

# Identification of circulating tumor DNA using a targeted 545-gene next generation sequencing panel in patients with gastric cancer

JING LAN<sup>1</sup>, YAPING LU<sup>2</sup>, YANFANG GUAN<sup>2</sup>, LIANPENG CHANG<sup>2</sup>, ZHENGYUAN YU<sup>3</sup> and HAIXIN QIAN<sup>1</sup>

<sup>1</sup>Department of General Surgery, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006;

<sup>2</sup>Research and Development Department, Geneplus-Beijing Institute, Beijing 102206; <sup>3</sup>Department of Oncology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006, P.R. China

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**Abstract.** Gastric cancer (GC) is characterized by unique genetic aberrations. Some of these mutations may be used to predict tumor prognosis or to guide patient therapy. Cell-free circulating tumor DNA (ctDNA) has been considered a promising alternative to biopsy to identify genome aberrations. However, no standardized methods to detect ctDNA variations in patients with GC are currently available. In the present study, the targeted sequencing of 545 genes was used to identify somatic alterations in tissues and matched plasma samples of nine patients with GC. Driver gene mutations were detected in matched tissues and plasma ctDNA. The mutated reads concordance rate of ctDNA in GC tissues with matched tissues was 45%. A true positive copy number gain of human epidermal growth factor receptor 2 in plasma from patients with GC was identified. Furthermore, the ctDNA fraction in plasma cell-free DNA (cfDNA) was positively correlated with metastasis lymph node number and with lactate dehydrogenase level. In conclusion, results from the present study suggested that targeted sequencing of plasma ctDNA may be considered a potential option for the clinical monitoring of GC.

## Introduction

Gastric cancer (GC) is a highly heterogeneous malignant disease characterized by a unique pattern of genome driver aberrations. Some of these aberrations are used to predict

development of the disease or guide therapy (1-3). For example, overexpression of human epidermal growth factor receptor 2 (*HER2*) is detected in GC and can be considered a novel therapeutic agent (4-6). However, the genome aberration profile can change throughout the course of therapy, and many patients with GC develop acquired drug resistance along with tumor evolution (7,8). Detecting variations prior to and during therapy is therefore crucial to improve patient outcome. However, repeated invasive tissue biopsies of GC are not feasible due to the clinical risk of tumor spread. Cell-free circulating tumor DNA (ctDNA) has attracted increasing attention and may be considered a potential tumor marker. In addition, its detection is convenient and non invasive (9). Analysis of ctDNA presents therefore a potentially clinical prospect in the treatment and auxiliary diagnosis of GC.

Numerous approaches, including the BEAMing (beads, emulsion, amplification, and magnetics) method, the Scorpion ARMS method that detects epidermal growth factor receptor aberration and the droplet digital polymerase chain reaction method that detect *HER2* amplification, have been successfully used to identify ctDNA aberrations in patients with various types of cancer (10-15). Furthermore, a previous study using next generation sequencing (NGS) to detect ctDNA in the bloodstream of patients with GC has identified concordant variations between ctDNA and tumor DNA (tDNA); however, this study only primarily focused on a small cohort of genes, including tumor protein p53 (*TP53*) (16). However, due to the high heterogeneity of GC, numerous genes may be involved and available for analysis. To explore the association between ctDNA and the clinical characteristics of patients with GC, the present study used a targeted capture sequencing method to detect variations at known hot-spot loci of 545 cancer-associated genes in tumor and plasmatic ctDNA from nine patients with GC.

## Materials and methods

**Patients and samples.** The present study was approved by the Ethics Committees of the First Affiliated Hospital of Soochow University. All patients provided written informed consent for the use of their blood and tumor samples. Nine patients diagnosed with advanced GC and received surgery or palliative surgery were involved in this study. Tumor staging was

*Correspondence to:* Dr Zhengyuan Yu, Department of Oncology, The First Affiliated Hospital of Soochow University, 899 Pinghai Road, Suzhou, Jiangsu 215006, P.R. China  
E-mail: strongeryy1985@163.com

Professor Haixin Qian, Department of General Surgery, The First Affiliated Hospital of Soochow University, 899 Pinghai Road, Suzhou, Jiangsu 215006, P.R. China  
E-mail: qianhaixinjiangsu@163.com

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performed according to the 7th American Joint Committee on Cancer (AJCC) TNM system (17). All samples and medical data used in this study have been irreversibly anonymized. Gastric tumor and plasma samples from nine patients with GC were analyzed (Table I). All 9 patients, including six men and three women, were diagnosed with adenocarcinoma. Smoking history was not assessed. Tumor tissues obtained from biopsies taken at diagnosis or during surgery were fixed in formalin at room temperature for 6–48 h, then embedded in paraffin as previously described (18). HER2 Immunohistochemistry (IHC) was carried out on formalin-fixed, 5- $\mu$ m thick, paraffin-embedded (FFPE) tissue sections (Ventana; Roche) using a pre-diluted antibody (ready to use) of monoclonal rabbit PATHWAY anti-HER2 (4B5; Bench Mark GX; Roche Diagnostics K.K.). Briefly, the FFPE sections were deparaffinized. After cell conditioning, it was incubated with primary monoclonal rabbit PATHWAY anti-HER2 at 37°C for 30 min. Counterstaining was performed by incubation with hematoxylin at room temperature for 8 min, followed by incubation with building reagent for 12 min. Staining was scored as follows: 0, no membrane staining or no reactivity; +1, cancer cell cluster with a barely/faint perceptible membranous reactivity; +2, tumor cell cluster with a weak to moderate complete, basolateral, or lateral membranous reactivity; +3, tumor cell cluster with a strong complete, basolateral, or lateral membranous reactivity. Tissues with a score of +3, or +2 in addition to fluorescence in situ hybridization (FISH) positivity, were considered as HER2 positive. Peripheral blood samples were collected from patients one week prior to surgery.

**Sample processing and DNA extraction.** Two types of samples were collected from each patient, tumor tissue (fresh and FFPE) and 20 ml peripheral blood (PB) prior to surgery. DNA was extracted from fresh tissue using E.Z.N.A. Tissue DNA kit (Omega Bio-Tek), and from FFPE tissue using QIAamp DNA FFPE Tissue kit (Qiagen) according to the manufacturer's instructions. EDTA tubes containing blood samples were centrifuged for 10 min at 1,000 x g at 4°C. Cell layer containing peripheral blood lymphocytes (PBLs) was collected and transferred into 1-ml Eppendorf tubes and stored at -20°C until further use. Supernatants were further centrifuged at 10,000 x g at 4°C for 10 min and plasma was collected and stored at -80°C until further use. DNA from PBLs was extracted using the E.Z.N.A. Blood DNA kit (Omega Bio-Tek), whereas ctDNA was extracted from at least 1 ml plasma using QIAamp Circulating Nucleic Acid kit (Qiagen) following the manufacturer's instructions. DNA was quantified with the Qubit 2.0 Fluorometer and the Qubit dsDNA HS Assay kit (Life Technologies; Thermo Fisher Scientific, Inc.) according to the recommended protocols.

**Sequencing library construction and target enrichment.** DNA (1  $\mu$ g) from tissue and PBLs was cropped into 300-bp fragments with a Covaris S2 ultrasonicator as previously described (19). Libraries of DNA from tissue, PBLs germline and circulating DNA were prepared with the KAPA Library Preparation kit (Kapa Biosystems) according to the manufacturer's protocol. A custom SeqCap EZ Library (Roche NimbleGen, Inc.) was designed for targeted capture. To explore the comprehensive genetic properties of GC, the capture probe was designed

Table I. Clinicopathological characteristics of the nine patients with gastric cancer.

Characteristics	Number (%)
Age (years)	
Mean (SD)	62.89 (9.27)
Median (range)	64 (46–77)
Sex	
Male	3 (33.33%)
Female	6 (66.67%)
Pathological diagnosis	
Gastric adenocarcinoma	9 (100%)
Tumor stage	
II	3 (33.33%)
III	5 (55.56%)
IV	1 (11.11%)

SD, standard deviation.

according to genomic regions (total approximately 1.7 Mb in size, data not shown) of the 545 genes most frequently mutated in gastric tumor and other common solid tumors. Capture hybridization was carried out according to the manufacturer's protocol.

**NGS sequencing.** Sequencing was carried out using Illumina 2x100 bp paired-end reads on an Illumina HiSeq 3000 instrument according to the manufacturer's recommendations and using TruSeq PE Cluster Generation Kit v3 and the TruSeq SBS Kit v3 (Illumina, Inc.).

**Analysis of sequencing data.** After removal of terminal adaptor sequences and low-quality data, reads were mapped to the reference human genome and aligned as previously described (19). The Genome Analysis Toolkit (<https://www.broadinstitute.org/gatk/>) and MuTect (20) were used to call somatic small insertions and deletions and single nucleotide variants by filtering PBL germline mutations. The following somatic mutations obtained were further filtered as follows: i) All mutations from tissues and plasma should present  $\geq 5$  and  $\geq 2$  mutated reads, respectively; ii) the frequency of mutations in tissue should be  $\geq 3\%$ ; and iii) mutated reads of each mutations should be observed on both strands. Copy number variations (CNV) were generated using Copy Number Targeted Resequencing Analysis (<http://contra-cnv.sourceforge.net>; version 2.0.3) (21). BreakDancer algorithm was used to detect tumor-associated structure variations (22). The final candidate variants were manually verified with the Integrative Genomics Viewer (IGV) browser (<https://software.broadinstitute.org/software/igv>) (23). COSMIC database (<https://cancer.sanger.ac.uk/cosmic>) was used to determine the occurrence of variants.

**Statistical analysis.** Pearson's correlation and one-way ANOVA followed by Bonferroni correction post-hoc test were performed using SPSS software (version 16.0; SPSS, Inc.) to

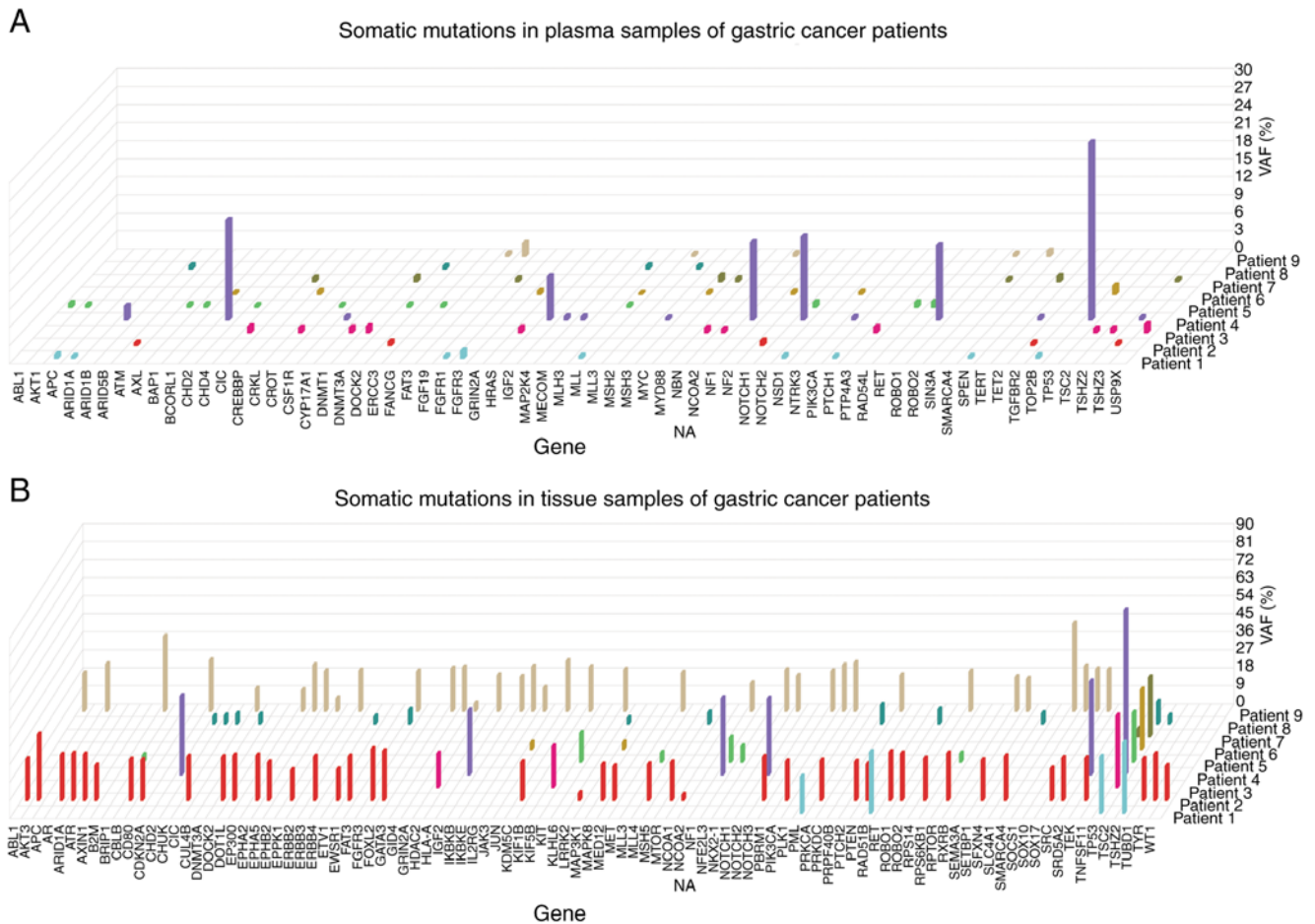


Figure 1. Somatic mutations in (A) tissues and (B) plasma samples from nine patients with gastric cancer. VAF, varied allele frequency.

analyze the correlation between ctDNA fraction and clinical characteristics, including metastasis lymph node number and lactate dehydrogenase (LDH) content, as previously described (24).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Sequencing coverage of the target region.** Of all nine paired samples, capture sequencing data demonstrated a mean coverage of 904x in tissues (ranging from 275x to 1,255x; data not shown) and of 1,375x in plasmas (ranging from 965x to 2,203x; data not shown). Furthermore, approximately 99% of the target region was covered at  $>20x$ . For each sample type, the gene coverage was uniformly distributed, with  $>167x$  in 92.73% of tissue genes and  $>500x$  in 87.71% of plasma genes (data not shown). In this case, mutations of most genes in both sample types could possess at least 5 support reads at a frequency of approximately 1% in plasma or approximately 3% in tissue.

**Somatic mutations in tissue and plasma samples.** Somatic mutations were detected in all tissues and matched plasma samples (100%). The number of non-synonymous somatic mutations detected in tissues ranged from 2 to 46, with a mean value of 16. The mean variant allele fraction (VAF) was 18.85%. In plasma, a total of 80 non-synonymous somatic

mutations were detected, with a mean VAF of 1.90%. Among all mutations, 32 mutations in tissues and 17 mutations in plasma were confirmed in COSMIC database.

Mutation spectrums of the 9 GC tissues revealed great inter-individual tumor genetic heterogeneity (Fig. 1). Notably, seven patients (78%) presented *TP53* gene mutations, which occurred at six different amino acid positions (p.T211Nfs\*5, p.C176F, p.P190L, p.R213\*, p.E271V and p.G245S). However, the structure variations were not detected in tissues and plasma samples.

**Mutation concordance between tissue and plasma.** In all detected non-synonymous somatic mutations, capture sequencing identified a total of eight concordant mutations in both tissue and plasma samples in four of the nine patients with GC (44%). Notably, in patient 4, who was the only patient diagnosed with distant metastasis, five out of six tumor-derived mutations were found in plasma ctDNA. In addition, the results from further analysis of plasma samples sequencing data demonstrated that 45% of mutation in tissue presented concordant mutation in the plasma ctDNA of all patients (Fig. 2).

**CNV amplification of *HER2* in FFPE and plasma samples.** Prior to sequencing, immunohistochemistry (IHC) was performed on GC FFPE to assess *HER2* expression. The results demonstrated that only two patients (22.22%) expressed *HER2* (Fig. 3). Based on capture sequencing, CNV of *HER2*

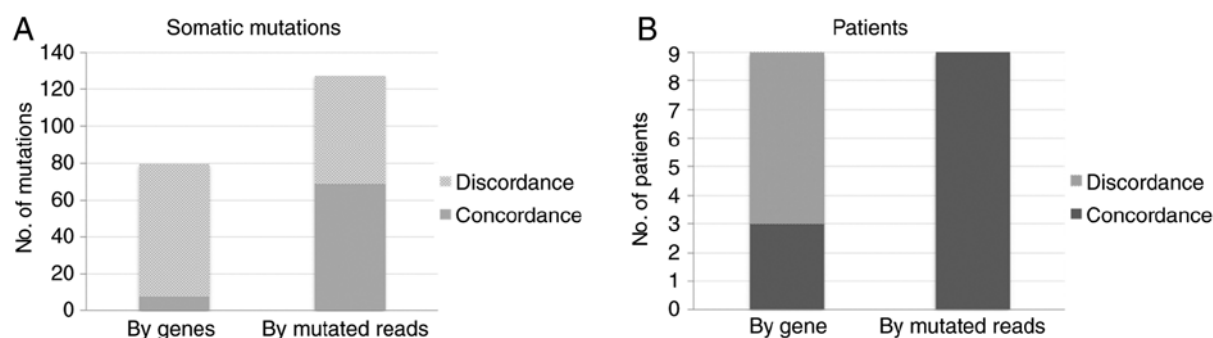


Figure 2. Concordant mutated (A) gene and (B) patients calculated by genes or mutated reads. No, number.

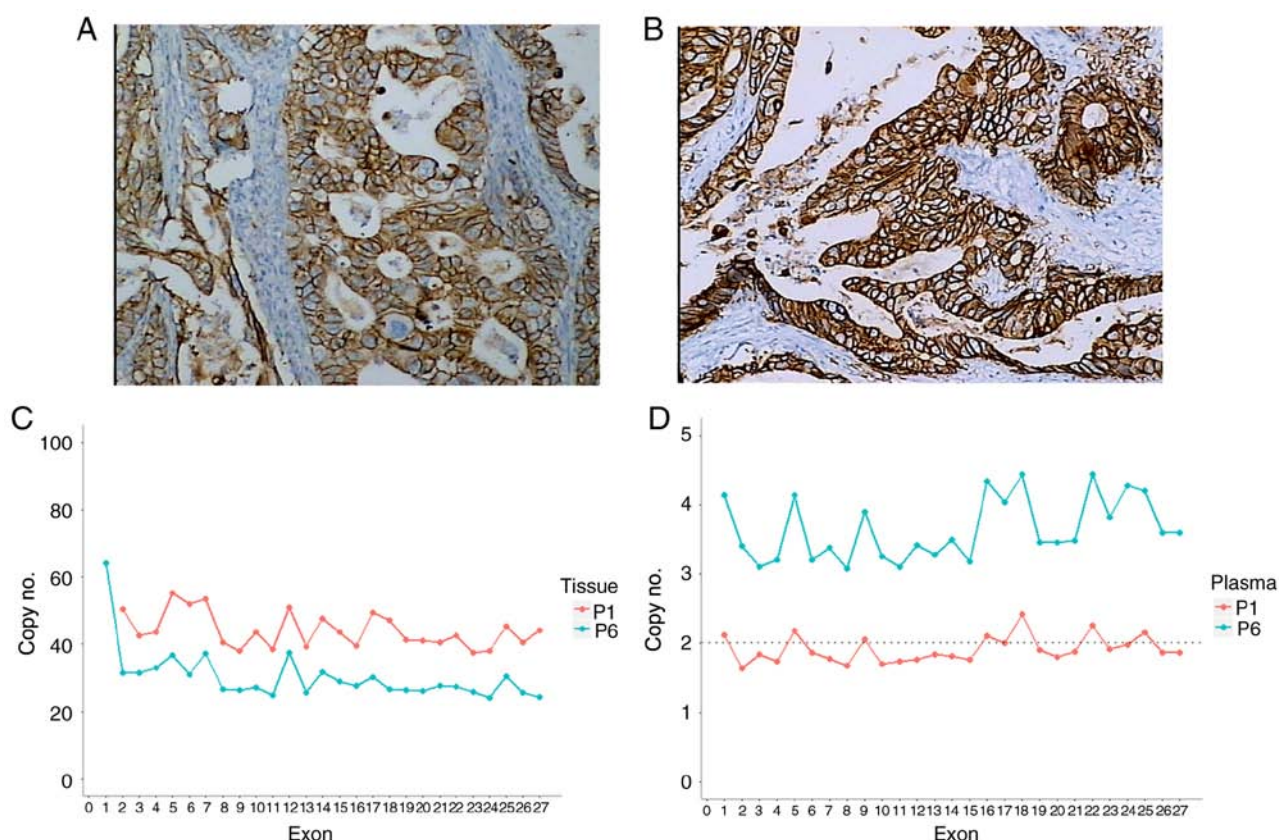


Figure 3. (A and B) Copy number variations of epidermal growth factor receptor 2 detected by immunohistochemistry and (C and D) capture sequencing. No, number; P1, patient 1; P6, patient 6.

was analyzed in tissue and matched plasma by comparing reads depth with PBL. Significant copy number gains of *HER2* in tissue samples was detected in these two patients (22.22%) [patient no. 1 (P1), copy no.=46.2,  $P<0.01$ ; patient no. 6 (P6), copy no.=30.3,  $P<0.01$ ]. Other CNV negative results were in accordance with IHC assess (25). Furthermore, only P6 presented a significant *HER2* gene amplification in plasma ctDNA ( $P<0.01$ ), and the fold-change of copy no. was only 3.6. In addition, analysis of plasma ctDNA from P1 demonstrated relative depth of all *HER2* exons that fluctuated around 2.

**Correlation between ctDNA fraction and clinical characteristics of patients with GC.** The correlation between ctDNA fraction and clinical characteristics of patients with GC was analyzed. Based on the number of metastasis lymph nodes,

patients were divided into two groups, a low metastasis lymph node (LMLN) group including N1 and N2 patients and a high metastasis lymph node (HMLN) group including N3 patients. The mean of ctDNA fraction in HMLN group was significantly higher than in LMLN group ( $P=0.03$ ; Fig. 4). In addition, the ctDNA fraction and the LDH level were positively correlated in all groups ( $r=0.85$ ;  $P=0.003$ ; Fig. 5).

## Discussion

Targeted capture sequencing is an economical and effective method used to explore the genomic characteristic (26-28). By using capture sequencing of 545 genes at a mean depth of 904x in tissues and 1375x in plasma, the present study reported numerous inter-individual molecular differences among



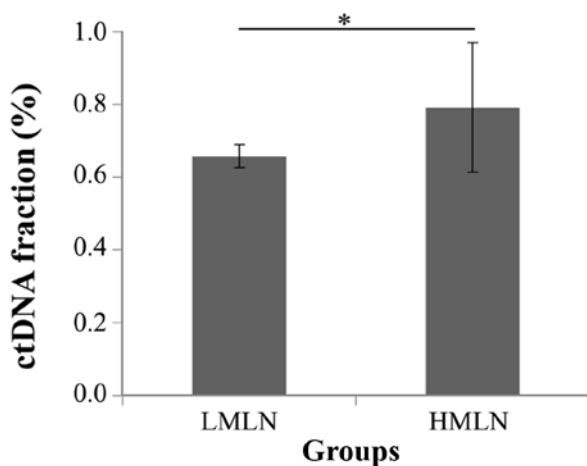


Figure 4. The mean of ctDNA fraction in LMLN group and HMLN groups. Values are expressed as the means  $\pm$  standard deviation of four separate experiments. \* $P < 0.05$ . ctDNA, cell-free circulating tumor DNA; HMLN, high metastasis lymph node; LMLN, low metastasis lymph node.

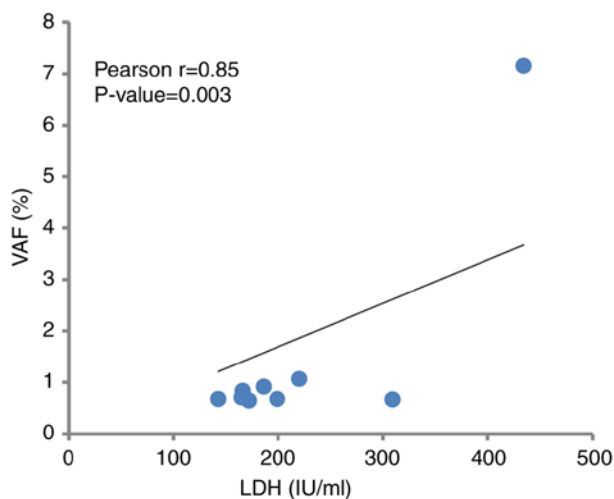


Figure 5. Pearson's correlation analysis between ctDNA fraction and LDH level. ctDNA, cell-free circulating tumor DNA; IU, international unit; LDH, lactate dehydrogenase.

patients with GC. Mutations frequently occurred on *TP53* gene and occurred at six different amino acid positions, which suggested that this PCR-based method could only be applied in a limited number of patients with hot-spot mutations. However, capture sequencing, whole exome sequencing or whole genome sequencing may be more suitable to identify cancer mutations, and would decrease the cost of sequencing (29).

The present study detected the mutation in plasma samples of patients with GC in a non-invasive way. The results demonstrate that 45% mutations in paired GC tissues presented concordant mutated reads in plasma samples from all 9 patients. Furthermore, additional *de novo* mutations in the DNA in the plasma can be induced by spatial heterogeneity of the lesion (30). A previous study reported that, in a case of metastatic breast cancer, multiregional tumor biopsies vary from each other, and that ctDNA present the mutations of both primary tumor and metastases (31). Similarly, a study revealed that ctDNA contains variations from heterogeneous regions in the primary lung

cancer lesion (32). Notably, in the only stage IV patient (P6) with distant metastasis from the present study, the consistency of mutations in plasma and tissue was of 83%, which may be due to the high ctDNA level of patients with distant metastasis (33). This result indicated that the non-invasive ctDNA detection may offer more benefit in late-stage patients.

One crucial purpose of molecular diagnosis in patients with cancer is to determine sensitive drug targets (34), including *HER2*, which could be specifically bound by herceptin, which is a monoclonal antibody used in anticancer therapy (35). The present study identified two true-positive *HER2* CNV in patients. Regarding plasma samples, despite the high dilution of cfDNA, the true positive *HER2* gain was detected in one case, which suggested that non-invasive ctDNA analysis in CNV is a viable method to determine target drugs for patients with GC. However, it is crucial to improve the sensitivity of ctDNA CNV detection.

The correlation between ctDNA fraction and clinical characteristics from patients with GC was determined. The results demonstrated that ctDNA fraction was abundant in patients with more metastasis lymph nodes. This result suggested that metastasis ability of tumor may be associated with ctDNA fraction in plasma. In addition, this result further explained the high ctDNA level observed in one case of stage IV GC (P6), which caused the high consistency of mutations between ctDNA and tDNA. Future studies should involve the detection of more clinical serum biomarker, including carcinoembryonic antigen, CA19-9 and *HER2* expression level (36,37). Detection of ctDNA as a biomarker has been considered a sensitive and specific method in the prognosis and monitoring of breast and colorectal cancers (38,39). However, ctDNA were not monitored for disease progression or remission, following surgery, and were not investigated following chemotherapy. This was an inevitable limitation of the present study. Since such investigation has not been made in GC, future study will involve ctDNA monitoring following treatment in GC. The results from the present study need to be further confirmed in a larger patient population. This could provide important findings on the use of ctDNA in GC. As a promising tool, the noninvasive detection of ctDNA may represent a promising tool in the individual treatment and monitoring of patients with GC.

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## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

## Authors' contributions

ZY and HQ designed the study. JL and ZY collected samples and clinical data. JL, YL, YG and LC performed the analysis

and interpretation of the data. JL, YL, YG and LC wrote the manuscript. All authors contributed to the drafting and revision of the manuscript.

### Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University. All patients provided written informed consent prior to the study start.

### Patients consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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