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ORIGINAL ARTICLE

# The Ratio of ssDNA to dsDNA in Circulating Cell-Free DNA Extract is a Stable Indicator for Diagnosis of Gastric Cancer

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## Abstract

Due to the different mechanisms of cell-free DNA production, the single-stranded DNA to double-stranded DNA ratio in blood maybe different between healthy individuals and gastric cancer (GC) patients. We aimed to explore the potential application of this ratio in GC diagnosis. The plasma cell-free DNA extracts from 118 healthy individuals and 106 GC patients were prepared. The levels of single-stranded DNA or double-stranded DNA in plasma, and the single-stranded DNA to double-stranded DNA ratio on the diagnostic efficiency for GC were assessed with ROC curve. The relationships between this ratio and the clinical characteristics of GC patients were analyzed. The ratios in 63 GC patients before and after surgery were compared. In healthy individuals, the single-stranded DNA to double-stranded DNA ratio was not affected by factors including age, gender and BMI, and subjected to normal distribution ($P = 0.1090$). GC patients had a lower value of this ratio than healthy individuals ($P < 0.0001$). Considering this ratio as a GC diagnostic indicator, the area under ROC curve (AUC) was 0.923[95% confidence interval (CI):0.880–0.955]. This ratio in unresectable GC was obviously lower than that in resectable GC ($P = 0.0045$). There was a rank correlation between this ratio and GC TNM staging (rho = −0.266, $P = 0.0058$), but it had no correlation with tumor size ($r = 0.14$, $P = 0.145$). Additionally, this ratio was not affected by hemolysis and repeated freeze-thaw of blood samples, and was significantly elevated after surgery($P < 0.0001$). The single-stranded DNA to double-stranded DNA ratio in plasma is a stable non-invasive indicator for GC diagnosis.

**Keywords** cfDNA · ssDNA to dsDNA ratio · Gastric cancer · Tumor biomarker · Non-invasive

## Introduction

Gastric cancer (GC) is one of the most common malignant tumors, and ranks as the 3rd most lethal cancer worldwide with approximately 1,033,701 new cases and 782,685 deaths in 2018 [1, 2]. In many Asian countries, particularly in eastern Asia like China, GC is the one of the most prevalent cancer in terms of the incidence and mortality rates [3, 4]. Since there are no specific symptoms for the patients with an early stage GC, most of the GC cases were diagnosed at the advanced stage and only less than 25% of patients survive for 5 years after diagnosis [5]. Although advanced surgical treatments can significantly improve the prognosis of patients with early stages of GC, this malignance is frequently diagnosed at an advanced stage and the patients have to undergo radiotherapy and chemotherapy, which often resulted in therapy resistances [6, 7]. Therefore, identification of novel biomarkers in gastric carcinogenesis are urgently needed to improve the early diagnosis, promote effective therapy and predict more accurate prognosis.

Cell-free DNA (cfDNA) contains complete tissue gene information, and is a potential marker for disease diagnosis, treatment and prognosis [8, 9]. Current hotspot application of cfDNA in tumor diagnosis have focused on obtaining tumor mutation and methylation information from cfDNA using

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various techniques. However, these techniques are expensive, and the differences in results are diverse [10–15]. Earlier studies have shown that quantitative measurement of cfDNA had diagnostic and prognostic role for multiple tumors [16]. Because quantitative analysis of cfDNA does not provide information about the biological characteristics and potential molecular targets, the in-depth study on quantitative measurement of cfDNA is almost given up. Although the usefulness of cfDNA quantitative analysis in tumor initiation and development is controversial, it still holds many advantages such as simplicity, speediness, non-invasiveness and cheapness [8, 10]. With the development of modern technology in molecular and cellular biology, accumulating studies have demonstrated that the quantitative measurement of cfDNA still have great values in cancer diagnosis, treatment and prognosis [17, 18].

cfDNA consists of double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA). Genomic instability caused by replication stress is an important cause of tumor igenesis, and genomic in stability in tumors often results in the production of large amounts of ssDNA and dsDNA [19–22]. These nascent DNA fragments are released into the blood by various routes, resulting in elevated levels of cfDNA in the plasma [23]. Unlike tumors, cfDNA in plasma of healthy individuals is mainly derived from normal apoptosis of lymphoid and myeloid cells [24, 25]. As the main digestive organ, the stomach of a GC patientis often under enormous replication stress [19, 23]. Due to the different mechanisms of cfDNA production under healthy and stressful conditions, we speculated that the ratio of ssDNA to dsDNA in blood may also be different between healthy individuals and tumor patients. In addition, although multiple studies have investigated the role of tumor-specific cfDNA in GC pathogenesis [26, 27], the implication on the ratio of ssDNA to dsDNA in circulating cfDNA for GC diagnosis has not been evaluated so far.

In this study, we compared the levels of ssDNA, the levels of dsDNA, and the ratios of ssDNA to dsDNA in plasma samples from 118 healthy individuals and 106 GC patients, and explored the impacts of experimental procedures including repeated freeze-thaw and hemolysis on the values of these cfDNA-associated parameters.

## Materials and Methods

### Sample Collection

The healthy individuals were recruited in the Health Care Center of the affiliated No.2 People's Hospital of Nanjing Medical University (Wuxi, China) between May, 2019 and October of 2019. The inclusion criteria for healthy individuals were as follows (meet all requirements): no abnormalities in tumor markers, hepatitis markers and routine physical examination including computed tomography (CT), upper gastrointestinal endoscopy, and abdominal ultrasonography. The exclusion criteria for healthy individuals were as follows (in any one condition): age < 30 years old, benign tumors, chronic inflammatory disease, autoimmune disease, and previous second-line therapies.

The diagnosis of GC was mainly based on clinical symptoms, imaging findings (such as CT, magnetic resonance imaging, and endoscopic ultrasonography), intraoperative biopsy and tumor markers. Pathological examination of the resected GC specimen was conducted, and the patients ($n$ = 63) at 28 days after surgery were recruited to this study as the post-operation group. The clinical or pathological staging of GC was performed according to the tumor node metastasis (TNM) classification. The tumor size of GC was expressed as in the sum of largest tumor diameters according to the RECIST 1.1 version.

A total of 224 subjects were recruited, including 106 patients with GC and 118 healthy individuals. Their information on age, gender, body mass index (BMI), cigarette smoking status and alcohol consumption was collected. Based on BMI values, these subjects were divided into three groups: <18.5, 18.5–23.9 and ≥ 24, as previously reported [28]. Based on cigarette smoking status, they were divided into three groups: never smoker, former smoker, and current smoker. Based on the alcohol consumption, they were divided into threegroups:0- < 5 g/day, 5-15 g/day and > 15 g/day as previously reported [29]. All participants provided written informed consent, and the study protocol was approved by the Ethics Committee of the affiliated No.2 People's Hospital of Nanjing Medical University (Wuxi, China).

### Preparation of Plasma

Fasting peripheral blood samples (8 ml /subject) were collected from the GC patients and healthy individuals using 4 mL EDTA vacuum tubes (BD Biosciences, Franklin Lakes, NJ, USA). The samples in EDTA vacuum tubes were processed within an hour from drawing the blood. The plasma was obtained after two rounds of centrifugation. Briefly, the blood samples were centrifuged at 1900×g at 4°Cfor 10 min. The supernatant was then transferred to a new centrifuge tube and centrifuged at16,000×g at 4°Cfor another 10 min. The plasma samples in the upper layers were collected and stored as 0.5 ml aliquots at −80 °C. A mixed plasma pool was made by mixing 118 plasma samples from healthy donors.

### cfDNA Extraction

Manual cfDNA extraction was performed using the Mini Cell-free DNA Extraction Kit of magnetic bead method (YUAN Biotechnology, Jiangsu, China) within two hours after thawing samples following the manufacturer's instructions.

Briefly, 1 mL of Lysis Adsorbent and 12.5 μL of Protease K were added into 0.5 mL of plasma, and they were mixed thoroughly. Samples were incubated with intermittent vortex at1,500rpm at 60°Cfor 10 min, and 10 μL of magnetic beads were added into the tube, which was subjected to intermittent vortex at 1,500 rpm at room temperature for another 10 min. On a magnetic frame, capturing and washing of the magnetic beads were finished. The cfDNA was eluted with 15 μL of Elution Buffer B provided by the kit, and samples were cryopreserved at −20 °C. The QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) was compared with the magnetic bead method in terms of cfDNA extraction efficiency. The cfDNA in 2 mL plasma was extracted with the Qiagen method following the manufacturer's instructions. The cfDNA was eluted in60μL Buffer AVE provided in the Qiagen kit, and samples were cryopreserved at −20 °C.

For accurate results, the mixed plasma pool was used as a quality control to monitor changes in extraction efficiency during each extraction. Plasma samples of randomly selected 20 patients were used for comparing the efficacy of the magnetic bead method and the Qiagen method in cfDNA extraction. The magnetic bead method was chosen for isolation of cfDNA from all the plasma samples.

## Quantification of cfDNA Concentrations

The concentrations of ssDNA and dsDNA in cfDNA extracts were examined using the Qubit ssDNA Assay Kit with a 50 pg-200 ng/μL of detection limit (Life Technologies, Carlsbad, California, US) and the Qubit 1 × dsDNA HS Assay Kit with a10pg-100 ng/μL of detection limit (Life Technologies) per the manufacturer's instructions, respectively. The quantifications of ssDNA and dsDNA through fluorometric assay were performed at 1 μL mode using the Qubit 3.0 Fluorometer (Life Technologies). For each assay, two replicates were performed. In order to ensure the accuracy of the measurement results, the CV values of the ssDNA and dsDNA concentrations in the quality control extract were required to be less than 5%.

## RNaseA and DNase I Digestion of cfDNA Extracts

The cfDNA extracts were isolated from the plasma samples of GC patients using the magnetic bead method. For RNase A digestion assay, 0.1 μL of RNaseA (10 mg/mL, TaKaRa, Kusatsu, Shiga, Japan) was added to 7 μL of the cfDNA extract, and the mixture was incubated at 37 °C for 3 min. For DNase I digestion, 0.5 μL of DNase I (5 U/μL, TaKaRa) was added to 5 μL of the same extracts, and the mixture was incubated at 37 °C for 10 min. The concentrations of ssDNA and dsDNA were then determined as described above.

## Analgesysis of cfDNA Fragments in Extracts and cfDNA Electrophoresis

The quality of cfDNA fragment in extracts was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Karlsruhe, Germany). For each extract,1 μL sample was examined using the Agilent High Sensitivity DNA Kit following the instructions of the manufacturer. The extracts of three GC patients with a high concentration of cfDNA were obtained with the magnetic bead method. The paired DNase I pre-digested and post-digested extracts samples were analyzed by electrophoresis with 1.5% agarose gel for 20 min. The electrophoresis results were photographed with the Gel Doc XR+ Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

## Repeated Freeze-Thaw and Hemolysis of Samples

For repeated freeze-thaw of samples, the plasma pool was rapidly frozen at −80 °C for 4 h, and taken out to get dissolved at 37 °C.This process was repeated 5 times, and the samples after each cycle of freeze-thaw were prepared. For hemolysis of samples, blood samples (2 mL each subject, 20 subjects) were rapidly frozen at −80 °C for 1 h, and taken out to get dissolved at 37 °C.This rapid freezing and thawing process was repeated 5 times to hemolyze samples. The cfDNA in post-freeze-thaw plasma samples was extracted using the magnetic bead method and quantitated as described above.

## Statistical Analysis

Quantitative variables were analyzed using Mann-Whitney U-test (statistical value was expressed as $U$) or Kruskal-Wallis test (statistical value was expressed as $H$), and paired quantitative variables were analyzed using Wilcoxon test (statistical value was expressed as $T$). The receiver operating characteristic (ROC) curve analysis was used to analyze the diagnosis efficiency. The point with the largest Youdent's index was selected as the optimal cut-off value according to the ROC curve. Multivariate unconditional logistic regression analysis models were also performed to assess the relationships between variables and GC occurrences. The relationship between the ssDNA to dsDNA ratio and tumor size was analyzed by linear regression. The above statistical $P$ values<0.05 were deemed as statistically significant. The normal distribution was analyzed by Shapiro-Wilk test (statistical value was expressed as $W$), and if $P > 0.05$, it was accepted as a normal distribution. Median range was reported as interquartile range (IQR). The area under ROC curve (AUC) and odds ratio (OR) were reported using a two-sided 95% confidence interval (CI). The statistical analysis was performed using the SPSS version 16.0 (SPSS Inc. Chicago, IL, USA).

## Results

### Comparison of Two Methods for cfDNA Extraction from Plasma Samples and Verification of cfDNA Extracts

In order to establish the methodology for efficient and effective cfDNA isolation, we first compared the magnetic bead method and the Qiagen column method using 20 samples randomly selected from the plasma samples of 106 GC patients. As shown in Supplementary Fig. 1a, the median level of ssDNA extracted by the magnetic bead method was 12.87(IQR:11.70–15.18) ng/μL, and the median level of ssDNA extracted by the Qiagen column method was5.86(IQR:5.27–6.58) ng/μL. There was a significant difference between the two results ($U = 0.00, P < 0.0001$). However, the median level of dsDNA extracted by the magnetic bead method (1.76(IQR:1.50–2.53)ng/μL) did not differ significantly from the median level of dsDNA extracted by the Qiagen column method(1.66(IQR:1.45–1.84) ng/μL) (Supplementary Fig. 1b). Therefore, we chose the magnetic bead method that resulted in a higher cfDNA yield for preparing all the remaining plasma cfDNA samples.

To verify the DNA nature of the extracts, we digested 20 isolated plasma cfDNA samples using RNaseA and DNaseI, and compared the concentrations of ssDNA and dsDNA in samples before and after digestion. After RNase A digestion, there were no significant changes in the concentrations of ssDNA($T = 88.50$, $P = 0.7983$) (Supplementary Fig. 2a) and dsDNA ($T = 88.00$, $P = 0.5459$) (Supplementary Fig. 2b). After DNase I digestion, the concentrations of ssDNA and dsDNA fell below the detection limit of the Qubit machine (Supplementary Fig. 2c and d). Moreover, we also selected 3 extracts to further evaluate the DNA content using Agilent 2100 bioanalyzer (Supplementary Figure 2e and 2f) and electrophoresis (Supplementary Fig. 2g). After DNase I digestion, the DNA fragments in all the extracts disappeared, suggesting the bona fide DNA nature of the cfDNA extracts.

### The Relationship between Clinical Characteristics and the Plasma ssDNA and dsDNA Levels As Well as the ssDNA to dsDNA Ratio in Healthy Individuals and GC Patients

We first examined the differences of the ssDNA levels, dsDNA levels, and the ssDNA to dsDNA ratio among the plasma samples from healthy individuals and GC patients that were categorized into various groups based on age, gender, BMI, cigarette smoking status, alcohol consumption, tumor size, existence of resected GC and TNM stage. In 118 healthy individuals, there was no significant difference in ssDNA and dsDNA levels and their ratios in each group, whether by age, BMI, cigarette smoking status or alcohol consumption grouping (all $P > 0.05$, Table 1). Although the levels of ssDNA ($U = 1273.00$, $P = 0.0163$) and dsDNA ($U = 1275.00$, $P = 0.0168$) in male healthy individuals were higher than that in female subjects (Table 1), the ratio of ssDNA to dsDNA did not differ between two genders ($U = 1413.00$, $P = 0.1005$). In healthy individuals, the ssDNA to dsDNA ratio followed normal distribution ($W = 0.9817$, $P = 0.1090$).

In 106 GC patients, there were no significant differences in ssDNA ($U = 1076.50$, $P = 0.0840$) and dsDNA ($U = 1155.50$, $P = 0.2234$) levels in two gender groups, but the ssDNA to dsDNA ratio of male was higher than that of female ($U = 1031.50$, $P = 0.0430$). In alcohol consumption groups, there were no significant differences in ssDNA level ($H = 4.3917$, $P = 0.1113$), but there were significant differences in dsDNA level ($H = 6.2443$, $P = 0.0441$) and the ssDNA to dsDNA ratio ($H = 9.1831$, $P = 0.0101$). There were no significant differences in ssDNA and dsDNA levels and their ratios between age, BMI and cigarette smoking status groups (all $P > 0.05$) (Table 2). After logarithmic transformation, results of the ssDNA and dsDNA levels still rejected normally distribution ($W = 0.9755$, $P = 0.0468$).

We also examined the impacts of the common parameters including age, gender, BMI, cigarette smoking status, alcohol consumption on the levels of ssDNA and dsDNA, as well as their ratios, in all the 224 subjects. It is interesting that the participants aged over 60 had significantly increased ssDNA and dsDNA levels while reduced ssDNA to dsDNA ratio compared with the participants aged 60 or less than 60 (Table 3). In addition, among all the healthy individuals and GC patients, the females had significantly lower plasma ssDNA and dsDNA levels than the males, while the ssDNA to dsDNA ratios between the two genders did not differ significantly. There were no significant differences in ssDNA and dsDNA levels and their ratios between BMI, cigarette smoking status and alcohol consumption groups (all $P > 0.05$) (Table 3).

### Potentials of the Plasma ssDNA and dsDNA Levels As Well as the ssDNA to dsDNA Ratio in GC Diagnosis and Prognosis

In GC patients, the plasma ssDNA to dsDNA ratio value in unresectable GC was obviously lower than that in resectable GC($U = 913.00$, $P = 0.0045$; Table 2). Additionally, there were significant differences in the ratio value among the patients with different TNM stages ($H = 7.8692$, $P = 0.0196$) (Table 2). Moreover, there was a certain rank correlation between the ratio and TNM staging, as the coefficient of rank correlation (rho) was −0.266 (95%CI: −0.435 ~ −0.0796,$P = 0.0058$). However, there was no correlation between the ratio and tumor size ($r = 0.14$, $P = 0.145$; data not shown). Our further multivariate unconditional logistic regression analysis showed that among all the variables investigated, the age and

**Table 1** Relationship between the clinical characteristics of healthy individuals and the concentrations of ssDNA and dsDNA, or the ssDNA to dsDNA ratios in plasma

| Characteristics | No. | ssDNA (ng/mL) Median (IQR) | *P value* | dsDNA (ng/mL) Median (IQR) | *P value* | ssDNA to dsDNA ratio Median (IQR) | *P value* |
|---|---|---|---|---|---|---|---|
| All | 118 | 7.65(6.06–9.06) | | 0.84(0.69–1.05) | | 8.75(8.42–9.06) | |
| Age (years) | | | | | | | |
| 31–40 | 14 | 6.48(5.19–8.22) | | 0.81(0.60–0.96) | | 8.49(8.27–8.92) | |
| 41–50 | 41 | 7.44(5.88–9.75) | | 0.84(0.66–1.14) | | 8.79(8.35–9.11) | |
| 51–60 | 43 | 7.98(6.36–9.66) | | 0.90(0.72–1.14) | | 8.75(8.43–9.12) | |
| 61–70 | 20 | 7.80(6.18–8.58) | 0.2792[a] | 0.87(0.69–0.96) | 0.4635[a] | 8.77(8.64–8.99) | 0.4312[a] |
| Gender | | | | | | | |
| Male | 66 | 8.01(6.30–10.02) | | 0.93(0.72–1.17) | | 8.66(8.33–9.02) | |
| Female | 52 | 7.38(5.94–8.16) | 0.0163[b] | 0.84(0.66–0.93) | 0.0168[b] | 8.79(8.58–9.09) | 0.1005[b] |
| BMI | | | | | | | |
| <18.5 | 18 | 7.95(5.91–10.74) | | 0.91(0.67–1.31) | | 8.62(8.42–8.95) | |
| 18.5–23.9 | 65 | 7.50(6.15–9.08) | | 0.85(0.70–1.06) | | 8.75(8.34–9.14) | |
| ≥24 | 35 | 7.68(6.02–8.49) | 0.8064[a] | 0.87(0.67–0.98) | 0.8495[a] | 8.77(8.52–9.06) | 0.6356[a] |
| Cigarette smoking status | | | | | | | |
| Never | 59 | 7.50(6.21–9.00) | | 0.84(0.69–1.08) | | 8.74(8.35–9.02) | |
| Former | 8 | 8.01(7.32–9.06) | | 0.93(0.84–0.99) | | 8.55(8.41–8.95) | |
| Current | 51 | 7.68(5.70–9.33) | 0.7234[a] | 0.84(0.66–1.05) | 0.6489[a] | 8.77(8.51–9.14) | 0.4594[a] |
| Alcohol consumption(g/day) | | | | | | | |
| 0-<5 | 16 | 7.50(6.42–8.37) | | 0.84(0.72–0.93) | | 8.97(8.49–9.08) | |
| 5–15 | 54 | 7.92(6.54–9.72) | | 0.90(0.72–1.08) | | 8.69(8.33–9.13) | |
| >15 | 48 | 7.32(5.58–8.61) | 0.3471[a] | 0.81(0.60–1.02) | 0.3283[a] | 8.75(8.48–9.05) | 0.5410[a] |

*P* value <0.05 indicates a difference. *P* value <0.01 indicates a significant difference. IQR, interquartile range. a, the comparison among the multiple groups was analyzed using the Kruskal-Wallis test. b, the comparison between the two groups was analyzed using the Mann-Whitney test

the ssDNA to dsDNA ratio were significantly related to GC, and the adjusted ORs were 1.2346 [95% CI: 1.1369–1.3407, $P < 0.0001$] and 0.0192(95% CI: 0.0014–0.2605, $P = 0.0030$), respectively (Table 4). Taken together, these results suggest that the ssDNA to dsDNA ratio in plasma is potentially an indicator for GC diagnosis, and lower ssDNA to dsDNA ratio predicts more advanced disease stages in GC.

We then compared the ssDNA levels, dsDNA levels and the ssDNA to dsDNA ratios in plasma samples from all the healthy individuals and GC patients. As shown in Fig. 1a and b, the ssDNA ($U = 3023.00$, $P < 0.0001$) and dsDNA($U = 2277.50$, $P < 0.0001$) levels in GC patients were significantly higher than that in healthy individuals (all $P < 0.0001$), while the ratio of ssDNA to dsDNA in GC patients was lower than that in healthy individuals($U = 958.00$, $P < 0.0001$) (Fig. 1c). Moreover, the distribution of the ssDNA to dsDNA ratios displayed a clear discrimination in the merged histogram plots of healthy individuals and GC patients (Fig. 1d). Furthermore, ROC curves were further summarized to clarify the diagnostic utility of the ssDNA to dsDNA ratio in GC (Fig. 2a). As shown in Table 5, the AUC of ssDNA to dsDNA ratio alone as a diagnostic tool was 0.923(95%CI: 0.880–0.955) with a sensitivity of 86.79% and a specificity of 90.68%. The AUC of the ssDNA to dsDNA ratio in combination with the ssDNA and dsDNA levels was 0.930 (95%CI: 0.889–0.960) with a sensitivity of 83.96% and a specificity of 94.07%. Among the healthy individuals, 11 out of 118 subjects had the ssDNA to dsDNA ratio lower than the cut-off value 8.154, while 92 out of 106 GC patients had the ratio lower than the cut-off value (Fig. 2b). In addition, the vast majority of the healthy subjects (89 out of 106) had a combined index higher than the cut-off value, whereas only a tiny portion of GC patients (8 out of 118) had a higher combined index (Fig. 2c).

We also examined the changes in plasma ssDNA and dsDNA levels, and their ratio in GC patients before and after surgery. We observed significantly reduced plasma ssDNA levels (Fig. 3a) and dsDNA levels (Fig. 3b) in GC patients after surgery. The median value of the ssDNA to dsDNA ratios in63 tumor resectable GC patients before surgery was 7.47(IQR:7.23–7.90), and the median value after surgery was 8.45(IQR:8.16–9.02). The ratio before surgery was significantly lower than that after surgery($T = -6.74$, $P < 0.0001$). However, the ssDNA to dsDNA ratio in 60 of 63tumor resectable GC patients experienced increases in varying degrees

**Table 2** Relationship between the clinical characteristics of GC patients and the concentrations of ssDNA and dsDNA, or the ssDNA to dsDNA ratios in plasma

| Clinical characteristics | No. | ssDNA (ng/mL) Median (IQR) | *P value* | dsDNA (ng/mL) Median (IQR) | *P value* | ssDNA to dsDNA ratio Median (IQR) | *P value* |
|---|---|---|---|---|---|---|---|
| All | 106 | 11.49(7.80–18.90) | | 1.53(0.99–2.61) | | 7.33(7.02–7.69) | |
| Age (years) | | | | | | | |
| ≤60 | 26 | 9.63(8.70–18.00) | | 1.35(1.01–2.53) | | 7.19(6.94–7.61) | |
| 60+ | 80 | 12.00(7.80–21.66) | 0.3215[b] | 1.58(0.99–3.08) | 0.3903[b] | 7.37(7.07–7.86) | 0.2201[b] |
| Gender | | | | | | | |
| Male | 64 | 12.60(8.73–19.86) | | 1.67(1.09–2.75) | | 7.45(7.07–7.89) | |
| Female | 42 | 9.51(6.90–16.02) | 0.0840[b] | 1.35(0.90–2.21) | 0.2234[b] | 7.26(6.86–7.62) | 0.0435[b] |
| Body Mass Index (BMI) | | | | | | | |
| <18.5 | 12 | 15.45(9.60–31.65) | | 2.14(1.35–4.21) | | 7.29(7.02–7.75) | |
| 18.5–23.9 | 58 | 11.04(7.62–19.68) | | 1.44(0.93–2.81) | | 7.34(7.10–7.67) | |
| ≥24 | 36 | 10.62(8.43–16.65) | 0.3680[a] | 1.45(1.01–2.18) | 0.3509[a] | 7.37(6.94–7.94) | 0.1466[a] |
| Cigarette smoking status | | | | | | | |
| Never | 14 | 11.67(9.54–17.40) | | 1.53(1.20–2.46) | | 7.44(6.94–8.10) | |
| Former | 41 | 10.20(7.71–18.36) | | 1.38(0.93–2.58) | | 7.24(7.01–7.65) | |
| Current | 51 | 11.76(7.80–25.89) | 0.7160[a] | 1.5 (1.08–3.48) | 0.8706[a] | 7.35(7.12–7.74) | 0.4981[a] |
| Alcohol consumption(g/day) | | | | | | | |
| 0-<5 | 14 | 9.27(6.36–14.94) | | 1.20(0.87–1.47) | | 7.58(7.31–8.37) | |
| 5–15 | 48 | 10.65(7.53–17.07) | | 1.3(0.93–2.22) | | 7.36(7.12–7.83) | |
| >15 | 44 | 14.58(8.97–30.39) | 0.1113[a] | 1.89(1.20–4.17) | 0.0441[a] | 7.22(6.77–7.54) | 0.0101[a] |
| Tumor size (mm) | | | | | | | |
| ≤20 | 24 | 10.77(6.93–17.19) | | 1.24(0.92–2.34) | | 7.59(7.22–8.19) | |
| 20–50 | 57 | 11.16(8.27–18.38) | | 1.57(1.05–2.51) | | 7.28(7.06–7.65) | |
| ≥50 | 25 | 14.46(8.52–31.80) | 0.4354[a] | 1.83(1.21–4.28) | 0.3151[a] | 7.14(6.66–7.73) | 0.0974[a] |
| Resected GC | | | | | | | |
| Yes | 63 | 10.44(7.62–17.37) | | 1.33(0.93–2.44) | | 7.47(7.23–7.90) | |
| No | 43 | 12.36(8.84–26.61) | 0.0261[b] | 1.68(1.18–3.47) | 0.0963[b] | 7.13(6.86–7.61) | 0.0045[b] |
| TNM | | | | | | | |
| I | 35 | 9.54(7.62–17.34) | | 1.23(0.93–2.40) | | 7.62(7.23–8.09) | |
| II | 32 | 11.46(8.79–17.88) | | 1.56(1.17–2.49) | | 7.32(7.04–7.60) | |
| III-IV | 39 | 12.60(8.37–28.86) | 0.5221[a] | 1.68(1.17–3.84) | 0.2672[a] | 7.25(6.88–7.57) | 0.0196[a] |

*P* value <0.05 indicates a difference. *P* value <0.01 indicates a significant difference. IQR, interquartile range. a, the comparison among the multiple groups was analyzed using the Kruskal-Wallis test. b, the comparison between the two groups was analyzed using the Mann-Whitney test

after surgery (Fig. 3c). Therefore, these results further support the notion that the ssDNA to dsDNA ratio in plasma can be used as a diagnostic and prognostic factor of GC.

## The ssDNA to dsDNA Ratio in Plasma is Anindicator with Good Stability

Experimental procedures like repeated freeze-thaw and hemolysis may impact the levels of ssDNA and dsDNA in plasma. To verify the effects of these procedures on cfDNA abundance, we artificially recapitulated the processes of repeated freeze-thaw cycles and hemolysis of plasma samples, and evaluated the changes in the ssDNA level, dsDNA level, and the ssDNA to dsDNA ratio. As shown in Fig. 4a, repeated freeze-thaw cycles in pooled plasma samples from healthy individuals and GC patients had almost no effect on the ssDNA and dsDNA levels and the ssDNA to dsDNA ratio. Hemolysis caused elevated levels of ssDNA ($T = 0.00$, $P < 0.0001$; Fig. 4b) and dsDNA($T = 0.00$, $P < 0.0001$; Fig. 4c) in plasma from GC patients, but the ssDNA to dsDNA ratio was essentially constant ($T = 103.00$, $P = 0.9563$; Fig. 4d) after hemolysis. Collectively, these results indicate that the ssDNA to dsDNA ratio in plasma is a stable indicator that is not influenced by repeated freeze-thaw cycles and hemolysis.

**Table 3** Relationship between the clinical characteristics of all subjects and the concentrations of ssDNA and dsDNA, or the ssDNA to dsDNA ratios in plasma

| Clinical characteristics | No. | ssDNA (ng/mL) Median (IQR) | *P value* | dsDNA (ng/mL) Median (IQR) | *P value* | ssDNA to dsDNA ratio Median (IQR) | *P value* |
|---|---|---|---|---|---|---|---|
| Age (years) | | | | | | | |
| ≤60 | 124 | 7.95(6.18–10.02) | | 0.90(0.71–1.26) | | 8.60 (8.02–9.02) | |
| 60+ | 100 | 10.35(7.46–17.37) | 0.0001 | 1.30(0.90–2.38) | 0.0001 | 7.58(7.20–8.40) | 0.0001 |
| Gender | | | | | | | |
| Male | 130 | 9.36(7.26–12.90) | | 1.14(0.82–1.68) | | 8.24(7.38–8.82) | |
| Female | 94 | 7.80(6.18–9.78) | 0.0031 | 0.91(0.68–1.36) | 0.012 | 8.33(7.29–8.91) | 0.9027 |
| Body Mass Index (BMI) | | | | | | | |
| <18.5 | 30 | 9.93(6.48–12.36) | | 1.20(0.71–1.62) | | 8.40(7.60–8.72) | |
| 18.5–23.9 | 123 | 8.40(6.71–11.61) | | 0.99(0.80–1.52) | | 8.24(7.358–8.90) | |
| ≥24 | 71 | 8.52(6.83–11.63) | 0.7519 | 1.00(0.81–1.50) | 0.7515 | 8.27(7.30–8.82) | 0.9840 |
| Cigarette smoking status | | | | | | | |
| Never | 100 | 8.04(6.75–10.68) | | 0.95(0.80–1.37) | | 8.27(7.38–8.82) | |
| Former | 22 | 9.99(7.68–12.48) | | 1.22(0.93–1.88) | | 8.16(7.23–8.63) | |
| Current | 102 | 8.73(6.36–12.36) | 0.2069 | 1.06(0.73–1.57) | 0.2372 | 8.29(7.35–8.94) | 0.6286 |
| Alcohol consumption(g/day) | | | | | | | |
| 0-<5 | 30 | 7.65(6.36–10.44) | | 0.89(0.81–1.25) | | 8.49(7.58–9.01) | |
| 5–15 | 102 | 8.79(6.96–11.58) | | 1.03(0.82–1.46) | | 8.27(7.38–8.77) | |
| >15 | 92 | 8.82(6.39–14.58) | 0.5231 | 1.10(0.76–1.77) | 0.4491 | 8.08(7.24–8.78) | 0.1302 |

## Discussion

GC remains one of the leading causes of cancer-related death, and exploration of the complex molecular mechanisms underlying GC occurrence and development is still urgent [7]. Currently, identification of sensitive and non-invasive biomarkers for GC diagnosis is a priority that helps significantly to increase quality of life and overall survival for GC patients. In this study, we focused on the abundancies of ssDNA and dsDNA in circulating cfDNA from blood samples of GC patients, and found that the ratio of ssDNA to dsDNA in plasma samples from GC patients was significantly lower than that from healthy individuals. Notably, this ratio did not differ significantly even after repeated freeze-thaw cycles and hemolysis of blood samples, which suggests that the ssDNA to dsDNA ratio in circulating cfDNA extract is a stable indicator for GC diagnosis.

**Table 4** Multi-factor unconditional logistic regression analysis of GC occurrence

| Factors | Crude OR | 95% CI | *P value* | Adjusted OR | 95% CI | *P value* |
|---|---|---|---|---|---|---|
| Age | 1.1693 | 1.1230–1.2175 | <0.0001 | 1.2346 | 1.1369–1.3407 | <0.0001 |
| Gender | 1.2006 | 0.7049–2.0448 | 0.5011 | 1.4955 | 0.3818–5.8587 | 0.5634 |
| BMI(<18.5) | 0.7471 | 0.3318–1.6824 | 0.4815 | 0.5986 | 0.0880–4.0728 | 0.5999 |
| BMI(≥24) | 1.1527 | 0.6424–2.0684 | 0.6338 | 2.6496 | 0.7125–9.8524 | 0.1459 |
| Cigarette smoking status (Former) | 2.5183 | 0.9684–6.5490 | 0.0582 | 0.7953 | 0.1075–5.8813 | 0.8225 |
| Cigarette smoking status (Current) | 1.4390 | 0.8250–2.5099 | 0.1997 | 2.3887 | 0.6381–8.9418 | 0.1960 |
| Alcohol consumption(5-15 g/day) | 1.0159 | 0.4493–2.2971 | 0.9698 | 0.2492 | 0.0402–1.5446 | 0.1355 |
| Alcohol consumption(>15 g/day) | 1.0476 | 0.4588–2.3923 | 0.9121 | 0.2251 | 0.0330–1.5365 | 0.1281 |
| ssDNA | 1.2897 | 1.1708–1.4207 | <0.0001 | 2.2514 | 0.2900–17.4759 | 0.4376 |
| dsDNA | 1.3054 | 1.1939–1.4273 | <0.0001 | 0.6511 | 0.1336–3.1740 | 0.5955 |
| ssDNA to dsDNA ratio | 0.0477 | 0.0229–0.0994 | <0.0001 | 0.0192 | 0.0014–0.2605 | 0.0030 |

Due to the small value of dsDNA levels, it is prone to result in a large numerical value of OR when performing multi-factors unconditional logistic regression analysis. Therefore, the OR values of the dsDNA row in Table 4 represent the results after multiplying the dsDNA levels by 10 times

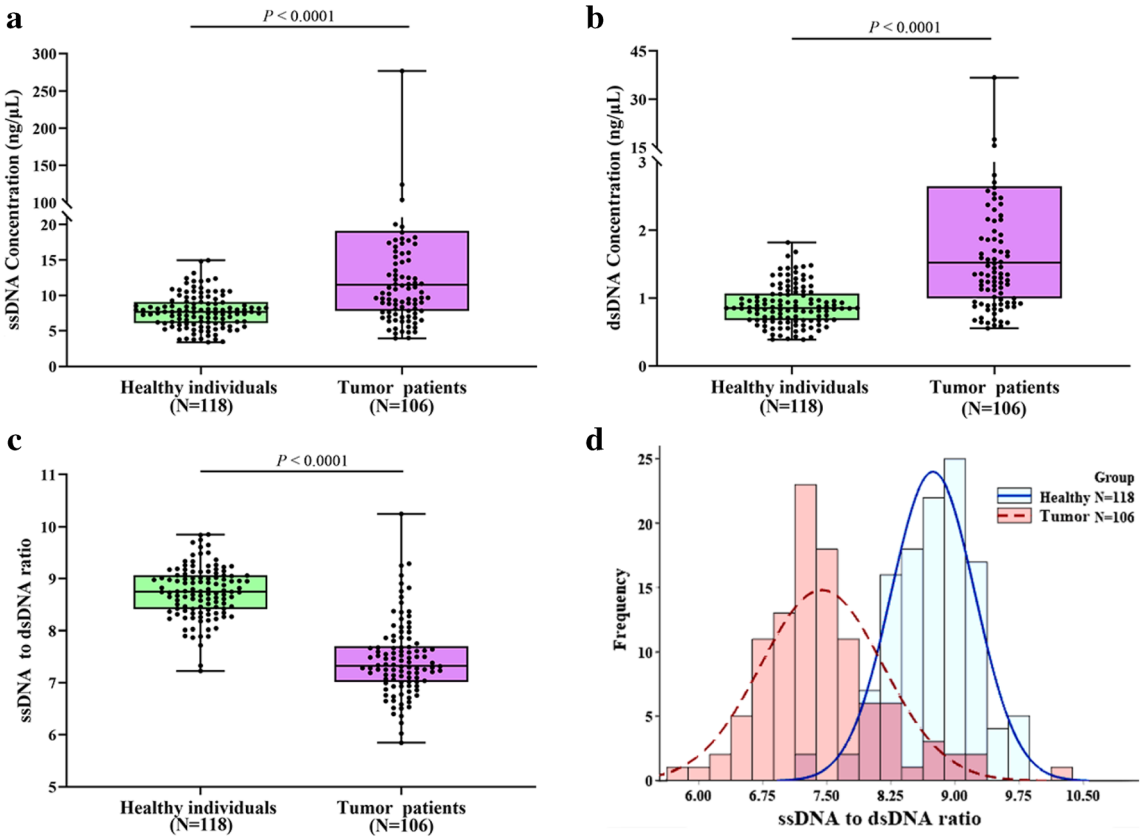


**Fig. 1** Comparison of the ssDNA levels, dsDNA levels and the ssDNA to dsDNA ratios in plasma samples from healthy individuals and GC patients. **a-c** The concentrations of ssDNA (**a**), dsDNA (**b**), and the ssDNA to dsDNA ratios (**c**) in plasma samples from healthy individuals ($n = 118$) and GC patients ($n = 106$) were compared. **d** The distributions of the ssDNA to dsDNA ratios in healthy individuals and GC patients were plotted in overlapping histograms.

Efficient and standardized extraction of cfDNA fragments is the basis of accurate quantitation of ssDNA and dsDNA in plasma. Different cfDNA extraction techniques usually lead to varied yields [30], and most of the isolation kits result in significantly underestimated amounts of ssDNA [30], which seriously affects the accurate application of cfDNA in disease diagnosis, especially when the ssDNA to dsDNA ratio is calculated. Previous reports have demonstrated that the magnetic bead method for cfDNA extraction shows higher extraction efficiency and quality than other column methods [15, 31]. The successful extraction of ssDNA is the key of this study, so we first compared two popular cfDNA extraction kits. The results showed that the magnetic bead method had remarkably higher ssDNA yield than the Qiagen column method, which was consistent with previous reports [15, 31]. Therefore, in this study, magnetic beads were used to extract cfDNA to further generate all the other data. The successful standardization of cfDNA extraction in plasma samples paved the way for subsequent applications of the ssDNA to dsDNA ratio in GC diagnosis.

The Qubit ssDNA Assay Kit is not specific for ssDNA detection, and it also detects dsDNA and RNA. Because plasma contains a lot of RNA types such as microRNA (miRNA), long non-coding RNA (lncRNA) and circular RNA (circRNA). To verify the nature of the cfDNA extract, we digested them using RNaseA and DNaseI, and compared the results of Qubit assays before and after digestions. Since we did not observe obvious declines on the concentrations of ssDNA and dsDNA after RNase A digestion, the extracted nucleic acids therefore belonged to DNA fragments. It is worth noting that we focused on total cfDNA isolation and the magnetic bead method can achieve a good cfDNA integrity. Thus, the isolation efficiencies of certain oncogene-specific cfDNA did not affect our conclusion, as the total ssDNA and total dsDNA were what we cared about, which represents one of the advantages of using the ssDNA to dsDNA ratio as an indicator. Additionally, without the PCR procedures for confirming the tumor-specific DNA, this indicator can save large amounts of labor and is easy to be fully automated.

In the processes of cfDNA isolation, hemolysis and plasma freeze-thaw often lead to clinically inaccurate evaluations of tumor-specific markers [32, 33]. However, our investigations demonstrated that plasma freeze-thaw had little effect on the ssDNA to dsDNA ratio. Although hemolysis led to increased levels of ssDNA and dsDNA, the ratio of ssDNA to dsDNA

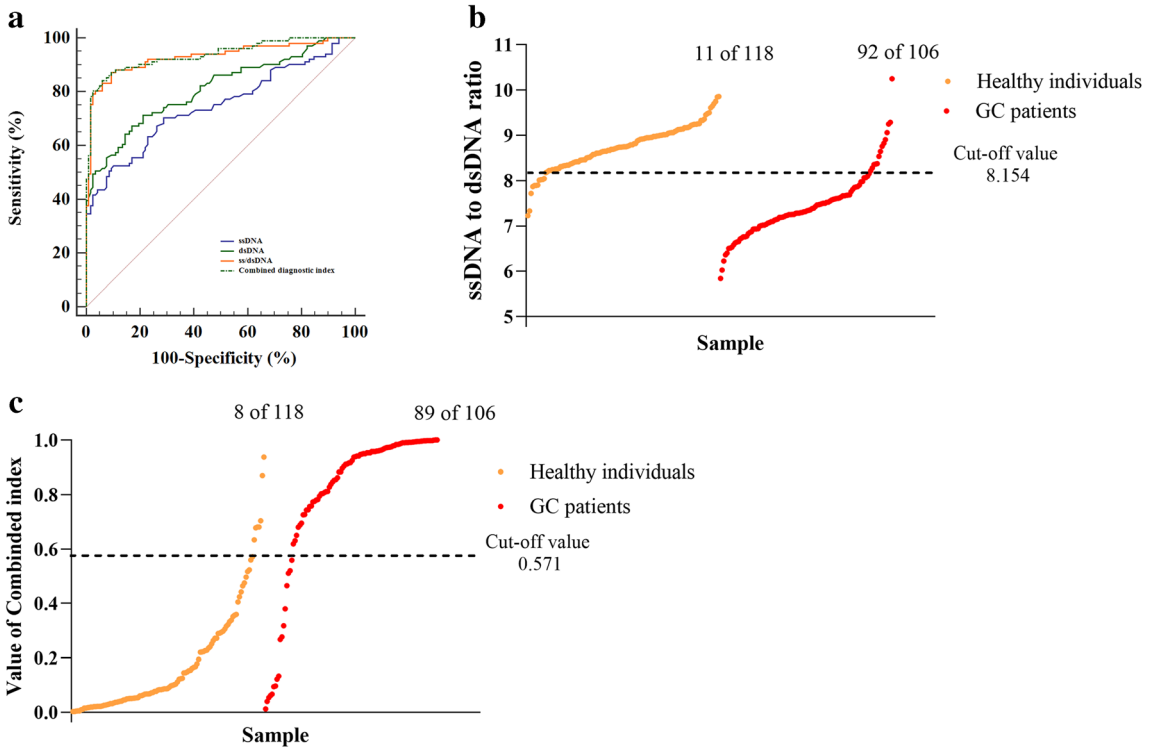


**Fig. 2** Diagnostic utility of the ssDNA and dsDNA levels and the ratios of ssDNA to dsDNA for GC. **a** ROC curves of the indicatedvariables. ssDNA, the ssDNA level in plasma; dsDNA, the dsDNA level in plasma; ss/dsDNA, the ratio of ssDNA level to dsDNA level in plasma; combined diagnostic index, the ssDNA to dsDNA ratio in combination with ssDNA and dsDNA concentrations in plasma. **b** Comparison on the distributions of the ssDNA to dsDNA ratios in plasma samples from healthy individuals and GC patients based on the cut-off value. **c** Comparison on the distributions of the combined index values from healthy individuals and GC patients based on the cut-off value

after hemolysis was constant. Traditional tumor biomarkers, such as carbohydrate antigen 19–9 (CA19–9), carbohydrate antigen 72–4 (CA72–4), carbohydrate antigen 12–5 (CA12–5) and carcinoembryonic antigen (CEA), were affected by many factors such as cigarette smoking status, alcohol consumption, age, and gender [34, 35]. We showed that the ssDNA to dsDNA ratio in healthy individuals was not affected by age, gender, BMI, cigarette smoking status and alcohol consumption, and was subjected to a normal distribution in healthy individuals. In GC patients, the ssDNA to dsDNA ratio was not subjected to the normal distribution and was significantly lower than that of healthy individuals. Furthermore, our multi-factor unconditional logistic regression analysis indicated that age and the ssDNA to dsDNA ratio in plasma were independent risk factor for GC. Since most cancers appear in adults at an advanced age [36], it is reasonable to identify the connection between age and risk of GC. To the best of our knowledge, this is the first report on revealing the connection between the ssDNA to dsDNA ratio in plasma and risk of GC. Considering the invasiveness and simplicity in methodology, this ratio is evidently a more ideal indicator than most traditional gastrointestinal tumor markers for GC diagnosis.

**Table 5** Diagnostic utility ofvarious variables for GC

| Variables | Cut-off values | Sensitivity (%) | Specificity (%) | AUC | 95% CI | Positive predictive value (%) | Negative predictive value (%) |
|---|---|---|---|---|---|---|---|
| ssDNA | >10.74 | 54.72 | 89.83 | 0.758 | 0.697–0.813 | 82.85 | 68.82 |
| dsDNA | >1.176 | 68.87 | 83.05 | 0.818 | 0.761–0.866 | 78.49 | 74.81 |
| ratio | <8.154 | 86.79 | 90.68 | 0.923 | 0.880–0.955 | 89.32 | 88.43 |
| combined index | >0.571 | 83.96 | 94.07 | 0.930 | 0.889–0.960 | 92.71 | 86.72 |

Ratio represents the ratio of ssDNA to dsDNA; combined index represents the ssDNA to dsDNA ratio in combination with ssDNA and dsDNA concentrations

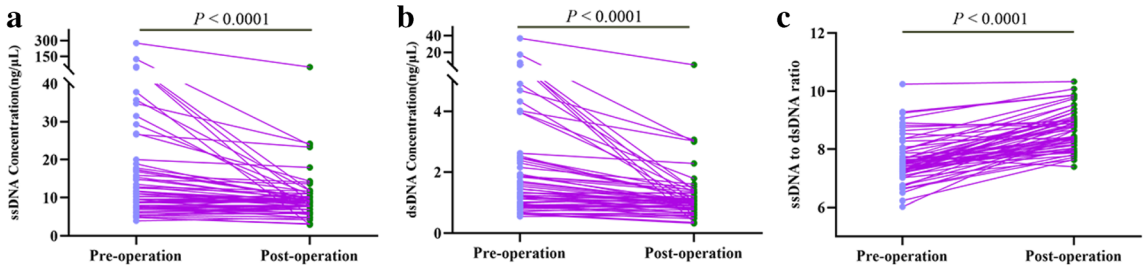


**Fig. 3** Comparisons on the ssDNA levels, dsDNA levels, and the ssDNA to dsDNA ratios in plasma samples from GC patients before and after surgery. **a-c** The ssDNA levels (**a**), dsDNA levels (**b**), and the ssDNA to dsDNA ratios (**c**) in paired cfDNA extract samples from GC patients ($n$ = 63) at the time points of pre-operation and post-operation were compared.

Diagnostic efficiency is an important indicator for evaluating diagnostic markers. We showed that the ssDNA to dsDNA ratio had markedly higher diagnostic specificity and sensitivity for GC than the level of ssDNA or dsDNA alone. We also showed that this ratio had no correlation with the GC size, but had a certain rank correlation with the TNM stage of GC. In addition, the ratio value of unresectable GC was significantly lower than that of resectable GC, which further suggests that the ssDNA to dsDNA ratio is also a potential indicator for judging GC metastasis and the possibility of tumor surgical resection. Furthermore, GC patients after surgery had a significantly elevated ratio of ssDNA to dsDNA in plasma, implying that this ratio is also a potential indicator for GC prognosis after tumor removal. However, the prognostic value of this ratio requires more further study on the patients over a long period of follow-up and a series of systemic evaluations. Taken together, according to the previously reported standards on measuring the accuracy of diagnostic systems [37], we believe that the ssDNA to dsDNA ratio in plasma is a stable indicator that can be used to distinguish the GC patients from healthy individuals, and the prognostic value of this ratio remains to be further investigated.

Although for the first time, this study uncovered the diagnostic value of the ssDNA to dsDNA ratio forGC, there were several limitations. First, because the fluctuation range of the ratio was small, the thresholds for cutoff values selected based on ROC analyses need to be validated in an independent cohort. Second, only one cancer type was studied in this article,

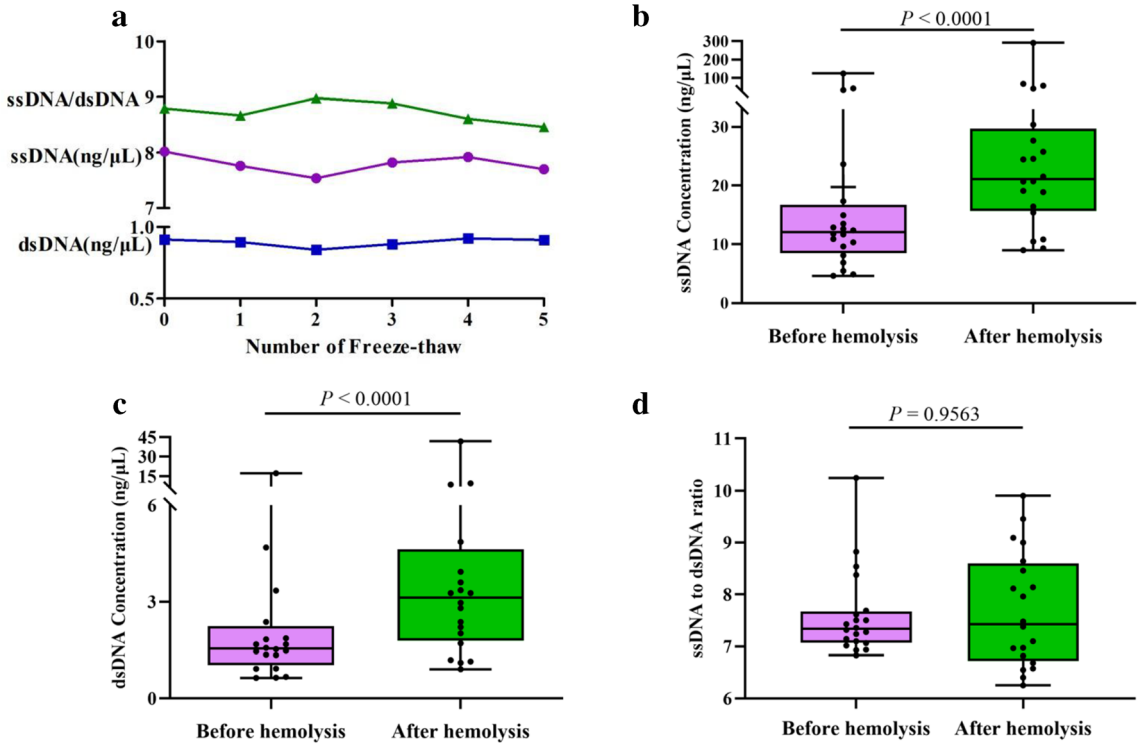


**Fig. 4** Repeated freeze-thaw cycles and hemolysis did not affect the ssDNA to dsDNA ratios in plasma samples. **a** The concentrations of ssDNA and dsDNA, as well as the ssDNA to dsDNA ratio in plasma samples pooled from healthy individuals and GC patients at the indicated time points of before and after 1–5 cycles of repeated freeze-thaw were measured. **b-d** The concentrations of ssDNA (**b**), dsDNA (**c**) and the ssDNA to dsDNA ratios (**d**) in plasma samples acquired before and after hemolysis were compared. $n$ = 20 randomly selected GC patients

and it is unknown whether the main finding of this study also applies to other cancer types. Third, due to the small sample size in a single institute, as well as the short duration and limited follow-up cases, the application of this ratio in diagnosis and prognosis of GC needs to be further explored with larger sample size and in more locations.

In conclusion, we revealed that the ssDNA to dsDNA ratioin plasma is a stable, non-invasive and cost-effective new indicator for GC diagnosis. Although it takes time to observe an immediate impact of a new indicator on the clinic, we believe that this study represents the first step towards achieving this long-term goal.

**Authors' Contributions** (I) Conception and design: Xuewen Huang.
(II) Administrative support: Xuewen Huang.
(III) Provision of study materials or patients: Qi Zhao, Yiqiu Xu.
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(V) Data analysis and interpretation: Xuewen Huang, Jie Pan, Lanfeng Shen, Dandan Yuan.
(VI) Manuscript writing: All authors.
(VII) Final approval of manuscript: All authors.
All authors have read and approved the manuscript.

**Data Availability** The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no competing interests.

**Ethics Approval and Consent to Participate** The study protocol was approved by the Ethics Committee of the affiliated No.2 People's Hospital of Nanjing Medical University (Wuxi, China) [(2019) Academic Review No. (Y-25)]. The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All participants provided written informed consent.

**Consent for Publication** Written informed consent to publish this information was obtained from study participants.

## References


1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68(6):394–424. https://doi.org/10.3322/caac.21492
2. Herszenyi L, Tulassay Z (2010) Epidemiology of gastrointestinal and liver tumors. Eur Rev Med Pharmacol Sci 14(4):249–258
3. Kim YI, Kim YW, Choi IJ, Kim CG, Lee JY, Cho SJ, Eom BW, Yoon HM, Ryu KW, Kook MC (2015) Long-term survival after endoscopic resection versus surgery in early gastric cancers. Endoscopy 47(4):293–301. https://doi.org/10.1055/s-0034-1391284
4. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J (2016) Cancer statistics in China, 2015. CA Cancer J Clin 66(2):115–132. https://doi.org/10.3322/caac.21338
5. Zeng H, Zheng R, Guo Y, Zhang S, Zou X, Wang N, Zhang L, Tang J, Chen J, Wei K, Huang S, Wang J, Yu L, Zhao D, Song G, Chen J, Shen Y, Yang X, Gu X, Jin F, Li Q, Li Y, Ge H, Zhu F, Dong J, Guo G, Wu M, Du L, Sun X, He Y, Coleman MP, Baade P, Chen W, Yu XQ (2015) Cancer survival in China, 2003-2005: a population-based study. Int J Cancer 136(8):1921–1930. https://doi.org/10.1002/ijc.29227
6. Ronellenfitsch U, Schwarzbach M, Hofheinz R, Kienle P, Kieser M, Slanger TE, Burmeister B, Kelsen D, Niedzwiecki D, Schuhmacher C, Urba S, van de Velde C, Walsh TN, Ychou M, Jensen K (2013) Preoperative chemo (radio) therapy versus primary surgery for gastroesophageal adenocarcinoma: systematic review with meta-analysis combining individual patient and aggregate data. Eur J Cancer 49(15):3149–3158. https://doi.org/10.1016/j.ejca.2013.05.029
7. Alsina M, Miquel JM, Diez M, Castro S, Tabernero J (2019) How I treat gastric adenocarcinoma. ESMO Open 4(Suppl 2):e000521. https://doi.org/10.1136/esmoopen-2019-000521
8. Skrypkina I, Tsyba L, Onyshchenko K, Morderer D, Kashparova O, Nikolaienko O, Panasenko G, Vozianov S, Romanenko A, Rynditch A (2016) Concentration and methylation of cell-free DNA from blood plasma as diagnostic markers of renal Cancer. Dis Markers 2016:3693096. https://doi.org/10.1155/2016/3693096
9. Burnham P, Kim MS, Agbor-Enoh S, Luikart H, Valantine HA, Khush KK, De Vlaminck I (2016) Single-stranded DNA library preparation uncovers the origin and diversity of ultrashort cell-free DNA in plasma. Sci Rep 6:27859. https://doi.org/10.1038/srep27859
10. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber B, Alani RM, Antonarakis ES, Azad NS, Bardelli A, Brem H, Cameron JL, Lee CC, Fecher LA, Gallia GL, Gibbs P, Le D, Giuntoli RL, Goggins M, Hogarty MD, Holdhoff M, Hong SM, Jiao Y, Juhl HH, Kim JJ, Siravegna G, Laheru DA, Lauricella C, Lim M, Lipson EJ, Marie SK, Netto GJ, Oliner KS, Olivi A, Olsson L, Riggins GJ, Sartore-Bianchi A, Schmidt K, Shih LM, Oba-Shinjo SM, Siena S, Theodorescu D, Tie J, Harkins TT, Veronese S, Wang TL, Weingart JD, Wolfgang CL, Wood LD, Xing D, Hruban RH, Wu J, Allen PJ, Schmidt CM, Choti MA, Velculescu VE, Kinzler KW, Vogelstein B, Papadopoulos N, Diaz LA Jr (2014) Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 6(224):224ra224. https://doi.org/10.1126/scitranslmed.3007094
11. Siravegna G, Marsoni S, Siena S, Bardelli A (2017) Integrating liquid biopsies into the management of cancer. Nat Rev Clin Oncol 14(9):531–548. https://doi.org/10.1038/nrclinonc.2017.14
12. Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshew T, Piskorz AM, Parkinson C, Chin SF, Kingsbury Z, Wong AS, Marass F, Humphray S, Hadfield J, Bentley D, Chin TM, Brenton JD, Caldas C, Rosenfeld N (2013) Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 497(7447):108–112. https://doi.org/10.1038/nature12065
13. Wu HC, Yang HI, Wang Q, Chen CJ, Santella RM (2017) Plasma DNA methylation marker and hepatocellular carcinoma risk prediction model for the general population. Carcinogenesis 38(10):1021–1028. https://doi.org/10.1093/carcin/bgx078
14. Liao W, Mao Y, Ge P, Yang H, Xu H, Lu X, Sang X, Zhong S (2015) Value of quantitative and qualitative analyses of circulating cell-free DNA as diagnostic tools for hepatocellular carcinoma: a meta-analysis. Medicine (Baltimore) 94(14):e722. https://doi.org/10.1097/MD.0000000000000722
15. Volckmar AL, Sultmann H, Riediger A, Fioretos T, Schirmacher P, Endris V, Stenzinger A, Dietz S (2018) A field guide for cancer diagnostics using cell-free DNA: from principles to practice and

clinical applications. Genes Chromosom Cancer 57(3):123–139. https://doi.org/10.1002/gcc.22517
16. Wei L, Wu W, Han L, Yu W, Du Y (2018) A quantitative analysis of the potential biomarkers of non-small cell lung cancer by circulating cell-free DNA. Oncol Lett 16(4):4353–4360. https://doi.org/10.3892/ol.2018.9198
17. Ponti G, Maccaferri M, Manfredini M, Kaleci S, Mandrioli M, Pellacani G, Ozben T, Depenni R, Bianchi G, Pirola GM, Tomasi A (2018) The value of fluorimetry (Qubit) and spectrophotometry (NanoDrop) in the quantification of cell-free DNA (cfDNA) in malignant melanoma and prostate cancer patients. Clin Chim Acta 479:14–19. https://doi.org/10.1016/j.cca.2018.01.007
18. Lee TH, Montalvo L, Chrebtow V, Busch MP (2001) Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma. Transfusion 41(2):276–282. https://doi.org/10.1046/j.1537-2995.2001.41020276.x
19. Macheret M, Halazonetis TD (2015) DNA replication stress as a hallmark of cancer. Annu Rev Pathol 10:425–448. https://doi.org/10.1146/annurev-pathol-012414-040424
20. Branzei D, Foiani M (2010) Maintaining genome stability at the replication fork. Nat Rev Mol Cell Biol 11(3):208–219. https://doi.org/10.1038/nrm2852
21. Zeman MK, Cimprich KA (2014) Causes and consequences of replication stress. Nat Cell Biol 16(1):2–9. https://doi.org/10.1038/ncb2897
22. Gaillard H, Garcia-Muse T, Aguilera A (2015) Replication stress and cancer. Nat Rev Cancer 15(5):276–289. https://doi.org/10.1038/nrc3916
23. Thierry AR, El Messaoudi S, Gahan PB, Anker P, Stroun M (2016) Origins, structures, and functions of circulating DNA in oncology. Cancer Metastasis Rev 35(3):347–376. https://doi.org/10.1007/s10555-016-9629-x
24. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J (2016) Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin. Cell 164(1–2):57–68. https://doi.org/10.1016/j.cell.2015.11.050
25. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, Knippers R (2001) DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 61(4):1659–1665
26. Zhang X, Wu Z, Shen Q, Li R, Jiang X, Wu J, Li D, Wang D, Zou C, Zhong Y, Cheng X (2019) Clinical significance of cell-free DNA concentration and integrity in serum of gastric cancer patients before and after surgery. Cell Mol Biol (Noisy-le-grand) 65(7):111–117
27. Park JL, Kim HJ, Choi BY, Lee HC, Jang HR, Song KS, Noh SM, Kim SY, Han DS, Kim YS (2012) Quantitative analysis of cell-free DNA in the plasma of gastric cancer patients. Oncol Lett 3(4):921–926. https://doi.org/10.3892/ol.2012.592
28. You Y, Teng W, Wang J, Ma G, Ma A, Wang J, Liu P (2018) Hypertension and physical activity in middle-aged and older adults in China. Sci Rep 8(1):16098. https://doi.org/10.1038/s41598-018-34617-y
29. Steevens J, Schouten LJ, Goldbohm RA, van den Brandt PA (2010) Alcohol consumption, cigarette smoking and risk of subtypes of oesophageal and gastric cancer: a prospective cohort study. Gut 59(1):39–48. https://doi.org/10.1136/gut.2009.191080
30. Sorber L, Zwaenepoel K, Deschoolmeester V, Roeyen G, Lardon F, Rolfo C, Pauwels P (2017) A comparison of cell-free DNA isolation kits: isolation and quantification of cell-free DNA in plasma. J Mol Diagn 19(1):162–168. https://doi.org/10.1016/j.jmoldx.2016.09.009
31. Sato A, Nakashima C, Abe T, Kato J, Hirai M, Nakamura T, Komiya K, Kimura S, Sueoka E, Sueoka-Aragane N (2018) Investigation of appropriate pre-analytical procedure for circulating free DNA from liquid biopsy. Oncotarget 9(61):31904–31914. https://doi.org/10.18632/oncotarget.25881
32. Lippi G, Salvagno GL, Montagnana M, Brocco G, Guidi GC (2006) Influence of hemolysis on routine clinical chemistry testing. Clin Chem Lab Med 44(3):311–316. https://doi.org/10.1515/CCLM.2006.054
33. Chang Y, Tolani B, Nie X, Zhi X, Hu M, He B (2017) Review of the clinical applications and technological advances of circulating tumor DNA in cancer monitoring. Ther Clin Risk Manag 13:1363–1374. https://doi.org/10.2147/TCRM.S141991
34. Mathurin P, Cadranel JF, Bouraya D, Bronstein JA, Collot G, Devergie B, Poynard T, Opolon P (1996) Marked increase in serum CA 19-9 level in patients with alcoholic cirrhosis: report of four cases. Eur J Gastroenterol Hepatol 8(11):1129–1131. https://doi.org/10.1097/00042737-199611000-00019
35. Shang X, Song C, Du X, Shao H, Xu D, Wang X (2017) The serum levels of tumor marker CA19-9, CEA, CA72-4, and NSE in type 2 diabetes without malignancy and the relations to the metabolic control. Saudi Med J 38(2):204–208. https://doi.org/10.15537/smj.2017.2.15649
36. Zali H, Rezaei-Tavirani M, Azodi M (2011) Gastric cancer: prevention, risk factors and treatment. Gastroenterol Hepatol Bed Bench 4(4):175–185
37. Swets JA (1988) Measuring the accuracy of diagnostic systems. Science 240(4857):1285–1293. https://doi.org/10.1126/science.3287615