Circulating cell-free DNA as a promising biomarker in patients with gastric cancer: diagnostic validity and significant reduction of cfDNA after surgical resection

Kyongchol Kim, Dong Gue Shin¹, Min Koo Park², Seung Hyuk Baik³, Tae Hee Kim⁴, Sanghee Kim⁵, SaeYoung Lee⁶

The Clinical Genome Center, Chaum Life Center, Cha University, Seoul, ¹Department of Surgery, Seoul Medical Center, Seoul, ²TCM Epigenetics and Genomics Lab, Seoul, ³Department of Surgery, Yonsei University College of Medicine, Seoul, ⁴Department of Medical Oncology, Seoul Metropolitan Dongbu Hospital, Seoul, ⁵College of Nursing, Yonsei University, Seoul, ⁶Department of Family Medicine, Mizmedi Hospital, Seoul, Korea

INTRODUCTION

Stomach cancer is the fourth most commonly diagnosed cancer with an average of 1 million patients newly diagnosed annually and was the second leading cause of cancer deaths worldwide in 2008 [1]. Prevalence and mortality are particularly high in East Asia, including South Korea [2]. The 5-year survival rate is more than 90% when cancer is detected as early gastric cancer (EGC), but it decreases to 30%–40% in cases of advanced gastric cancer (AGC) [3]. Thus, early detection and development of gastric cancer biomarkers are important for the treatment and survival of gastric cancer. Biomarkers such as carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 19-9, and CA 72-4 have been investigated for years, but they are not recommended for screening and follow-up of gastric cancer in the National Comprehensive Cancer Network guidelines due...
to low sensitivity and specificity [4]. This limit of conventional biomarkers requires development of novel cancer biomarkers.

As a cancer cell develops, circulating free DNA (cfDNA) is released into the blood stream with various physiologic events such as micrometastasis, necroptosis, apoptosis, and secretion [5]. cfDNA from the physiologic apoptosis of cells can be detected in healthy subjects [6] and is slightly prone to be increased in cases of trauma, sepsis, and other diseases such as systemic lupus syndrome, pulmonary embolism, and myocardial infarction [7]. However, cfDNA in the plasma of cancer patients is 2–3 times higher than in normal healthy groups in various cancer studies [8]. Many studies suggest that circulating tumor cells or cfDNA can be alternative biomarkers for early detection of cancers, and predict prognosis and efficacy of therapies [8]. This cfDNA is also increased in benign preneoplastic tumors. Previous studies demonstrate that cfDNA is proportionally increased in benign colon polyps and colon cancers individually compared to normal groups [9]. Decreased cfDNA is mainly attributed to reduced tumor size by an operation and also rapid clearance by plasma nuclease such as DNaseI [10]. Previous studies demonstrated a half-life of cfDNA in plasma that ranges from minutes to hours. CfDNA can be a useful biomarker for the prediction of prognosis or cancer relapse. For example, one study demonstrated that the 2-year survival rate was 48% in cfDNA-detected groups and 100% in cfDNA-nondetected groups among colon cancer patients [11].

As many research outcomes point out cfDNA as a significant marker, cfDNA could be a good marker for early detection and follow-up treatment in gastric cancer. The purpose of this study was to determine the implication of cfDNA as a useful biomarker to detect EGJ, to predict tumor burden and to detect residual tumor after surgery.

**METHODS**

A cross-sectional case-and-control study design was applied to two Korean hospitals, located in a metropolitan area, between October 2012 and March 2013.

Thirty gastric cancer patients who underwent a gastrectomy with a curative intent in Seoul Medical Center, and 34 age-matched healthy controls who visited for regular health check-ups in MizMedi Hospital, were recruited. A healthy control was defined as a subject who was not diagnosed of any cancerous condition in the past and currently does not have a serious illness, such as severe infection, sepsis, or trauma.

The case group is defined as patients who had been diagnosed with gastric cancer, which was pathologically confirmed by an endoscopic biopsy. Patients underwent either a laparoscopy or open surgery. Patients who underwent a palliative resection or who had prior chemotherapy, distant metastasis and double-primary cancer, were excluded. Data obtained for each patient included age, sex, body mass index (BMI), tumor marker (CEA, CA 19-9), tumor size, histologic type, T stage, N stage, gastric cancer stage classified according to the seventh edition of the American Joint Committee on Cancer staging criteria [12], and the preoperative and post-24-hour operative serum cfDNA level. In the control group, healthy people who underwent an endoscopy for cancer screening were selected in order to obtain data.

Many procedures, such as phenol-chloroform extraction, salting-out, magnetic beads, and triton/heat/phenol protocols [13] have been used to isolate cfDNA. To successfully isolate cfDNA from plasma, we empirically found that the efficacy of the extraction procedures is the key issue. We compared the efficacy of several commercial cfDNA-isolation kits based on column-based systems such as QIAamp DNA Micro kit (Qiagen, Valencia, CA, USA), Nucleospin Plasma XS (Macherey-Nagel GmbH & Co. KG, Düren, Germany), and G-spin Total DNA Extraction kit (Intron biotechnology, Seongnam, Korea). Comparing plasma DNA yield and extent of DNA concentration variation from sample to sample, the QIAamp DNA Micro kit showed least variety in the amount of cell-free DNA among the 3 kits (data not shown). We reached the conclusion that the QIAamp DNA Micro kit provided robust and reliable cfDNA isolation. We routinely used 1.5-mL plasma as starting material, and the DNA was extracted according to the protocols instructed by the supplier. All blood samples were centrifuged once for 10 minutes at 4,000×g. From the withdrawn plasma, 200 μL of plasma were dispensed to 7 microcentrifuge tubes per sample. Twenty-μL proteinase K and 4 μL of an RNase A stock solution were added to each tube. Then, 200 μL of Buffer AL was added to the sample and mixed thoroughly to yield a homogeneous solution. The tube was incubated at 56°C for 10 minutes. Absolute ethanol (200 μL) was added to each reaction tube and then mixed by pulse-vortexing. For each sample, a 7 microcentrifuge mixture was then applied to the same QIAamp mini spin column to increase the recovery yield and centrifuged at 9,000 rpm for 1 minute. Then the filtrate was discarded. Afterwards, 500 μL of Buffer AW1 was carefully added to the spin column and centrifuged at 9,000 rpm for 1 minute. Here again, the filtrate was discarded. Then 500 μL Buffer AW2 was added to the spin column and centrifuged at 13,000 rpm for 1 minute. The QIAamp mini spin column was applied to a new collection tube and centrifuged at 13,000 rpm for 1 minute again. Finally, the spin column was placed in a clean 1.5-mL microcentrifuge tube and 50-μL Buffer AE was added. After incubation at room temperature for 5 minutes, it was then centrifuged at 13,000 rpm for 1 minute to elute the DNA.

Descriptive and comparative analyses were done. Fisher exact test, Wilcoxon rank sum test, and the Kruscal-Wallis test were used to compare demographics and clinical characteristics. Post hoc multiple comparisons were calculated using Tukey method.
A receiver-operating characteristic (ROC) curve was generated to assess the cfDNA level as a diagnostic biomarker. A cutoff point was chosen, and then sensitivity, false-positive rate, and 95% confidence intervals were calculated. A linear-regression model was used to adjust the covariants (including age and sex) affecting cfDNA. All tests were analyzed by IBM SPSS ver. 20.0 (IBM Co., Armonk, NY, USA).

This study was conducted prospectively after receiving permission from the local Institutional Review Board (No. 2012-056). All patients participated voluntarily with written informed consents.

**RESULTS**

The mean age was 66.72 ± 13.16 years in 30 gastric cancer patients, and 63.79 ± 6.76 years in 34 age-matched healthy subjects (P = 0.256). Aging cfDNA increases in gastric cancer patients (P < 0.01), but age and cfDNA are not correlated in the healthy control group (P = 0.969). CfDNA in females was higher than males in gastric cancer patients (P = 0.01), but not in the healthy control group (P = 0.598). CfDNA in nonsmokers was higher than smokers (P = 0.033) in gastric cancer patients, but not in the healthy control group (P = 0.375). Drinking alcohol, BMI, and Helicobacter pylori infection are not associated with cfDNA both in gastric cancer patients and the healthy control group (Table 1).

![Fig. 1. Comparison of circulating free DNA among healthy subjects (white), early gastric cancer (EGC) groups (gray), and advanced gastric cancer (AGC, black) groups.](image)

**Table 1. Mean levels of cfDNA according to clinical characteristics in gastric cancer and healthy control group**

| Variable                   | Case (n = 30) | Control (n = 34) |
|----------------------------|--------------|-----------------|
|                            | No. | Mean±SD | P-value | No. | Mean±SD | P-value |
| Age (yr)                   |     |         |         |     |         |         |
| <65                        | 13  | 102.31 ± 12.59 | <0.001  | 22  | 78.90 ± 8.55  | 0.969   |
| ≥65                        | 17  | 119.71 ± 8.28  | 0.010   | 12  | 78.78 ± 7.17  | 0.598   |
| Sex                        |     |         |         |     |         |         |
| Male                       | 23  | 110.00 ± 14.13 | 0.033   | 15  | 79.71 ± 6.85  | 0.375   |
| Female                     | 7   | 119.29 ± 7.88  | 0.010   | 19  | 78.13 ± 9.11  | 0.598   |
| Smoking                    |     |         |         |     |         |         |
| Current                    | 13  | 105.15 ± 12.88 | 0.033   | 4   | 82.25 ± 6.55  | 0.263   |
| Ex-smoker                  | 3   | 114.33 ± 19.34 | 0.033   | 4   | 81.27 ± 3.45  | 0.334   |
| None                       | 14  | 118.21 ± 10.09 | 0.033   | 26  | 78.35 ± 8.23  | 0.334   |
| Alcohola)                  |     |         |         |     |         |         |
| Severe                     | 8   | 105.12 ± 13.55 | 0.334   | 2   | 84.22 ± 18.71 | 0.334   |
| Mild to moderate           | 2   | 108.50 ± 14.84 | 0.334   | 7   | 75.30 ± 12.84 | 0.334   |
| None                       | 13  | 117.00 ± 12.55 | 0.334   | 15  | 76.06 ± 11.45 | 0.334   |
| Body mass index (kg/m²)    |     |         |         |     |         |         |
| <23                        | 19  | 114.00 ± 12.86 | 0.334   | 20  | 75.9 ± 7.4   | 0.189   |
| ≥23                        | 11  | 109.00 ± 14.41 | 0.334   | 14  | 80.14 ± 8.12 | 0.189   |
| H. pylori infection         |     |         |         |     |         |         |
| None                       | 10  | 116.30 ± 9.742 | 0.209   | 20  | 80.35 ± 6.12 | 0.348   |
| Yes                        | 8   | 107.88 ± 17.25 | 0.209   | 4   | 73.75 ± 11.78 | 0.348   |
| Not available              | 12  |          |         |     |         |         |

a) Total number of cases with alcohol history checked were 23 cases in gastric cancer patients group and 24 cases in healthy control group.

cfDNA, circulating free DNA; SD, standard deviation; H. pylori, Helicobacter pylori.
Comparison of cfDNA between healthy individuals and gastric cancer patients

CfDNA was proportionally increased between healthy subjects, EGC patients, and AGC patients (79.78 ± 8.12 ng/mL, 106.88 ± 12.40 ng/mL, and 120.23 ± 10.08 ng/mL, P < 0.001) (Fig. 1, Table 2). Fig. 2 shows the ROC curves of cfDNA between the cancer patients and healthy controls. The area under the curve is 0.991. As the cutoff value of cfDNA is defined to 90 ng/mL, sensitivity, specificity, positive-prediction value, and negative-prediction value are 96.67%, 94.11%, 93.54%, and 96.96%, respectively.

Table 2 shows clinical data that represent tumor burden. cfDNA is higher in the AGC group than the EGC group, and this is significant after adjustment by age, sex, and smoking (P = 0.004 and P = 0.035, respectively). CfDNA in the group with tumor sizes 5–9.9 cm is higher than in the group with tumor sizes <4.9 cm (P = 0.280. Tukey multiple-comparison test). Differentiation is not associated with cfDNA. The association between cfDNA and T stage is less significant (P = 0.065) but becomes significant after adjustment by age, sex and smoking (P = 0.037).

Table 2. Correlation between cfDNA and clinico-pathological data, which represents the invasiveness of gastric cancer

| Variable                      | No. | Mean ± SD       | P-value unadjusted | P-value adjusted<sup>a</sup> |
|-------------------------------|-----|-----------------|--------------------|-----------------------------|
| Cancer progression            |     |                 |                    |                             |
| Early gastric cancer          | 16  | 106.88 ± 12.40  | 0.004              | 0.035                       |
| Advanced gastric cancer       | 14  | 120.23 ± 10.08  |                    |                             |
| Tumor size (cm)               |     |                 |                    |                             |
| ≤4.9                          | 18  | 107.17 ± 12.89  | 0.005              | 0.045                       |
| 5–9.9                         | 9   | 120.89 ± 6.69   |                    |                             |
| ≥10                           | 3   | 128.00 ± 0.00   |                    |                             |
| Differentiation               |     |                 | 0.701              | 0.779                       |
| Differentiated                | 18  | 112.11 ± 14.02  |                    |                             |
| Undifferentiated              | 12  | 114.09 ± 11.99  |                    |                             |
| T stage                       |     |                 | 0.065              | 0.037                       |
| T1                            | 16  | 108.06 ± 13.20  |                    |                             |
| T2, 3                         | 6   | 110.83 ± 0.96   |                    |                             |
| T4                            | 8   | 121.38 ± 13.42  |                    |                             |
| N stage                       |     |                 | 0.344              | 0.096                       |
| N0                            | 22  | 110.00 ± 13.23  |                    |                             |
| N1, 2                         | 3   | 121.50 ± 6.36   |                    |                             |
| N3                            | 5   | 119.00 ± 15.58  |                    |                             |
| TNM stage                     |     |                 | 0.076              | 0.048                       |
| I                             | 20  | 108.50 ± 12.93  |                    |                             |
| II, III                       | 5   | 118.50 ± 8.69   |                    |                             |
| IV                            | 5   | 127.00 ± 1.41   |                    |                             |
| Curative resection<sup>b</sup>|     |                 | <0.001             | 0.016                       |
| Yes                           | 24  | 109.13 ± 13.17  |                    |                             |
| No                            | 6   | 124.37 ± 5.00   |                    |                             |
| CEA<sup>c</sup>              |     |                 | 0.641              | 0.979                       |
| ≤5                            | 21  | 110.52 ± 13.44  |                    |                             |
| >5                            | 6   | 113.50 ± 14.26  |                    |                             |

cfDNA, circulating free DNA; SD, standard deviation; CEA, carcinoembryonic antigen.

<sup>a</sup>Generalized linear-regression model; variables of age, sex, and smoking were adjusted. <sup>b</sup>Curative resection means R0 resection (no residual tumor), R1 (microscopic residual tumor), nd R2 resections (macroscopic residual tumor) are included in the noncurative resection groups. <sup>c</sup>Total number of cases with preoperative CEA level checked were 27 cases.
DISCUSSION

There is an increased incidence of stomach cancer cases in Korea, indicating a need for an applicable biomarker for better screening and management. According to the Korean National Cancer Incidence Database, in 2010, 202,053 incidents of cancer cases and 72,046 deaths were identified in Korea. The most prevalent cancer was thyroid cancer (36,021 cases), and the next most prevalent cancer was stomach cancer (total 30,092 cases: 2,017 males and 9,913 females). A total of 10,032 patients died due to stomach cancer in 2010. The crude rate of cancer incidence was 259.9 per 100,000 in the general Korean population. The age-standardized incidence rate of gastric cancer per 100,000 populations was 41.8 (males 62.3, females 24.9) in 2010 in Korea [14]. Also, Park et al. [15] reported that the incidence of second primary cancer (SPC) in male cancer survivors was 603.2 per 100,000 person-years, which was about 2.3 times compared to the incidence of the general population.

Thus, the incidence of gastric cancer and the SPC risk of cancer survivors in South Korea are much higher than in other countries. Although health screening for early diagnosis and follow-up tools are high, the use of conventional tumor markers (CEA, CA 19-9, CA 72-4, etc.) has many limits due to low sensitivity and specificity. So, more sensitive and specific biomarkers should be used for stomach cancer detection in Korea.

In this pilot study, we evaluated the efficacy of cfDNA as a biomarker that discriminates cancer patients from healthy subjects. The main finding of this study was that the plasma level of cfDNA is able to differentiate tumor burden such as tumor size, depth of invasion, and tumor stage. In our study, some demographic characteristics of patients with gastric cancer, such as age, sex and smoking, are associated with plasma-mean cfDNA levels. An unexpected result is that the levels of cfDNA are higher in the nonsmoking groups than the smoking groups. However these variables are not different in the healthy control group (Table 1). This may be due to the fact that the cancer type in the particular variables such as aged, female, non-smoker is more aggressive than others in this small sampled study.

Many studies have demonstrated that cfDNA levels have discriminatory power to differentiate cancer patients from healthy controls. As a diagnostic tool, sensitivity and specificity of cfDNA between cancer patients and healthy controls varies. Kamat et al. [16] demonstrated that a cutoff value of 4,500 GE/mL of cfDNA yielded a sensitivity of 87% and a specificity of 87% among 164 women with invasive epithelial ovarian carcinoma. 49 with benign ovarian neoplasms, and 75 age-matched controls. Another study showed cfDNA from plasma in metastatic colorectal cancer patients yields a specificity of 97% and a sensitivity of 31% [17]. Our study showed a much higher diagnostic value both in sensitivity and specificity compared to previous studies.

Furthermore, cfDNA can be a possible biomarker to differentiate the invasiveness of tumors. Increased serum cfDNA levels in relationship to tumor size are predictive of distant metastasis of esophageal squamous cell carcinoma [18]. Agostini et al. [19] demonstrated that cfDNA in breast cancer patients is associated with lymph-node involvement, but not with tumor stage and vascular invasion. The level of cfDNA was associated with malignant tumor size, lymph-node involvement, stage, and grade, as well as Her2/neu and Topoisomerase Iα expression, in breast cancer patients [20]. This study also demonstrated that the levels of cfDNA in plasma are proportionally associated with parameters representing the invasiveness of gastric tumors such as clinical cancer type (EGC vs. AGC), tumor size, and tumor stage, but not with histological differentiation and lymph-node involvement. In contrast to the cfDNA in this study, CEA and CA 19-9 are not associated with tumor stage and histological tumor type (data...
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