Fragmentation Analysis of Plasma DNA Reveals Its Prognostic Value in Gastric Cancer

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ABSTRACT

Background: *Gastric cancer (GC) is a common cause of cancer-related deaths. The poor clinical outcome in GC patients is partially associated with a lack of appropriate diagnostic and prognostic biomarkers. In the present study, we evaluated the diagnostic and prognostic values of cell-free DNA (cfDNA) integrity and the concentration of circulating nucleosomes (cNUCs).*

Methods: *In the study, 40 GC patients and 55 GC-free individuals were enrolled. Cell-free DNA integrity was calculated as the ratio of concentration of the longer ACTB (beta-actin) gene fragment to that of the shorter ACTB fragment, measured using quantitative PCR. Circulating nucleosomes were measured by an ELISA-based approach.*

Results: *We found that cfDNA integrity is higher in GC patients than in the control subjects (relative median values 0.51 vs. 0.38, respectively, P = .56) indicating prominent abundance of longer fragments in the patients. The patients with larger tumors (T3-4) had significantly higher cfDNA integrity than those with T1-T2 tumors. We also found GC patients to have higher concentrations of cNUCs in their plasma (relative median values 3.64 vs. 3.1). Importantly, the patients with high cfDNA integrity (i.e., lower fragmentation) had longer overall survival rates at 3 years than those with lower cfDNA integrity (76.5% vs. 38.9%, P = .02).*

Conclusion: *Cell-free DNA fragmentation has a prognostic value. However, it has no diagnostic value in GC.*

Keywords: *Circulating nucleosome, DNA integrity, gastric cancer, plasma*

INTRODUCTION

Currently, gastric cancer (GC) is the fifth most common malignant disease worldwide and the third most common cause of cancer-related deaths, although the incidence has been declining in the developed countries.[1] The poor clinical outcome of GC patients, with 5-year survival rates less than 30%, can be partially attributed to diagnosis at advanced stages of the disease, as currently, there is no clinically proven biomarker for the early detection and diagnosis of GC. Further understanding of cancer biology and the development of reliable diagnostic biomarkers are urgently needed, and will improve the clinical outcomes for GC patients.

The main goal of biomarker discovery is to identify novel molecules, ideally obtained by non-invasive methods. Liquid biopsy is a minimally invasive approach for detection of the relevant diagnostic, prognostic, and predictive biomarkers in body fluids in cancer patients,[2] and applies to circulating tumor cells, circulating nucleic acids including cfDNA (cell-free DNA), noncoding RNAs (microRNAs, lncRNAs and others), exosomes, and nucleosomes.[3] Circulating cfDNA is increasingly being accepted as a biomarker for cancer as it carries information about the dynamics of cancer-specific genetic and epigenetic alterations.[4] Point mutations, aberrant DNA methylation, cNUCs (circulating nucleosomes), and histone modifications, circulating mitochondrial DNA, and microsatellite alterations are the examples of blood-based biomarkers related to cfDNA in cancer patients.[5] The contents of apoptotic or necrotic cells are supposed to be the main source of cfDNA in plasma or serum. Analyses of DNA fragmentation patterns and nucleosome occupancy or tissue-specific methylation patterns indicated that cfDNA in healthy subjects is derived mainly from hematopoietic cells, whereas in cancer patients, increased DNA levels originated from the tumor of origin.[6] Therefore, tumor-specific mutations can be detected in the plasma of cancer patients, albeit in varying proportions.

Cell-free DNA can exist in the circulation as unbound DNA, as histone-bound DNA in nucleosomes, or as DNA

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packed in the apoptotic bodies. Circulating nucleosomes have been the subject of research in the last 2 decades. Elevated levels of cNUCs are observed in the impaired inflammatory responses such as in cancer, cerebral stroke, rheumatoid arthritis, Crohn's disease, and sepsis.[7] Hemodialysis experiments have shown that half of the mononucleosomes were removed after 4 minutes.[8] In cancer patients, cNUCs are detected at increased levels due to the large rates of cell turnover and cell death that occur during chemotherapy.[2,9]

DNA integrity is the fragmentation analysis of cfDNA, and is detected by the ratio of concentration of longer DNA fragments to shorter fragments in plasma or serum. Most of the studies have measured the cfDNA integrity by quantitative PCR; however, quantitative-denaturing high performance liquid chromatography or atomic force microscopy have been utilized to investigate the fragmentation of cfDNA.[10] It is well documented that cfDNA differs in size in cancer patients.[11,12] Tumor cells release longer DNA fragments (>200 bp) by necrosis. However, the shorter fragments (<200 bp) in circulation are assumed to be derived from the apoptotic death of cells in healthy individuals.[13] Numerous studies have assessed the diagnostic, and prognostic relevance of cfDNA integrity in various cancer types, including hepatocellular carcinoma, breast, colorectal, thyroid, and prostate cancer.[13-18]

The data on cfDNA integrity in GC is limited.[19,20] In the present study, we investigated the diagnostic and prognostic values of cfDNA integrity and cNUCs in patients with GC. Differing from the above-mentioned articles which included apparently healthy subjects as controls, we enrolled endoscopy-verified cancer-free individuals as controls. We report here that cfDNA integrity has a prognostic value in GC.

MAIN POINTS

- *The plasma DNA fragmentation is lower in gastric cancer (GC) patients than in control subjects, with low diagnostic potential.*
- *Circulating nucleosome (cNUC) levels are increased in GC patients, with low diagnostic potential.*
- *Patients with larger tumors (T3-T4) have lower plasma DNA fragmentation than those with smaller tumors.*
- *Plasma DNA integrity is a prognostic marker in gastric cancer, and high DNA integrity (i.e., lower fragmentation) is associated with longer survival.*

MATERIALS AND METHODS

Study Subjects and Sample Processing

This study included 95 participants aged between 30 and 80 years who were recruited from the General Surgery Department of the Istanbul Faculty of Medicine. The demographic characteristics (age and gender) of the individuals in the group with GC, the inflammation group, and the control group in this study did not differ. The exclusion criteria for individuals were a past cancer history or second malignancies, severe coronary disease, myelosuppression, or autoimmune diseases. Of 95 participants, 40 patients had pathologically confirmed resectable GC, and 55 individuals were GC-free (control group). The sample size analysis showed that these numbers were sufficient to achieve a significance level at 0.05. Within the control group, 31 individuals had inflammatory disease and the remaining 24 had no sign of inflammation in the stomach, as seen in gastroscopy. From a subset of the GC patients (n = 21), we also collected blood samples within 7-12 days after surgery. This study was approved by the Ethics Committee of Istanbul Faculty of Medicine (2014/1180). Written informed consent was obtained from all participants.

Ten milliliters of venous blood samples were collected into EDTA tubes before gastroscopy from all participants. Blood was centrifuged at 680 g for 20 minutes within 2 hours after being drawn. Plasma fractions were separated and stored in aliquots at −80°C until further use.

Measurement of DNA Integrity

Plasma DNA was extracted from 240 µL plasma using the NucleoSpin Plasma XS kit (Macherey-Nagel, Germany) according to the instructions, and eluted in 50 µL water and stored at −20°C. Plasma DNA was employed in quantitative PCR (qPCR) to amplify the *ACTB* gene, using the following primer pairs: ACTB106 F: 5′-TCGTGCGTGACATTAAGGAG-3′; R: 5′-GGCAGCTCGTAGCTCTTCTC-3′, ACTB384 F: 5′-GCTATCCCTGTACGCCTCTG-3′; R: 5′-AGGAAGGAAGGCTGG AAGAG-3′. qPCR was carried out in the LightCycler 480 instrument (Roche) using the QuantiTect SYBR Green PCR Kits (Qiagen), in accordance with the manufacturer's instructions. The conditions for real-time PCR were as follows: A hot start at 95°C for 10 min followed by 40 cycles at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. DNA integrity was calculated as the ratio of concentration of the 384 bp fragment to that of the 106 bp fragment. As the 384 : 106 ratios were not normally

distributed in the study group, we defined the median value as a threshold to specify the subgroups with "low" and "high" fragmentation.

Measurement of Circulating Nucleosomes

The commercially available Cell Death Detection ELISA PLUS Kit (Roche Diagnostics, Mannheim, Germany) was employed to quantify cNUCs in the plasma, according to the manufacturer's instructions. Their concentrations were calculated using a standard curve of nanograms per milliliter.

Statistical Analysis

The obtained numerical values were analyzed for normality distribution using the Shapiro–Wilk test. The differences between the study groups regarding the DNA integrity index and cNUCs, and their association with clinical variables were determined by the Mann–Whitney *U*-test. Receiver operating characteristic (ROC) curves were generated to assess the diagnostic power of the study parameters. The statistical power of the study was 99.9% on post hoc analysis, thereby eliminating type II error. The univariate survival analysis was based on the Kaplan–Meier method. The comparison between the survival curves was analyzed using the log-rank test. The overall survival was defined as the time between the date of surgery and the last date of follow-up or the date of GC-related death. The differences were considered statistically significant when the probability level (*P*) was below .05.

RESULTS

Gastric Cancer Patients Have Higher Plasma cell-free DNA Integrity Index and Circulating Nucleosomes Than the Levels in Control Subjects

The plasma DNA fragmentation was studied using the *ACTB* gene fragments. The distribution of the ACTB384/ACTB106 ratios in the study groups is shown in Figure 1A. The median values of the ACTB384/ACTB106 ratios were 0.38 for the control group and 0.51 for the GC patients. Despite this notable difference between the groups, the difference was statistically not significant ($P = .56$). These results showed that GC patients had higher levels of longer DNA fragments in their blood circulation compared with the levels in the control subjects. Within the control group, we found no difference between the individuals with inflammation and those without inflammatory disease (0.42 vs. 0.38). The cNUC concentrations were also higher in the plasma of GC patients than in the control subjects. The relative median value of cNUCs was 3.11 in the control group, and 3.64 in cancer patients ($P = .2$) (Figure 1B). Even if higher in GC patients than in control subjects, DNA integrity and cNUCs had no diagnostic value in GC, as demonstrated by the ROC curve (Figure 1C), with the area under curve rates of 0.59 and 0.56, respectively.

Beyond the diagnostic analysis, we looked at any association of cfDNA integrity and cNUCs with the clinical characteristics of GC patients (Table 1). The patients with larger tumors (T3-4) had significantly higher cfDNA integrity than the patients with T1-T2 tumors, indicating increased release of longer necrotic DNA fragments from the larger tumors. Another intriguing association of cfDNA integrity was observed with perineural invasion. Patients with perineural invasion had significantly higher DNA integrity index and significantly higher levels of cNUCs. Additionally, DNA integrity was significantly higher in the patients with low carcinoembryonic antigen (CEA) ($\leq$5 ng/mL) than those with higher serum CEA values.

In the next analysis, in a subset of GC patients (N = 21), we compared the baseline (pre-surgery) levels of cNUCs with levels after surgery, where the changes in plasma cNUCs levels from pre- to post-surgery in individual patients are depicted in Figure 1D. We found a considerable decline of cNUCs levels from 4.11 to 3.24 in the entire subgroup (Figure 1E, $P > .05$), indicating that the cNUCs level may be a gross indicator of the tumor burden declining after surgical removal of the tumor.

DNA Fragmentation Has Prognostic Value in Gastric Cancer Patients

Finally, we assessed the prognostic value of cfDNA integrity and cNUCs in GC patients who were followed up to 39 months. We defined the median value of cfDNA integrity as a threshold to specify the subgroups with "low" and "high" DNA integrity. The 3-year survival rates were significantly higher in the patients with high cfDNA integrity than in those with low integrity (76.5% vs. 38.9%, $P = .02$). The Kaplan–Meier survival curve in Figure 1F clearly shows that increased fragmentation of plasma DNA was associated with shorter overall survival.

DISCUSSION

Detection of clinically relevant biomarkers will improve the outcome of GC patients. The integrity of cfDNA and cNUCs have widely been studied in various malignancies. However, the data regarding this are very limited for

Table 1. Association of DNA Integrity Index and Nucleosomes With Clinical Variables

	DNA Integrity			Nucleosomes		
Characteristics	No of Patients	Median	*P*	No of Patients	Median	*P*
Age (years)[a]						
≥60	20	0.44	.55	20	4.1	.7
<60	18	0.56		18	2.66	
Gender[a]						
Male	27	0.44	.45	27	2.99	.59
Female	11	0.58		11	3.34	
Tumor size (cm)[b]						
≥5	24	0.62	.39	23	2.99	.55
<5	10	0.43		11	3.95	
CEA (ng/mL)[c]						
≤5	27	0.63	.01	26	4.11	.08
>5	4	0.13		4	1.14	
CA 19-9 (ng/mL)[c]						
≤34	28	0.51	.9	27	2.99	0.89
>34	2	0.5		2	2.97	
Tumor localization[d]						
Cardia+Fundus	6	0.75	.2	6	3.75	.4
Antrum+Corpus	30	0.39		30	3.4	
Lymphatic met. (N)[e]						
N0	6	0.6	.8	7	3.95	.36
N1&N2&N3	27	0.58		26	4.14	
Primary tumor (T)[f]						
T1-2	8	0.16	.01	9	2.44	.5
T3-4	27	0.63		26	4.13	
Venous invasion[b]						
No	16	0.65	.37	17	4.1	.6
Yes	18	0.39		17	2.98	
Lymphocyte invasion[g]						
No	19	0.29	.84	20	4.03	.76
Yes	13	0.58		13	2.98	
Perineural invasion[b]						
No	12	0.21	.03	13	2.44	.04
Yes	22	0.65		21	4.17	
Mesenteric tumor nodules[b]						
No	29	0.63	.06	29	4.11	.28
Yes	5	0.19		5	1.78	

[a]Not available for 2 patients; [b]not available for 6 patients; [c]not available for 9 patients; [d]not available for 4 patients; [e]not available for 7 patients; [f]not available for 5 patients; [g]not available for 8 patients.
A statistical significance of $P < .05$ is highlighted in bold.
CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.

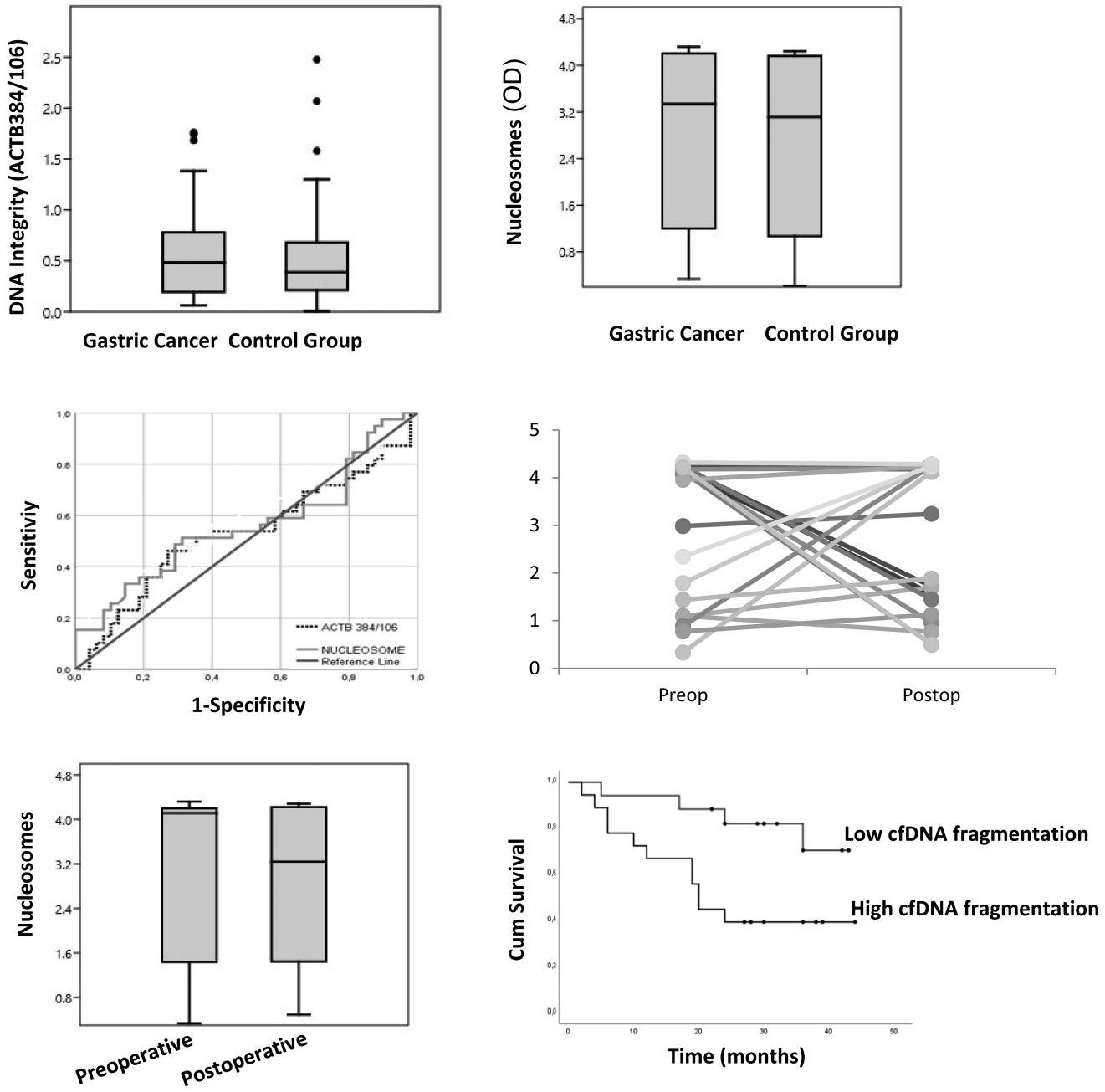

Figure 1. Box plots in A and B display plasma DNA integrity and cNUC levels in cancer patients and control subjects, respectively. The ROC curve in C shows the diagnostic power of plasma DNA integrity. In D and E, cNUC levels are shown in a subset of GC patients at baseline and within 7-12 days after surgery. Survival analysis is shown in F. The median levels along with ranges, and the 25th and 75th percentiles are shown in the box plots.

GC. In the present study, we investigated the diagnostic and prognostic values of cfDNA integrity and cNUC levels in the patients with resectable GC. We found higher DNA integrity (i.e., lower fragmentation) in GC patients, with no statistical significance. The lack of statistical significance is likely to be associated with the relatively small sample size in our study. There are 2 reports in the literature on the cfDNA integrity in GC.[19,20] Similar to our findings, both studies found higher cfDNA integrity in GC patients, with statistically non-significant results. In

contrast to these studies, the strength of our study was the inclusion of gastroscopy-verified cancer-free control subjects in the study, rather than the apparently healthy controls. Despite higher integrity of plasma DNA in GC, the ROC analysis revealed no diagnostic value in GC, as also reported in colorectal cancer[21] (Figure 1C).

Even cfDNA integrity possesses a limited diagnostic value, and has been found to be useful in the evaluation of cytotoxic/immunotherapy treatments in various cancer types.[22-24] This suggests that cfDNA integrity reflects the tumor burden. In line with this, we found that the ACTB384/106 index was higher in patients with larger tumors (T3-4) compared with the index in smaller tumors (Figure 1D, Table 1). The cfDNA can be released into blood circulation via apoptotic or necrotic death of both cancer cells and healthy cells.[25] The higher DNA integrity in GC patients in our study provides a clue that most of the cfDNA might be derived from necrotic cancer cells. It is supposed that an elevated release of long necrotic fragments occurs from tumor cells of larger lesions which may suffer from low oxygenation, thereby inducing necrotic cell death.[26] Furthermore, higher cfDNA integrity was associated with perineural invasion, an established independent predictor of overall survival and disease-free survival in GC.[27] We found that patients with lower CEA values had higher cfDNA integrity. However, this finding should be interpreted cautiously, as the number of patients in the CEA subgroups was imbalanced.

To our knowledge, this is the first report on the association of cfDNA fragmentation with clinical outcome in GC. A low ACTB384/106 ratio, meaning higher plasma DNA fragmentation, was significantly associated with shorter overall 3-year survival (Figure 1F). Prognostic relevance of cfDNA integrity has been shown in different cancer types including breast, non-small-cell lung, prostate, colorectal, and renal cell cancers, and lymphoma.[18,28-33] In most of these studies, like our findings, the abundance of longer fragments in plasma has been found to be associated with favorable outcomes in cancer patients. As GC patients generally have a poor clinical outcome, with 5-year survival rates being less than 30%, plasma DNA integrity at baseline might be used to identify patients with a possible poor outcome. Additionally, this marker could be combined with other prognostic markers such as tumor size, lymph node metastasis, or invasion depth, to accurately predict clinical outcome after surgical treatment.

The increased level of circulating nucleosomes in cancer patients appears to possess a very limited diagnostic value. However many studies revealed its potential utility in the evaluation of the efficacy of cytotoxic therapy.[34,35] We found mildly increased levels of cNUCs in GC patients compared with the levels in the control group. We evaluated cNUCs pre- and post-operatively in a subset of patients, and found that plasma levels of nucleosomes were decreased after surgical tumor removal. This finding reveals that considerable portion of cNUCs was likely derived from tumor cells and that cNUC level, like cDNA integrity, is a gross indicator of tumor burden in GC patients. Higher cNUC levels were also found to be associated with perineural invasion.

In conclusion, this exploratory study is the first report describing the prognostic value of cfDNA integrity in GC, and will be a basis for the further investigation of the ACTB384/106 integrity index as a prognostic tool in future studies with larger sample sizes. If validated in further studies, cfDNA integrity may be implemented in the management of patients with GC, along with established prognostic factors.

Ethics Committee Approval: This study was approved by the Ethics Committee of Istanbul Faculty of Medicine (2014/1180).

Informed Consent: Written informed consent has been obtained from all the participants involved in this study.

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