Advantages and Limitations of Monitoring Circulating Tumor DNA Levels to Predict the Prognosis of Patients Diagnosed With Gastric Cancer

Wan He1*, Jingxin Yang2*, Xiao Sun3*, Shunda Jiang1, Jinchan Jiang1, Ming Liu4, Tianhao Mu4, Yingmei Li4, Xiaoni Zhang4, Jingxian Duan4,5 and Ruilian Xu1

1Department of Oncology, Shenzhen People’s Hospital, Shenzhen, China, 2Center of Medical Genetics, Shenzhen Maternity & Child Healthcare Hospital, Shenzhen, China, 3Department of Gastrointestinal Surgery, Shenzhen People’s Hospital, Shenzhen, China, 4HaploX Biotechnology, Co., Ltd., Shenzhen, China, 5Department of Biomedical and Health Engineering, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China.

ABSTRACT: Next-generation sequencing-based genomic profiling facilitates biomarker detection by cell-free DNA (cfDNA) liquid biopsy. However, the efficiency of mutation calling and the prognostic value of cfDNA biomarkers are disputed. We investigated 24 patients with gastric cancer in this study, using a 605-gene sequencing panel to sequence their plasma cfDNA and tumor tissue DNA. The mutation concordance between plasma cfDNA and tumor tissue DNA was 70.6% in stage IV gastric cancer and 30.2% in stage III gastric cancer, indicating insufficient mutation detection rates in stage III and early-stage cancer. When compared with total cfDNA load and blood tumor mutation burden (bTMB), the variant allele frequencies (VAF) of commonly mutated genes are highly accurate in representing disease burden. Further, VAF are a better prognostic indicator compared with serum biomarkers including carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), cancer antigen 125 (CA125), and alpha-fetoprotein (AFP). The use of cfDNA in molecular profiling of patients allows prediction of patient survival and clinical response, as well as the development of personalized therapy regimens.

KEYWORDS: Gastroenterology, cfDNA, molecular characterization, prognosis, surveillance

Key Summary
- The vast difference in cfDNA mutation detection efficiency between stages III and IV of gastric cancer shows that the mutation spectrum of stage I-III cancer revealed by cfDNA is not representative under the current sequencing settings.
- The cfDNA level is a more sensitive prognostic indicator compared with TMB, and the prognostic value of TMB depends largely on the VAF of cfDNA.
- The cfDNA VAF of frequently mutated genes serves as a good prognostic indicator, and is superior to serum biomarkers.

Introduction
Gastric cancer is a public health threat in China, ranking third in terms of incidence and mortality, with 47.9 new cases and 37.4 new deaths per 10,000 people.2 The prevailing risk factors for gastric cancer include Helicobacter pylori infection, alcohol intake, obesity, smoking, and low socioeconomic status.3 The pathology frequently involves modification of patients’ genomic landscape.

Liquid biopsy uses body fluid samples to decipher tumor genetics. It is a less invasive approach compared with needle biopsy or surgery, and reduces the risk of metastasis caused by punctures. However, it is not clear whether the cancer biomarkers identified by tissue sample sequencing can be applied directly to liquid biopsy. The mutation consistency between tissue samples and plasma samples ranged from 69% to 94% depending on the target sequencing settings.4-7 Several studies have reported that cell-free DNA (cfDNA) levels can be used to monitor tumor recurrence in various cancer types.8,9 The aim of the study is to investigate the efficiency and reliability of cfDNA in monitoring disease progression, and validate the prognostic value of cfDNA by comparing it with other prognostic indicators.
Methods

Patients and samples

From June 2017 to March 2021, 24 patients with histologically proven stomach cancer were included in this study at Shenzhen People's Hospital (Shenzhen, China). The study was approved by the Ethics Committee of Shenzhen People's Hospital (Shenzhen, China) under the approval number SYLLS201522. All patients provided written informed consent before enrollment. In this retrospective clinical study, 22 formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples and 39 whole peripheral blood (10 mL) samples were obtained. Serum carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), cancer antigen 125 (CA125), and alpha-fetoprotein (AFP) levels were measured at multiple time points via immunostaining. The clinical responses of the patients were evaluated according to the RECIST 1.1 guideline. We collected preoperative or pretreatment whole blood samples from 7 patients who received standard or adjuvant therapy. The samples were collected before and at the end of a regime.

DNA extraction and quantification

Aliquots of 10-20 mL whole blood samples were collected from each patient. The debris and plasma in peripheral blood lymphocytes (PBL) were separately harvested and stored. Genomic DNA (gDNA) from PBLs was extracted using the RelaxGene Blood DNA System (TianGen Biotech Co., Ltd., China). Cell-free DNA (cfDNA) was extracted from at least 2 mL plasma using the QIAamp Circulating Nucleic Acid kit (QIAGEN) following the manufacturer’s instructions. Tumor tissues measuring $0.5 \text{ cm} \times 0.5 \text{ cm}$ were collected following surgical resection. Tumor DNA was extracted using the QIAamp DNA FFPE tissue kit (Qiagen) following the manufacturer’s instructions.

Library construction

FFPE tumor tissue DNA and genomic DNA from PBLs were first sheared by the enzyme NEBNext dsDNA Fragmentase. The size selection of the DNA fragments (150-250 bp) was performed using Ampure XP beads (Beckman Coulter, Inc., Brea, CA, USA). The KAPA Library Preparation kit (Kapa Biosystems, Inc., Wilmington, MA, USA) was used for library construction, according to the manufacturer’s protocol.

Targeted capture and sequencing

Targeted capture was performed using a custom set of biotinylated DNA probes containing all the exon and some intron regions of 468 cancer-related genes, and SNP loci of 137 treatment-related genes, encompassing 1.31 Mb of sequencing panel (Roche NimbleGen). The libraries were sequenced using 150 bp paired-end runs on the Illumina NovaSeq 6000 system (Illumina).

Raw data processing and alignment

Raw sequencing data were pre-processed by fastp v0.12.6 version 0.18.0, followed by sliding window trimming. Clean reads were aligned to the hg19 genome (GRCh37) using Burrows Wheeler Aligner v0.7.15 r1140 under default settings. The Gencore v0.12.0 was used to remove duplicate reads. Samtools v0.1.19 was applied to generate pileup files for properly paired reads with mapping quality $\geq 60$.

Mutation calling, filtering and annotation

Single nucleotide variants (SNVs) and short insertions/deletions (indels) were identified by VarScan2 v2.3.8. The average sequencing depth after deduplication was around 1000 (all samples $\geq 750 \times$) for tumor tissue DNA and 2500 (all samples $\geq 1500 \times$) for cfDNA. The variant allele frequency (VAF) threshold was set to 0.1% in cfDNA and 2% in FFPE tumor tissue DNA. The cutoff values were selected based on previous publications. All SNVs/indels were annotated using ANNOVAR v2018-04-16. CNVkit v0.9.3 was used for detection of copy number variation (CNV). The blood tumor mutational burden (bTMB) was estimated by cfDNA based on the total number of SNVs and indels with greater than 0.5% VAF per million bases. Visualizations of groups of mutations were carried out in R using the package maftools.

Statistical analysis

Statistical analysis as shown in Figures 1, 5 and 6 was performed using GraphPad Prism v8.0. Normality tests, F test, and $t$-test were conducted as described in Figure captions. Statistical significance was evaluated as described by the figure captions. $*** P < .001$, $** P < .01$, * $P < .05$, ns = not significant (P > .05). Numerical data in this study were presented as mean ± S.E.M. The mutation concordance rate was calculated as the number of shared mutations divided by the sum of mutations identified in plasma samples and tumor tissue samples.

The statistical power and sample size were calculated with the software G*Power 3.2. Based on our study design, we set a 2-tailed $t$-test with 80% power and the type I error probability ($\alpha$) of .05. The calculated total sample size was 26 with the actual power of 0.82. To adjust the sample size, we revisited published studies with a comparable study design. Our sample sizes are similar to those reported in previous publications.

Results

The mutation concordance between cfDNA and tumor tissue DNA was low in stage III cancer

Twenty-four patients who were diagnosed with gastric adenocarcinoma were enrolled in this study at Shenzhen People's
Paired plasma samples and fresh tissue samples were collected at first diagnosis. Follow-up plasma samples were collected at the beginning and the end of each course of treatment. The mean age of the patients was 54.33 ± 2.95 (28–75), and 50% (12/24) of them were male (Table 1). One (4.2%, n = 24) stage I patient, 9 (37.5%, n = 24) stage II patients, 11 (45.8%, n = 24) stage III patients, and 3 (12.5%, n = 24) stage IV patients were enrolled in this study.

Figure 1. Mutation landscape of DNA extracted from tumor tissue and peripheral blood samples: (A) OncoPrint showing all mutations and copy number variations identified in tumor tissue samples of all patients. Mutation type and copy number gain or loss were color coded. (B) OncoPrint showing cfDNA mutations in patients with stage IV cancer, (C) as in (B), but involving patients with stage III cancer, (D) percentage of total mutations detected in plasma samples, tumor tissue samples, or both are shown in the boxplot, with red denoting stage IV cancer and blue representing stage III cancer. Statistical comparison was conducted by Mann-Whitney test, *denotes P < .05, **indicates P < .01, data expressed as mean ± S.E.M., and (E) as in (D), but showing the percentage of mutations that were solely identified in tumor tissue samples or plasma samples.
stage III patients, and 14 (58.3%, \( n = 24 \)) stage IV patients were enrolled. One stage III patient and one stage IV patient harbored high microsatellite instability (MSI), while the others were stable microsatellites. The mean overall survival of the patients was 15.60 ± 2.10 (1.37-41.574) months.

To compare the mutation concordance between cfDNA and tumor tissue DNA (tDNA), we collected preoperative or pretreated whole blood samples and corresponding tumor tissue samples. The frequent mutations of cfDNA and tDNA are displayed separately in Figure 1. The frequently mutated genes detected in tDNA were TP53 (64%), ARID1A (23%), and SYNE1 (23%, Figure 1A, Supplemental Table 1). In terms of CNV, most of the genes exhibited copy number amplification, including BRCA1/2, CCND1/3, CDKN1/2A, FGFR3/4, and FGFR2/3 (Supplemental Figure 1). Copy number loss was only observed in 2 patients (FGFR3 and CDKN2A), while no structural variants were identified.

The mutation spectrum of cfDNA captured from stage IV cancer patients showed high concordance with that of paired tDNA (Figure 1B). In contrast, the cfDNA in stage I & III cancer exhibited a different mutation spectrum (Figure 1C). Unique mutations were found in both tissue and plasma samples. The tDNA of patients with stage IV and III cancer carried 86.8% ± 7.8%, and 95.2% ± 4.8% of total mutations, respectively. However, only 32.6% ± 12.1% of total mutations were identified in the cfDNA of patients with stage III cancer, which is significantly lower than the mutation detection rate of cfDNA in patients with stage IV cancer (83.8% ± 8.1%, \( n = 21 \), \( P < .001 \), Figure 1D). The mutation concordance between matched cfDNA and tDNA was evidently higher in stage IV cancer than in stage III cancer (70.6% ± 10.3% vs 30.2% ± 11.7%, \( n = 21 \), \( P < .05 \)), suggesting the need for caution using cfDNA to guide clinical procedures for the management of stage III and earlier-stage cancer.

In stage III cancer, 65.5% ± 13.2% of mutations were solely detected in tDNA, while only 4.3% ± 4.3% of mutations were exclusively found in cfDNA. This tendency was reversed in stage IV cancer, with only 16.1% ± 8.0% of mutations identified in tDNA, whereas 13.3% ± 7.8% of mutations identified solely in cfDNA (Figure 1E). The low mutation concordance in patients with stage III cancer may be attributed to the low VAF of cfDNA. The mean VAF cfDNA of stage III cancer samples was 0.6 ± 0.2 (ranging from 0.1 to 41.4), significantly lower than in stage IV cancer samples (4.5 ± 0.9, ranging from 0.15 to 21.16, \( P < .0001 \), \( n = 21 \)). Accordingly, the cfDNA more accurately reflects the molecular profile of late-stage cancers that release a sufficient amount of ctDNA into the circulation system.

Disease burden can be assessed by cfDNA level, tumor mutation burden, and maximum variant allele frequency

To evaluate the prognostic value of cfDNA in gastric cancer, we analyzed the mutation spectrum and VAFs of cfDNA in patients providing plasma samples at the beginning and the end of each regimen. The cfDNA level, tumor mutation burden (TMB), and maximum VAFs were compared to identify the parameter that accurately reflected the disease burden. Among the 7 patients who were monitored for 6-23 months, 4 showed consistent fluctuation in cfDNA level and TMB (Figure 2). P13 showed a decrease in cfDNA level and TMB (Figure 2A), while
P5 and P18 experienced a relapse of disease after the initial decrease (Figure 2B and C). P11, however, exhibited an increase in disease burden along with the treatment (Figure 2D). However, the cfDNA level and TMB were inconsistent in 3 patients (Figure 3A–C), with 2 cases reporting the TMB below the detection threshold (Figure 3A and C) and 1 case showing inverted trends of cfDNA level and TMB (Figure 3B).

The 2 groups of patients were not different in terms of staging, lymph node metastasis, overall survival (OS), molecular subtypes, and treatment regimens. One factor that may contribute to the inconsistency between cfDNA load and TMB is the maximum VAF. The maximum VAF of the cfDNA-TMB consistent group was 6.8 ± 4.8, far exceeding the maximum VAF of the cfDNA-TMB inconsistent group (1.4 ± 1.3, Figure 3C).
To determine whether the difference in mutation detection rates was caused by varied tumor shedding, we compared the cfDNA concentration between stage III and IV gastric cancers. The mean cfDNA concentration of stage III was $26.74 \pm 6.03$ ng/mL, which was not significantly different from the cfDNA concentration in stage IV ($20.28 \pm 3.47$ ng/mL, $n=22$, $P=.36$, Figure 3E), demonstrating that the low technical sensitivity was not caused by differential tumor shedding. However, the changes in VAF of frequently mutated genes appear to be a more sensitive parameter for monitoring compared with the changes in total cfDNA (Figures 2 and 3). Therefore, we further assessed the prognostic value of VAF of frequently mutated genes.

The cfDNA VAF of frequently mutated genes represents a good prognostic indicator

The serum biomarkers and cfDNA loads of 6 patients was monitored at 2 to 4 time points, and the changes in disease burden of the patients were traced. The results showed that the fluctuations in VAF of frequently mutated genes more accurately reflect the progression of disease compared with the serum biomarkers. The VAFs of 3 frequently mutated genes (top gene mutations at each time point) were plotted against time, and compared with the changes in canonical serum biomarkers including CEA, CA19-9, CA125, and AFP. During the 6 to 23 months of follow-up, the symptoms of 2 patients were relieved (Figure 4A and B), whereas 2 patients experienced disease progression (Figure 4C and D), and 2 other patients reported possible relapse of disease after the initial alleviation (Figure 4E and F). The increase or decrease in VAF of the 3 frequently mutated genes reflected disease progression or alleviation with high fidelity. In 5/6 (83.3%) cases, the change of VAF accurately depicted the fluctuations in disease burden. In P18 (Figure 4E), the drop in VAF was consistent with the partial response to neoadjuvant chemotherapy and the alleviation following surgical resection. Although the disease stopped in July 2020, a novel TP53 mutation with very low...
VAF (1.53%) was detected in the cfDNA measured in March 2021, indicating tumor relapse, which was confirmed by follow-up clinical examination in 2022. This increase in disease burden was also reported based on serum CEA, CA19-9, and cfDNA levels, although the fluctuation was within the normal range and may be too subtle to be identified by imaging.

The prognostic value of the serum markers CEA, AFP, CA19-9, and CA125 was comparatively poor. The consistencies between CA125, CA199, AFP, and CEA fluctuations and changes in disease burden were 50.0% (3/6), 33.3% (2/6), 16.7% (1/6), and 16.7% (1/6), respectively. The cfDNA load precisely reflected the disease burden in 66.7% (4/6) patients, showing a moderate accuracy. Collectively, the VAF of frequently mutated genes is a better prognostic indicator compared with serum markers and cfDNA level. Moreover, monitoring the mutation status of all driver genes instead of the initially identified mutations provides insight into tumor metastasis, given that new loci of mutations were reported in the 3 patients that exhibited disease progression (Figure 4C, D, and F).

**cfDNA-based molecular characterization associated with patient prognosis**

Until our last follow-up on April 1, 2021, 54.2% (13/24) of patients survived, while 41.7% (10/24) of patients died, and 1 patient lost contact with the research group. The mean overall survival (OS) of the patients was 15.6 ± 2.1 months (ranging from 1.4 to 41.6 months. Two or more lines of treatment were administered to 83.35% (20/24) of the patients including...
surgical removal (62.5%, 15/24), chemotherapy (83.3%, 20/24), targeted therapy (58.3%, 14/24), immunotherapy (58.3%, 14/24), and radiotherapy (4.2%, 1/24, Figure 5A). Among the 14 patients who received targeted therapy, 12 carried mutations targeted by the drug (Supplemental Table 2).

As each patient was assigned a unique treatment strategy based on their clinical features, it is hard to analyze how the treatment strategy affected patient survival. Both bTMB (log-rank test $P = .62$; multivariate analysis using Cox proportional hazards model yielded a hazard ratio of 0.39, 95% confidence interval 0.70–1.22, $P = .84$, Figure 5B) and maximum VAF (log-rank test $P = .21$; multivariate Cox proportional hazards regression analysis yielded a hazard ratio 0.92, $P = .58$, Figure 5C) did not correlate with patient survival. However, the molecular characterization of the patients showed a sophisticated impact on the OS of the patients regardless of the treatment choice.

Both The Cancer Genome Atlas (TCGA) and Asian Cancer Research Group (ACRG) revealed the proposed molecular subtypes of gastric cancer. However, the enrolled patients were mostly EBV-negative and microsatellite stable, while the epithelial-to-mesenchymal transition status, TP53 activity, and genomic stability could not be assessed based on the targeted sequencing results. Therefore, we examined the impact of mutation status of frequently mutated genes (mutations shared by at least 4 patients) on patient survival. No statistically significant difference was observed between the wild type group and the mutant group carrying TP53 (log-rank test $P = .99$; multivariate Cox proportional hazards model hazard ratio 0.93, 95% confidence interval 0.22–3.89, $P = .93$, Figure 5D), ARID1A (log-rank test $P = .62$; multivariate Cox proportional hazards model hazard ratio 0.75, 95% confidence interval 0.06–7.23, $P = .75$, Figure 5E), and SYNE1 (log-rank test $P = .76$; multivariate Cox proportional hazards model hazard ratio 0.91, 95% confidence interval 0.19–4.41, $P = .91$, Figure 5F). In contrast, patients carrying CDKN1C and CDKN2A/C mutations showed an OS of $8.3 \pm 3.5$ months, while the OS of the wild type group reached $16.6 \pm 2.3$ months (log-rank test

Figure 5. Prognosis of gastric cancer patients. (A) Patient OS and the treatments received are shown in the plot, $n = 23$, 1 patient lost contact with the research group. (B) Kaplan-Meier survival analysis of patients with low (bTMB = 0, $n = 8$), medium (bTMB 0.76-3.82, $n = 8$), and high (bTMB 5.34-10.69, $n = 7$) bTMB. (C) Kaplan-Meier survival analysis of patients with low (MVAF = 0, $n = 7$), medium (MVAF 0.01-0.04, $n = 8$), and high (MVAF 0.08-0.4, $n = 8$) MVAF. (D) Kaplan-Meier survival analysis of patients with or without TP53 mutation. (E) Kaplan-Meier survival analysis of patients with or without ARID1A mutation. (F) Kaplan-Meier survival analysis of patients with or without SYNE1 mutation. (G) Kaplan-Meier survival analysis of patients with or without CDKN1C mutation. Statistical analysis was conducted using log-rank test. Patient number at risk table was shown below the plot.
Mutations of CDKN1C, CDKN2A, and CDKN2C have been identified. The genes encode a series of tumor suppressors and potential tumor suppressors that regulate cell cycle and cell proliferation similar to other proteins encoded by CDKN gene. Due to the limited patient size, it is impossible to reliably analyze the effect of CNV of CDKN genes on patient survival. To validate our findings and further investigate the question, we downloaded the DNA sequencing data of patients with gastric cancer from the TCGA database.

We analyzed the correlation between OS and CDKN1/2/3 gene mutations or CNV using the TCGA data comprising 295 patients with primary gastric adenocarcinoma. Patients with or without CDKN3, CDKN2D, CDKN2A, and CDKN1 showed no difference in OS (24.4 ± 3.1 months vs 20.2 ± 0.1 months, log-rank test \( P = .23 \), multivariate Cox proportional hazards model hazard ratio 0.97, 95% confidence interval 0.33-2.83, \( P = .96 \)). We further performed correlation analysis of the CNV data. The copy number of the chromosome region containing CDKN2A and CDKN2B exhibited a negative correlation with patient OS (Spearman’s correlation \( P = .05, r = -.118 \)). The copy number amplification does not facilitate patient survival. Consistent with our finding, aberrations in CDKN genes tend to compromise the prognosis of patients with gastric cancer. Such molecular characterizations associated with patient prognosis can be identified by cfDNA, which contributes to the determination of treatment regimens based on the predicted patient survival.

**Discussion**

The study reviewed the advantages and limitations of cfDNA in monitoring the progression of late-stage gastric cancer. The vast difference in the efficiency of cfDNA mutation detection between stages III and IV showed that the mutation spectrum of the stages I-III cancer revealed by cfDNA is not representative under the setting of 1500× depth and the lower detection threshold of 0.1%. The staging of cancer has restricted the utility of cfDNA in mutation detection. Studies demonstrated that the mutation concordance between plasma samples and tissue samples was 32% when patients in all cancer stages were included,26 which reached 83.4% if only patients with metastatic cancer were enrolled.27 The low mutation concordance is also attributed to the technical limitations of NGS, as the mutation concordance was over 70% when assessed by PCR.4,28 Increasing the DNA input and sequencing coverage depth can improve the performance and robustness of NGS tests. High coverage and read depth can be achieved by improving the capture efficiency and stability of enriched DNA, NGS library conversion, and amplification. However, this will also increase the cost of sequencing, thus limiting the use of NGS in clinical settings. Our study showed that the mean sequencing depth of 1500× at the lower detection threshold of 0.1% failed to ensure detection stringency and sensitivity. Consistent with our

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Figure 6. The phylogenetic trees of p5 and p10: (A) the phylogenetic trees of p5, including the tumor tissue and 3 cfDNA samples and (B) the phylogenetic trees of p10. The tumor tissue and 3 cfDNA samples are also included.
foundings, a previous study showed that the mean fragment depth of 4000× may yield around 50% sensitivity at the VAF cutoff of 0.1%.18

The cfDNA or ctDNA level was widely utilized in prognostic monitoring. The ctDNA clearance was reported to predict prognosis under different treatment regimens.29 Our results showed that ctDNA level was a more sensitive prognostic indicator compared with TMB, and the prognostic value of TMB depends strongly on the VAF of cfDNA. The VAF of frequently mutated genes was the best prognostic indicator compared with the cfDNA level, TMB, and serum markers. The detection threshold of 0.1% or less was based on predetermined valid results. Our study proved that the accuracy of monitoring by VAF of frequently mutated genes reached 83.3% at the detection threshold of 0.1%. Notably, the monitoring of disease burden should be based on all genes and not merely on the initially identified mutations, due to the frequent emergence of novel mutations observed in metastatic and recurrent tumors.

There are many plausible hypotheses that could explain the heterogeneity in cancer. The clonal evolution model proposed that mutations occur over time and may confer a selective advantage that allows sub-clones of cancer cells to outcompete other clones.30 Our findings showed that in some patients only selective mutation levels were increased after treatment (Figure 3). This observation indicates that the tumors comprised heterogeneous clones of cells, some clones responded to treatment while the others did not. ctDNA detects subclones that are resistant to current treatment. Different from total progression, this clonal progression manifests the selective advantage of certain mutations, which causes secondary drug resistance. More importantly, our data showed that ctDNA test could detect the clonal progression before tumor progression can be visualized by medical imaging. This clonal dynamic detected by ctDNA could partially explain secondary drug resistance, guide the design of treatment regimen based on the mutation spectrum of the progressive clone, and monitor disease progression ahead of imaging.

The cfDNA can also be used in the molecular characterization of patient subgroups. We identified a series of CDKN gene aberrations associated with poor prognosis. CDKN1/2/3 consists of genes that are transcribed to tumor suppressors or regulators of cell cycle. CDKN1A encodes the cell cycle inhibitor p21, which is triggered by p53 in response to DNA damage, and leads to cell cycle inhibition in G1 phase.31 CDKN2A encodes p16 and p14, both of which are CDK4 inhibitors that regulate cell cycle.32,33 CDKN2B is located adjacent to CDKN2A on chromosome 9p21, and are frequently deleted together in various cancer types. CDKN2B is also transcribed to a CDK4 inhibitor named p15, which regulates cell growth and cell cycle G1 phase.34 CDKN3 also encodes a cyclin-dependent kinase inhibitor that binds primarily to CDK1 and CDK2 to regulate the cell cycle.35 Mutant and aberrant expression of CDKN genes is frequently encountered in cancer and is associated with patient survival.36-39 Copy number loss and large deletion of the CDKN2A/B also lead to poor prognosis.33,34 Consistently, our study showed that mutations of CDKN1C/2A/2C and CNV of CDKN2A/B were associated with poor prognosis in gastric cancer. Identifying the molecular features of patients contributes to the prediction of patient survival, which in turn guides the treatment strategy based on the therapeutic time window available.

The major limitation of study is the small sample size, which may lead to unstable results. Although statistical methods were adjusted to address the issue, a larger cohort is required to validate the findings.

Conclusion
In this study, the clinical application of cfDNA was discussed regarding the accuracy of mutation calling, advantages over serum biomarkers, and its role in predicting prognosis. The mutation concordance between cfDNA in plasma samples and tumor DNA in tissue samples was markedly lower in patients with stage III gastric cancer compared with stage IV at the VAF detection threshold of 0.1%. Among the parameters that reflect disease burden, VAFs of frequently mutated genes are the most reliable indicator in predicting the prognosis of patients with better accuracy compared with serum biomarkers. One limitation of the study is the small sample size, highlighting the need to validate the results in a larger clinical study.

Declarations

Ethics approval and consent to participate
The studies involving human participants were reviewed and approved by the Ethics Committee of Shenzhen People’s Hospital. The patients/participants provided their written informed consent to participate in this study.

Consent for publication
Not applicable.

Author contributions
WH, JD, and XS contributed equally to this work. JD and RX designed the study. WH, SJ, JJ and RX designed the treatment strategy and collected clinical samples. ML, XZ, and JD performed the sequencing and data analysis. XS was responsible for tissue sample collection. JY and XS were involved in patient recruitment. JY guided the study design. All authors were involved in the construction of the manuscript. JD and RX proofread the manuscript.

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Availability of data and materials
The raw sequence data reported in this study have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2017) in BIG Data Center (Nucleic Acids Res 2018), Beijing Institute of Genomics (BIG), Chinese Academy of Sciences. The data accession number HRA001030 provides data access at http://bigd.big.ac.cn/gsa-human.

Supplemental material
Supplemental material for this article is available online.

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