Once diagnosed as having advanced disease, systemic and local recurrences are common despite complete tumor resection and extensive lymphadenectomy; consequently the survival rates are extremely poor (3). Therefore, to have a reasonable chance of achieving a cure in esophageal cancer, the primary disease must be detected at an early stage.

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Serum tumor markers have proved to be valuable and convenient for the detection of various primary and recurrent carcinomas. However, due to their low sensitivity (approximately 30%), markers such as squamous cell carcinoma related antigen (SCC) and cytokeratin 19 fragment (CYFRA) have little practical use in the early detection of patients with esophageal squamous cell carcinomas (4, 5). Recently, assays of tumor-associated circulating DNA and RNA in blood samples have been shown to be valuable for detecting and monitoring various carcinomas (6). These assays utilize enriched nucleic acid fragments in the peripheral blood to detect tumor-specific abnormalities in patients with cancer. However, a significant drawback to these assays is that they are restricted to patients with specific nucleic acid abnormalities in their primary tumor. In contrast to of these assays using tumor-specific genetic alterations, a few recent studies have focused on measuring the absolute circulating DNA concentration for a possible new biomarker (7-10).

In this study, we set out to measure the amount of circulating DNA and also DNA integrity, by quantitative real-time polymerase chain reaction (qPCR), in plasma samples from patients with esophageal cancer. We then analyzed the relationships between the results and various clinicopathological findings to assess the diagnostic value of these markers for screening and monitoring esophageal cancer.

Patients and Methods

Sample collection. Plasma samples were collected from 24 patients with esophageal squamous cell carcinoma, and from 21 healthy controls. Five ml peripheral blood samples were preoperatively collected from patients after obtaining informed consent. Immediately after collection, the blood samples were subjected to the isolation of cell-free nucleic acids by a 3 spin protocol (1500 rpm for 30 min, 3000 rpm for 5 min and 4500 rpm for 5 min) to prevent cross-contamination from cellular nucleic acids. Following centrifugation, the plasma samples were stored at –80˚C until further processing. Cell-free genomic DNA was isolated from

400 μl of the plasma samples by the QIAamp blood mini kit (Qiagen, Hilden, Germany). The final elution was performed in a 60 μl of AE buffer of the QIAamp blood mini kit. Resected cancer specimens were fixed in buffered formalin and embedded in paraffin for pathological examination using standard methods. Macroscopic and microscopic classification of the tumors was based on the Unio Internationalis Contra Cancrum (UICC)/TMN staging system(11).

Quantitative polymerase chain reaction (qPCR) for plasma DNA. qPCR was carried out with a QuantiTect SYBR Green PCR kit (Qiagen) on a Light Cycler System (Roche Diagnostics, Mannheim, Germany). qPCR was performed using two different primer sets for the beta-actin gene, amplifying short (102 bp) and long (253 bp) products. The primer set for the short product was as previously described(12). The recently designed reverse primer for the long product was 5'-AGAACCTGCAGAGTTCCA-3'. The standard reaction volume was 20 μl and contained 10 μl of SYBR Green Master Mix, 1 μl of each primer (20 μM) and 5 μl of the extracted template DNA samples. Samples were incubated for 15 minutes at 95˚C after which time the amplification was carried out for 50 cycles under the following conditions: 95˚C for 15 seconds, 58˚C for 20 seconds, 72˚C for 20 seconds and 86˚C for 5 seconds (when fluorescence was measured). Melting curve analysis was performed directly after PCR. The DNA levels were calculated on a standard curve constructed with the use of genomic DNA extracted from the leukocytes of healthy individuals (Novagen, Wisconsin, USA), and converted into the absolute plasma DNA concentration accounting for the total volume of the elution buffer (60 μl) and the volume of the plasma extracted (400 μl). The standard reference DNA was amplified for each reaction. DNA integrity was calculated as the ratio of the concentrations in each assay: the concentration of the 253 bp fragments /the concentration of the 102 bp fragments.

Statistical analyses. The Mann-Whitney *U*-test was used to compare the differences in the concentrations of cell-free DNA and the DNA integrity between the cancer group and the healthy control group. Receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) were used to assess the feasibility of using plasma DNA concentration and integrity as diagnostic tools for detecting gastric cancer in patients.

Results

Evaluation of qPCR for measuring DNA concentrations. To evaluate the appropriateness of this assay, the specificity of these primer sets by conventional PCR was first confirmed, and then the amplification of 10-fold serial dilutions of genomic DNA extracted from healthy human leukocytes by real-time PCR (our submitted data) was monitored. With these primer sets, specific amplification products were obtained, and the linearity of real-time PCR was confirmed in both the 102 bp and 253 bp assays over the range of 1-10000 ng/ml. The concentrations of all extracted DNA samples in this study were within this range.

Circulating plasma DNA concentration in esophageal cancer patients and controls. The twenty four patients included eight with stage I, eight with stage II, seven with stage III, and one with stage IV esophageal cancer. Since the primer set for the short fragment can amplify almost all of the circulating DNA released by apoptosis and/or necrosis, the results from the 102 bp assay represent the absolute amount of DNA. The median absolute plasma DNA concentration in the esophageal cancer patients was 13.7 (range:1.3-97.3) ng/ml, which was significantly higher than the 3.20 (range:1.2-15.6) in the healthy controls ($p<0.001$, Mann-Whitney u-test) (Figure 1A). The plasma long DNA fragment concentration was also significantly higher in the esophageal cancer patients than in the healthy controls (median concentrations (range): 1.7 (0.0068-57.5) *vs.* 0.21 (0.016-1.4) ng/ml, $p<0.001$, Figure 1B). The correlations between the plasma concentrations of the DNA fragments and the clinicopathological findings were therefore examined. Although the difference was not significant, patients with larger tumors (>4 cm) had a higher level of circulating DNA fragments than those with smaller tumors ($p=0.07$). However, no association was found between the DNA concentration and the other clinicopathological findings, such as histology, nodal status, or disease-state (Table I). The ROC curves for the plasma DNA concentration are

Table I. *Relationship between plasma DNA concentrations of 102 bp fragment and clinicopathological factors.*

	Number of cases	Concentrations (ng/ml)		
		Median	Range	*p*-value
Age (year)				
<65	10	12.555	(2.426-25.4)	
65≤	14	13.685	(1.271-97.3)	0.639
Tumor size (cm)				
<4	13	10.450	(1.271-97.3)	
4≤	11	17.070	(8.124-44.8)	0.078
Histology				
differentiated	20	13.685	(1.271-44.8)	
Undifferentiated	4	12.071	(5.997-97.3)	0.938
Depth (T)*				
1	13	13.230	(1.271-97.3)	
2≤	11	14.140	(5.997-25.4)	0.434
Lymph node (N)*				
negative	11	8.554	(1.271-30.3)	
positive	13	15.110	(5.997-97.3)	0.213
Lymphatic invasion				
+	8	12.555	(2.426-97.3)	
−	16	13.685	(1.271-44.8)	0.501
Venous invasion				
+	10	15.110	(1.271-25.4)	
−	14	13.340	(5.997-97.3)	0.519
Stage				
I	8	8.339	(1.271-25.4)	
II, III, IV	14	14.625	(5.997-97.3)	0.586

*TNM staging.

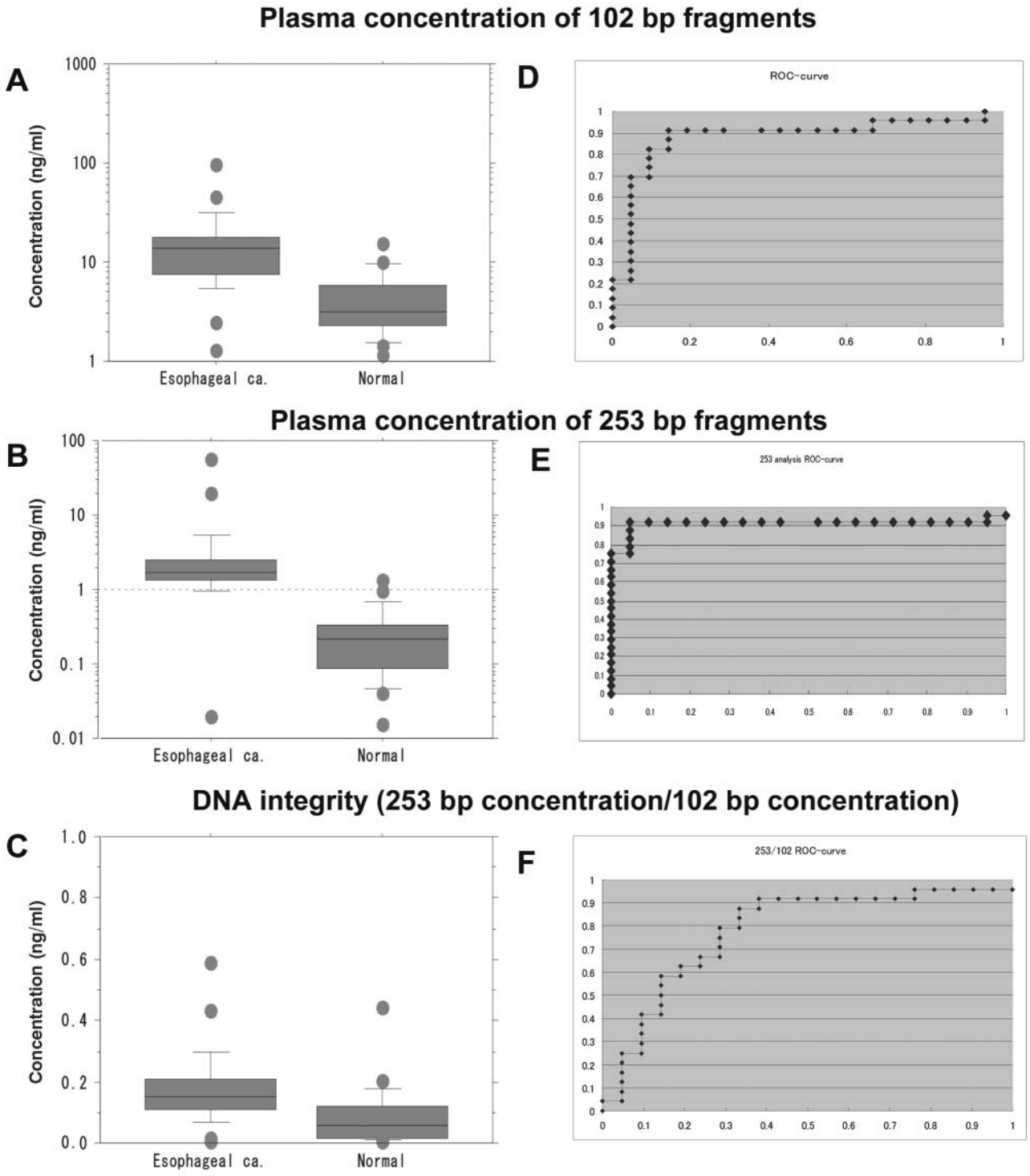

Figure 1. *Box plots (A, B, C) and receiver-operating characteristic (ROC) curves (D, E, F) of DNA concentrations and DNA integrity in esophageal cancer patients and controls. The plasma DNA concentrations were significantly higher in the esophageal caner patients in both the 102 bp (A, $p<0.001$) and 253 bp (B, $p<0.001$) assays, and the difference was more significant in the 253 bp assay. The DNA integrity (253 bp concentration/102 bp concentration) was also higher in the esophageal cancer patients than in control individuals (C, $p=0.001$). The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median, respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles, respectively. The area under the ROC curve were 0.83 (D), 0.91(E) and 0.78(F) in distinguishing esophageal cancer patients versus control individuals.*

shown in Figure 1D and E, and the area under the ROC curve (AUC) was 0.83 (short) and 0.91 (long) for the esophageal cancer patients *versus* the healthy controls.

Plasma DNA integrity in esophageal cancer patients and controls. The median plasma DNA integrity in the esophageal cancer patients tended to be higher than in the healthy controls (15.0% *vs.* 5.7%, $p=0.001$) (Figure 1C). No correlation was observed between the plasma DNA integrity and clinicopathological findings (data not shown). The ROC curve for the plasma DNA integrity is shown in Figure 1F and the area under the ROC curve was 0.78 for the esophageal cancer patients *versus* the healthy controls.

Discussion

Leon *et al.* were the first to report that cancer patients have a much higher concentration of circulating DNA fragments than those without cancer (13). Some of the circulating DNA fragments are thought to be of tumor origin, and the discovery that tumor cells can release DNA fragments has opened up the possibility for non-invasive cancer diagnosis. Over the past few years, many experiments have shown that tumor-related alterations, such as point mutations, microsatellite alterations and hypermethylated sequences, can be detectable in plasma/serum samples, and these assays can be used as complementary tools for cancer diagnosis (6, 14-16). Despite having been proven to be useful as tools, these techniques have limitations for the early detection of malignancies because not all carcinomas are currently associated with known specific DNA abnormalities, and the low sensitivity and the complexity of the method makes it less attractive for clinical applications.

Recently, real-time PCR technology has made it easier to quantify the small amounts of circulating nucleic acids in the plasma/serum samples from cancer patients. Several investigators have reported detailed studies concerning the absolute circulating DNA concentration in various cancer patients, and they have found that this simple assay might be useful for the early detection of various diseases, such as lung carcinoma, breast carcinoma, colon carcinoma, hepatocellular carcinoma, ovarian carcinoma, and melanoma (7-10).

In this study, the median plasma DNA concentrations, measured by the quantitative real-time PCR method, in esophageal cancer patients were significantly higher than those in healthy controls. The difference was more significant in the long DNA assay, although there was overlap between them in both the short and long assays. In addition, the DNA integrity tended to be higher in the esophageal cancer patients than that in the controls. The difference in the DNA integrity analysis, however, was not as significant as those in the concentration assays. Among these three assays in our study, the long DNA concentration assay showed the most significant difference for distinguishing between esophageal cancer patients and healthy controls, and showed 92% sensitivity and 95% specificity if the cut-off DNA concentration was set at 0.96 ng/ml. These results indicate the diagnostic usefulness of the DNA concentration for the detection and monitoring of esophageal cancer patients.

The origin and mechanism responsible for the increase in circulating DNA in cancer patients are not well understood. However, circulating DNA fragments are believed to be released from both normal and tumor cells into the circulation mostly by necrosis and/or apoptosis (17, 18). Whereas most of cell death in normal tissues is through apoptosis which results in short fragments of 185-200 bp, necrotic cell death frequently occurs in tumor tissue, resulting in various lengths of longer DNA fragments (17, 18). The greater difference in the long DNA concentration in our study might be correlated with the difference in the length of the DNA fragments through each cell death pathway. Our present findings would be consistent with previous reports concerning other types of cancer, including ovarian, endometrium, colorectal and breast carcinomas (19, 20). The combination of both assays, the absolute circulating DNA concentration and its integrity, could be the reason for the high specificity and sensitivity.

Various factors have been reported to affect the results of the circulating DNA concentration, such as the organs involved with cancer, the type of sample (plasma or serum), and the methods used for blood sampling and processing (6, 21). In fact, we have confirmed that the circulating DNA concentration was lower in patients with gastric cancer than in those with esophageal cancer, and was affected by the blood processing procedures (data not shown). Furthermore, a recent study has demonstrated that the DNA concentrations in serum samples are significantly higher than those in matched plasma samples, mainly being generated by the lysis of white blood cells (22). Therefore, various factors must be considered in order to compare and evaluate the results of quantitative DNA assay.

Although the simple quantitative analysis of circulating DNA fragments will only have limited value as a diagnostic marker, this new biomarker might have potential clinical use in combination with other clinical parameters and other qualitative molecular markers for screening patients at high risk of various carcinomas, and for detecting recurrent disease during long term follow-up after an apparently curative operation. In addition, the assay may be useful in predicting the efficacy of non-surgical therapies, because the DNA concentration is also a sensitive indicator of cellular damage of cancer cells by chemo-and/or radiotherapy, which results in the release of cellular DNA into the circulation.

Conclusion

The plasma DNA concentration and its integrity assayed, by a real-time PCR technique, can serve as new diagnostic markers for the screening and monitoring of patients with esophageal cancer.

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