Quantification of Plasma Cell-free DNA in Patients with Gastric Cancer

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Abstract. *Background: The circulating DNA concentration and integrity was examined by a quantitative polymerase chain reaction (qPCR) in the plasma from patients with gastric cancer and their diagnostic value for the detection of gastric cancer assessed. Patients and Methods: Plasma samples were collected preoperatively from 53 patients with gastric cancer and 21 healthy controls. qPCR was performed using two different primer sets for the beta-actin gene, amplifying short and long segments. DNA integrity was calculated as the ratio of concentrations in both assays. Results: The DNA concentrations in the short and long assays of the gastric cancer patients were significantly higher ($p=0.03$ and $p<0.0001$, respectively) than those of the control group. The DNA integrity was also higher in cancer patients than that of the controls, however the difference was not significant ($p=0.07$). Conclusion: The plasma DNA concentration assay may serve as a new diagnostic marker for the screening and monitoring of patients with gastric cancer.*

Stomach cancer is one of the most prevalent types of cancer in the world today (1). Although recent advances in diagnostic techniques have increased the early detection of gastric cancer, patients with advanced disease frequently develop recurrent disease, which is associated with extremely poor survival rates (2, 3). Most recurrent cases are incurable, such as those with peritoneal dissemination and/or multiple liver metastases. To improve the cure rates in gastric cancer, the primary tumors must be detected at early stage and recurrent disease must be minimal, or clinically occult at the initiation of aggressive chemotherapy.

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Several studies have recently shown that a simple quantitative circulating DNA test, using a real-time polymerase chain reaction (PCR) technique, may identify patients with lung, prostate, breast and colorectal cancer, and may also predict recurrence (4-6). The circulating DNA fragments are released from both normal and tumor cells into the circulation, mostly by necrosis and/or apoptosis. Cell death in normal tissues is mainly through apoptosis which characteristically results in the cleavage of DNA into 185-200 bp fragments. On the other hand, tumor necrosis frequently occurs in addition to apoptosis in cancer tissues, which generates DNA fragments longer than 200 bp (7, 8). Based upon the difference in the length of the DNA fragments, a few studies have recently reported the usefulness of DNA integrity as well as DNA concentration as a potential marker for cancer detection (9-11).

In this study, we measured the amount of circulating DNA and also DNA integrity in plasma samples from patients with gastric cancer using quantitative real-time PCR and analyzed the relationships between the results and clinicopathological findings to assess the diagnostic value of these markers for the detection of gastric cancer.

Patients and Methods

Patients and samples. Plasma samples were collected from 53 patients with gastric cancer, from 21 healthy controls. A 5 ml peripheral blood sample was preoperatively collected from patients after obtaining informed consent. Immediately after collection, the blood samples were 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) to prevent the contamination of cellular nucleic acids. Plasma samples were then stored at –80˚C until further processing. Cell-free genomic DNA was isolated from 400 μl of each plasma sample using the QIAamp DNA blood mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The final elution was performed in 60 μl of AE buffer of the QIAamp DNA blood mini kit.

The resected gastric cancer specimens 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 IGCC/TMN staging system (12).

Quantitative polymerase chain reaction (qPCR). Quantitative PCR was carried out with a QuantiTect SYBR Green PCR kit (Qiagen) on a LightCycler System (Roche Diagnostics, Mannheim, Germany). qPCR was performed using two different primer sets for the β-actin gene, amplifying short (102 bp) and long (253 bp) products. The primer set for short products was as described elsewhere (9), and the reverse primer for the long products was the newly designed 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 isolated template of DNA sample. Samples were incubated for 15 min at 95˚C; after which amplification was carried out with 50 cycles under the following conditions: 95˚C for 15 sec, 58˚C for 20 sec, 72˚C for 20 sec and 86˚C for 5 sec (measuring fluorescence). Melting curve analysis was performed directly after PCR. The DNA levels were calculated from a standard curve constructed with the use of genomic DNA extracted from leukocyte from a healthy individual (Novagen, Wisconsin, USA). Each sample was analyzed in triplicate. The absolute plasma DNA concentration was calculated from the extracted DNA concentrations by total volume of elution buffer (60 μl) and volume of plasma extracted (400 μl) with the equation reported by Lo *et al.* (13). The standard reference DNA was amplified for each reaction. DNA integrity was calculated as the ratio of concentrations in each assay: concentration of 253 bp fragments/concentration of 102 bp fragments.

Statistical analyses. The Mann-Whitney test was used to compare the difference in DNA concentration and integrity between the cancer group and the healthy group. Receiver-operating-characteristic (ROC) curves and an 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 concentration. To evaluate the appropriateness of this assay, we first tested the specificity of these primer sets by conventional PCR and then monitored the amplification by real-time PCR of a 10-fold serial dilution of genomic DNA extracted from healthy human leukocytes. With these primer sets, single, specific amplification products were obtained and the linearity of real-time PCR was confirmed for both the 102 bp and 253 bp assays (Figure 1). The concentrations of all the extracted DNA samples were proportional according to the PCR assays.

Circulating plasma DNA concentrations in gastric cancer patients and controls. The 53 gastric cancer patients included 27 with stage I, 4 with stage II, 6 with stage III and 16 with stage IV. Since the primer set for the short beta-actin DNA fragment can amplify almost all of the circulating DNA released by apoptosis and/or necrosis, the result of the 102 bp assay represents the absolute amount of DNA present.

The absolute plasma DNA concentration in the gastric cancer patients (median: 5.71 ng/ml, range 0.855-303 ng/ml) was significantly higher than that in the healthy controls (median: 3.20 ng/ml, range 1.16-15.6 ng/ml) ($p=0.03$, Mann-Whitney test) (Figure 2). The plasma DNA concentration in the long fragment assay, which amplified a 253 bp β-actin segment, was also significantly higher in the gastric cancer patients than in the healthy controls (median concentration (range): 0.470 (0.0599-41.4) *vs.* 0.212 (0.0155-1.35) ng/ml, $p<0.0001$; Figure 2). This difference was more prominent for the 253 bp than for the 102 bp analysis. We, therefore, examined the association between the plasma concentrations of the long DNA fragment and the clinicopathological findings. Patients with advanced-stage disease had a significantly higher DNA concentration than those with early-stage disease. However, no association was found between the DNA concentration and the other clinicopathological findings, such as nodal status or histology (Table I).

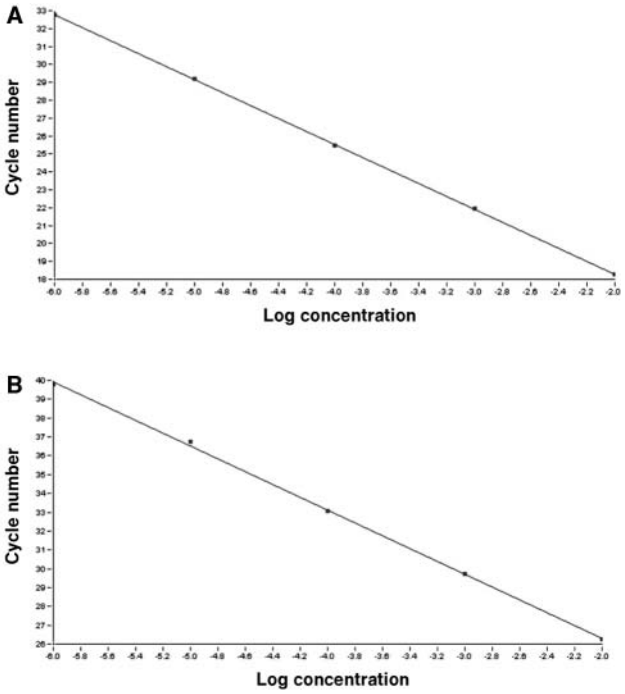

Figure 1. *Standard curves of 102 bp (A) and 253 bp (B) portion of the beta-actin gene fragments using genomic DNA. A ten-fold serial dilution of genomic DNA was used to generate the standard curves. Linearity was confirmed within these concentrations, ranging from 5 pg to 50 ng using a 20 μl assay volume (the errors were 0.02 and 0.05, respectively).*

The ROC curve for the plasma DNA concentrations of the long DNA fragment is shown in Figure 3 and the area under the ROC curve (AUC) was 0.75 for the gastric cancer patients *versus* the healthy controls. On the other hand, the median plasma DNA integrity in the gastric cancer patients tended to be higher than in the healthy controls (7.1% *vs.* 5.7%, $p=0.07$); however, the difference was not significant (Figure 2).

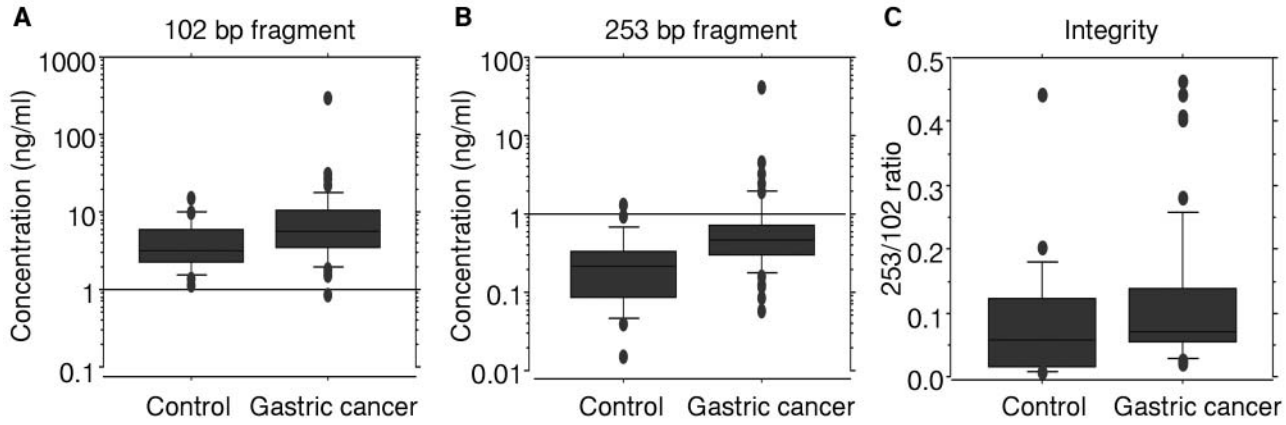

Figure 2. *Box plots of the DNA concentrations (A, B) and DNA integrity (C) in gastric cancer patients and controls. The plasma DNA concentrations were significantly higher in the gastric caner patients in both the 102 bp (A, p=0.03) and 253 bp (B, p<0.0001) assays; the difference was more significant in the 253 bp assay. On the other hand, the integrity assay did not show a significant difference between the gastric cancer patients and control individuals (C, p=0.07). 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.*

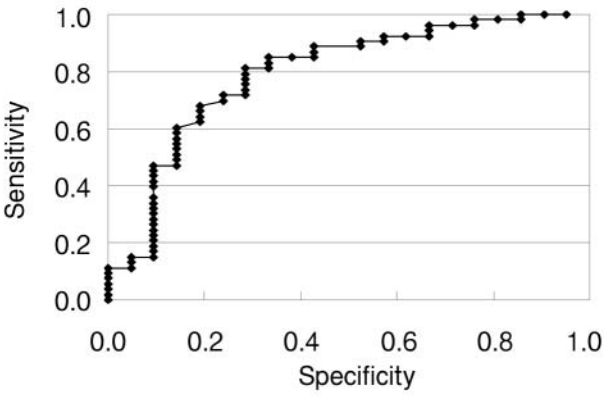

Figure 3. *A receiver-operating characteristic (ROC) curve of the long DNA fragment (253bp) assay in detecting gastric cancer patients. The area under the ROC curve was 0.75 in distinguishing gastric cancer patients versus control individuals.*

Table I. *Relationship between the plasma DNA concentration of 253 bp fragment and clinicopathological factors.*

	Number of cases	Concentrations (ng/ml)		*p*-value
		Median	Range	
Gender				
male	37	0.467	(0.0599-41.4)	0.399
female	16	0.572	(0.123-4.61)	
Age (year)				
<65	24	0.368	(0.0599-3.29)	0.172
≥65	29	0.488	(0.162-41.4)	
Size (cm)				
<4	25	0.470	(0.0599-4.61)	0.504
≥4	28	0.488	(0.0857-41.4)	
Depth (T)*				
1	24	0.426	(0.0599-1.98)	0.264
≥2	29	0.537	(0.123-41.4)	
Lymph node (N)*				
negative	27	0.386	(0.0599-41.4)	0.147
positive	26	0.555	(0.127-4.61)	
Stage				
I, II	31	0.384	(0.0599-4.61)	0.0066
III, IV	22	0.623	(0.260-41.4)	

*TNM staging.

Discussion

It has long been known that circulating cell-free nucleic acid fragments are increased in the plasma/serum of patients with various types of cancers (14). Based on these findings, several studies have identified tumor-specific alterations in the plasma/serum nucleic acids of cancer patients. These alterations include mutations in various genes such as K-*ras* and *p53*, microsatellite instability, loss of heterozygosity, aberrant methylation of several genes and also tumor-specific cell-free mRNA (15-19). The assays capable of detecting tumor-specific alterations, however, have several drawbacks that limit the early detection of cancer. First, the plasma/serum tumor-specific genetic assays necessitate identical genetic alterations in the primary cancer tissues. Furthermore, the detections of these alterations in blood samples are lower than those in cancer tissues. The low sensitivity and complexity of these assays make them less attractive for clinical use.

Instead of tumor-specific genetic alterations, several recent studies have focused on measuring the absolute circulating DNA concentration (4-6). Although various assays have been employed for quantifying DNA concentrations, quantitative real-time PCR is the simplest and most sensitive method for accurate quantification. Using quantitative methods, several authors have demonstrated that the measurement of the circulating DNA concentration may be a valuable tumor marker for the detection of various types of cancer, such as lung, prostate, breast and colorectal (4-6). On the other hand, other researchers have reported that the circulating DNA concentration assay is neither sensitive nor specific enough for the diagnosis of some kinds of cancer (20, 21). Various factors have been reported to affect the results of the circulating DNA concentration, such as the organs involved with the cancer, the sample type (plasma or serum) and the method of blood sampling and processing. We confirmed that the circulating DNA concentration was lower in patients with gastric cancer than those with esophageal cancer and moreover was affected by the blood processing procedures (data not shown). Therefore, these different factors must be considered and standardized in order to compare and evaluate the results of the quantitative DNA assay.

In this study, we examined the plasma DNA concentration of short and long fragments in gastric cancer patients by a quantitative real-time PCR method. To our knowledge, this is the first report presenting the circulating DNA concentration as a potential tumor marker of gastric cancer. The median concentrations were significantly higher in gastric cancer patients compared to normal controls in both the short and long assays. These results indicate the diagnostic usefulness of the plasma DNA concentration assay for the detection and monitoring of gastric cancer patients. Moreover, the difference was more significant in the longer segment assay, although we used only two primer sets because the efficiency of real-time PCR decreases in assays amplifying products longer than 300 bp. This finding may indicate that we might observe an even more statistically significant difference between cancer patients and control individuals in the DNA concentration of longer DNA fragments.

The origin and mechanism accounting for the increase in the circulating DNA in cancer patients is not well understood. In general, circulating DNA fragments are thought to be released from both normal and tumor cells into the circulation mostly by necrosis and/or apoptosis. Most cell death in normal tissues is through apoptosis, which results in DNA fragments less than 200 bp, whereas some cell death in cancer tissues is through necrosis, which results in DNA fragments of various lengths, including long DNA fragments (7, 8). The difference in the length of the DNA fragments due to each type of cell death pathway opens up a new and interesting field of diagnostics in cancer. Several studies have reported that DNA integrity is a potentially new tumor marker for the detection of ovarian, endometrial, colorectal, periampullary and breast cancer (9-11). Although the difference was not statistically significant, the plasma DNA integrity was somewhat more likely to be higher in the gastric cancer patients than in the normal controls in the present study. Plasma DNA concentrations with an even longer fragment assay may demonstrate a significant difference between the gastric cancer patients and the control group, and would also be valuable in detecting gastric cancer. Further studies are needed to clarify the diagnostic value of DNA integrity in gastric cancer patients.

The plasma cell-free DNA is not an ideal single tumor marker, because the results showed that elevated free DNA levels could be detected only in a subset of cancer patients. Its combination with other established markers, such as CEA, may constitute a better prognostic factor. New molecular assays using peripheral blood have several potential clinical uses: i) screening of patients at high risk for gastric cancer, ii) estimating the completeness of the surgical resection of gastric cancer, iii) detecting recurrent disease during long term follow-up after an apparently curative operation. In addition, the assays may be useful in predicting the efficacy of non-surgical therapies, such as chemotherapy and radiotherapy, because the DNA concentration should be a sensitive indicator of cellular damage of not only normal cells but also cancer cells which results in the release of cellular DNA fragments into the circulation.

Conclusion

The plasma DNA concentration assays, using the real-time PCR technique, may serve as new diagnostic markers for the screening and monitoring of patients with gastric cancer.

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