Clinical application of circulating tumor DNA in the genetic analysis of patients with advanced GIST

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Running title: ctDNA detection in advanced GIST patients

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Abstract

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumor of digestive tract. In the past, tissue biopsy was the main method for the diagnosis of GISTs. While circulating tumor DNA (ctDNA) detection by next-generation sequencing (NGS) may be a feasible and replaceable method for diagnosis of GISTs. We retrospectively analyzed the data for ctDNA and tissue DNA detection from 32 advanced GIST patients. We found that NGS obviously increased the positive rate of ctDNA detection. ctDNA detection identified rare mutations that were not detected in tissue DNA detection. Tumor size and Ki-67 were significant influencing factors of the positive rate of ctDNA detection and concordance between ctDNA and tissue DNA detection. In all patients, the concordance rate between ctDNA and tissue DNA detection was 71.9%, with moderate concordance. But the concordance was strong for patients with tumor size>10cm or Ki-67>5%. Tumor size, mitotic figure, Ki-67 and ctDNA mutation type were the significant influencing factors of prognosis, but only tumor size and ctDNA mutation type were the independent prognostic factors for advanced GIST patients. We confirmed that ctDNA detection by NGS is a feasible and promising method for the diagnosis and prognosis of advanced GIST patients.

Keywords: Gastrointestinal Stromal Tumor; ctDNA; Genetic Analysis; Diagnosis; Prognosis.

Introduction

Gastrointestinal stromal tumors (GISTs) originating from interstitial cells of Cajal

(ICC) are the most common abdominal soft-tissue sarcomas (1). Advances in genetic diagnosis and the development of tyrosine kinase inhibitors (TKIs) have yielded great improvements in the treatment of patients with GISTs. The introduction of imatinib has increased the median survival of advanced GIST patients from 10–20 months to 51–57 months (2). Mutations of KIT and platelet-derived growth factor receptor alpha (PDGFRA) are the main focuses of genetic analysis, which is a critical method for diagnosis and targeted therapy in patients with GISTs.

Circulating tumor DNA (ctDNA), small fragments of extracellular DNA released by apoptotic tumor cells, contains information on the specific somatic mutations in tumor cells (3). The detection of ctDNA in peripheral blood, referred to as "liquid biopsy", is a promising method for the early diagnosis and prognostic evaluation of human malignancies (4). Compared with traditional tissue biopsy, liquid biopsy of ctDNA is noninvasive, simpler and safer. The ctDNA level is associated with tumor burden, indicating a potential role of ctDNA as an informative, inherently specific, and highly sensitive biomarker in the diagnosis and prognosis of patients with various types of cancers (5-7). ctDNA detection has also been used to determine genotype in patients with GIST, which is beneficial for guiding targeted therapy (8).

The positive rates of methods such as BEAMing (beads, emulsions, amplification and magnetics) and PCR for detecting ctDNA mutations are insufficient (8, 9). With the development of next-generation sequencing (NGS), a technology capable of simultaneously sequencing millions of DNA fragments without previous sequence knowledge, increases in the positive rate of ctDNA mutation detection have

become feasible (10). NGS has been widely used in genetic analysis of patients with various tumors including GIST because of the advantages of higher reliability and lower costs (10, 11).

National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines recommend genetic analysis to guide targeted therapy in patients with GISTs (12). ctDNA detection represents a safer alternative to high-risk traditional tissue biopsy for the genetic analysis of GIST patients. A previous study demonstrated the reliability of ctDNA detection by NGS for diagnosing genotype in GIST patients (13), but the association between ctDNA and tissue DNA detection has not been examined. The aim of our study was to evaluate the feasibility of ctDNA detection by NGS, the concordance between ctDNA and tissue DNA detection, and independent prognostic factors in patients with advanced GISTs.

Patients and Methods

Patients

We retrospectively analyzed the data of genetic analysis of ctDNA and tissue DNA detection from 32 advanced GIST patients admitted in the First Affiliated Hospital of Nanjing Medical University from March 2015 to February 2017. Inclusion criteria are as below: patients with confirmed GISTs, good physical condition and normal function in important organs. Exclusion criteria are as below: patients could not undergo percutaneous CT-guided puncture and biopsy because of any cause, simultaneously with other tumors, with dysfunction of important organs, being

pregnant or in lactation period, simultaneously joined in other clinical trials. Our study was approved by The Institutional Ethical Board of the First Affiliated Hospital of NJMU (Ethical number: 2013-SR-142). All patients have been informed of sample collection and signed informed consent. Registration number of the study was ChiCTR-RNC-14004667. Risk levels of patients were estimated according to Armed Forces Institute of Pathology (AFIP) criteria (14).

Methods

Sample collection

Fresh tumor tissues (no less than 1cm, 1-3 strips) obtained by 1mm puncture needle or formalin fixed paraffin embedded (FFPE) blocks/sections were collected from each patient. Diagnosis of GISTs and tumor purity had been confirmed in pathology department of our hospital. Necrotic tissues of tumor samples should be less than 30%. 8mL peripheral blood (no less than 8mL) collected from each patient was stored in EDTA-coated tubes (BD Biosciences). Plasma was extracted using a pre-cooling centrifugal machine (Eppendorf 5424, 1800g, 4℃, for 10min) within 2h since blood collection and stored at 4℃, then transferred to the testing laboratory within 48h. Tumor tissue samples and blood samples were collected at the same time. All detections were performed in Nanjing Geneseeq Biotechnology Incorporation according to instructions reviewed and approved by the institutional ethical board of the hospital.

DNA extraction and quantification

Following FFPE blocks being de-paraffinized with xylene for twice, DNA was

extracted using a QIAamp DNA FFPE Tissue Kit (Qiagen) according to manufacturer's instructions. Fresh tissue DNA and plasma DNA were extracted using a DNeasy Blood & Tissue kit (Qiagen) according to manufacturer's recommendations. Extracted DNA was purified and qualified employing the Nanodrop2000 (Thermo) and then quantified by Qubit3.0 (Life Technology) with a dsDNA HS Assay Kit (Life Technology) according to manufacturer's protocols. A260/280 and A260/A230 values were recorded. DNA purity Criteria are as follows, A260/280 value was 1.5～2.0 and A260/A230 value was 1.8～2.3.

Fragment distribution of plasma DNA was analyzed using Analyser 2100 Bio-analyzer (Agilent Technology) with an Agilent High Sensitivity DNA kit (Agilent Technology) according to manufacturer's instructions. Small fragmented DNA was specifically selected from ctDNA samples adopting the Agencourt AMPure XP beads (Beckman Coulter) method according to manufacturer's protocols.

Sequencing library establishment

Genomic DNA was sheared into 350bp fragments using M220 instrument (Covaris). Then end-repairing, A-tailing and adaptor-adding were sequentially performed in genomic DNA fragments and small fragmented ctDNA adopting a q-PCR instrument (BioRad) according to manufacturer's recommendations, followed by fragment selection in genomic DNA with Agencourt AMPure XP beads method. Sequencing libraries were established with a KAPA Hyper Prep kit (KAPA Biosystems) and amplified by PCR according to manufacturer's protocols.

Target enrichment of gene fragments

xGen blocking oligos (Integrated DNA Technology, 1nmol/μL in TE buffer) and human cot-1 DNA (Life Technology) were used as blocking reagents. Capture reactions were performed using NimbleGen SeqCap EZ Hybridization and Wash Kit (Roche) and Dynabeads M-270 Streptavidin (Life Technology) in accordance with manufacturer's protocols. Illumina p5 (5'-AATGATACGGCGACCACCGA-3') and p7 (5'-CAAGCAGAAGACGGCATACGAGAT-3') primers were used in amplifying captured libraries in KAPA HiFi HotStart ReadyMix (KAPA Biosystems). Amplified libraries were purified with the Agencourt AMPure XP beads method and quantified by qPCR with a KAPA Library Quantification kit (KAPA Biosystems). Analyser 2100 Bio-analyzer (Agilent Technology) was employed to determine the size of library fragments. Target-enriched libraries were sequenced with HiSeq4000 NGS platform (Illumina) according to manufacturer's instructions. Sequencing depth was no less than 600× mean coverage by non-PCR duplicate read pairs in tissue samples. While in ctDNA samples, sequencing depth of majority of samples was no less than 3000× mean coverage by non-PCR duplicate read pairs in spite of different sequencing depths achieved for the assessment.

Sequencing

Sequencing reagents were prepared with HiSeq 4000 PE Cluster Kit (Illumina) according to user guidelines. Paired-end sequencing was performed in sequencing process. The 416 panel (with sequencing length of 150bp) produced by Nanjing Geneseeq Biotechnology Incorporation was used as DNA capture probe of tumor-related genes. HiSeq 4000 (Illumina) was employed as sequencing platform in

this study. Data collection software (Illumina) was used to control sequencing process and perform the analysis of real-time data.

Bioinformatics analysis

Original image data acquired from HiSeq 4000 sequencing platform were transferred by base calling analysis into raw sequence data, which contained sequence information and corresponding sequencing quality information. Quality control was performed using Trimmomatic (Illumina). Comprehensive information of tissue DNA and ctDNA mutations, including point mutation, fusion, amplification, deletion and insertion mutations, was determined by bioinformatics analysis, which further performed quality control of sequencing in mapping process.

Statistical analysis

Chi-square test was used in univariate analysis of ctDNA positive rate and concordance detection. Concordance analysis was performed by Kappa concordance test using SPSS 20 and SAS 9.3. Log-rank test was employed in univariate analysis of PFS. Multivariate Cox regression was performed to estimate independent prognostic factors of advanced GIST patients. PFS was defined from the date of being selected for this study to the date of disease progression or death. P value<0.05 was considered statistically significant.

Results

Clinicopathological characteristics of patients

The patients included 19 males (59.4%) and 13 females (40.6%). Ten patients (31.2%)

were>70-year of age, and 22 (68.8%) were≤70-year of age. The average age was 62.8 years (range, 42–83-years), and the median age was 64 years. The primary tumor site was the stomach in 18 patients (56.3%) and non-stomach in 14 patients (43.7%). The tumor size was >10 cm in 23 patients (71.9%) and ≤10cm in 9 patients (28.1%). The number of mitotic figures per 50 high-power fields (HPF) was >5 in 12 patients (37.5%) and ≤5 in 20 patients (62.5%). The recurrence risk level was low for 5 patients (15.6%), intermediate for 6 patients (18.8%), and high for 21 patients (65.6%). At the beginning of the study, 5 patients (15.6%) had previously received imatinib, and 27 (84.4%) had received no treatment. Immunohistochemistry detected Ki-67>5% in 19 patients (59.4%) and ≤5% in 13 patients (40.6%). The cell morphology was spindle cells in 27 patients (84.4%), epithelioid cells in 1 patient (3.1%), and mixed in 4 patients (12.5%). (Table 1)

After the beginning of the study, most patients received imatinib continuously. The dosages were as follows: 25 patients (78.1%) received 400mg daily, and 3 patients (9.4%) received 600mg daily; 4 patients (12.5%) received other treatments (including 1 patient received 200mg daily, 1 patient didn't receive imatinib, and 2 patients received imatinib purchased from India). (Supplementary figure 1a) At last follow-up, 8 patients had undergone surgery (including 5 underwent surgery after neoadjuvant treatment and 3 underwent direct surgery), whereas the remaining 24 patients had not undergone surgery. (Supplementary figure 1b)

Advantages of ctDNA detection by NGS

Among the patients, ctDNA mutation detection was positive in 18. The positive rate of ctDNA mutation was 56.3% (18/32), obviously higher than the previously reported rates of ctDNA tests using other technologies such as BEAMing (16.7%, 5/30) and PCR (39.5%, 15/38) (8, 9). This result indicates that NGS can remarkably increase the positive rate of ctDNA mutation detection. (Figure 1a)

Furthermore, in one patient, KIT exon14 mutation was detected by the ctDNA test, whereas a negative result was obtained in tissue DNA detection. This result reveals that ctDNA detection by NGS can identify gene mutations (even rare mutations) in cases in which the results of tissue DNA detection are negative. ctDNA detection may thus indicate tumor abnormalities more comprehensively than tissue biopsy.

Correlation between the number of ctDNA mutations and tumor size

The ability of ctDNA analysis to detect mutations was limited when the tumor size≤ 10cm, whereas ctDNA detection typically revealed one or more mutations when the tumor size>10cm. The number of ctDNA mutations was obviously higher in patients with tumor size＞10cm than in patients with tumor size≤10cm. These findings support a positive correlation between the number of ctDNA mutations and tumor size. (Figure 1b)

Univariate analysis of influencing factors of the positive rate of ctDNA detection

The positive rate of ctDNA detection was significantly higher for tumor size>10cm

than for tumor size≤10cm [73.9% (17/23) vs. 11.1% (1/9), P=0.004]. In addition, the positive rate of ctDNA detection was significantly higher for Ki-67>5% than for Ki-67≤5% [78.9% (15/19) vs. 23.1% (3/13), P=0.002]. (Table 1, Supplementary figure 2) Therefore, tumor size and Ki-67 were important influencing factors for the positive rate of ctDNA detection in advanced GIST patients.

Univariate analysis of influencing factors of concordance between ctDNA and tissue DNA detection

The concordance rate between ctDNA and tissue DNA detection was significantly higher for tumor size>10cm than for tumor size≤10cm [87.0% (20/23)vs. 33.3% (3/9), P=0.006]. Furthermore, the concordance rate between ctDNA and tissue DNA detection was significantly higher for Ki-67>5% than for Ki-67≤5% [89.5% (17/19) vs. 46.2%(6/13), P=0.015]. (Supplementary table 1, Figure 2a and b) Tumor size and Ki-67 were therefore significant influencing factors of concordance between ctDNA and tissue DNA detection in patients with advanced GISTs.

Analysis of concordance between ctDNA and tissue DNA detection

The ctDNA test detected KIT exon9 mutation in 2 patients, KIT exon11 mutation in 14 patients, KIT exon14 mutation in 1 patient, PDGFRA exon18 mutation in 1 patient and normal genotype in 14 patients. By comparison, the tissue DNA test detected KIT exon9 mutation in 5 patients, KIT exon11 mutation in 18 patients, PDGFRA exon18 mutation in 2 patients, and normal genotype in 7 patients. The outcomes of ctDNA

and tissue DNA detection were concordant for 71.9% (23/32) patients, including 2 patients with KIT exon9 mutation, 14 patients with KIT exon11 mutation, 1 patient with PDGFRA exon18 mutation and 6 patients with normal genotype. Evaluation of the concordance by the kappa concordance test yielded a weighted kappa value of 0.489 (P<0.001), indicating moderate concordance. (Table 2)

Among the 23 patients with tumor size>10cm, the outcomes between ctDNA and tissue DNA detection were concordant for 20 patients, including 2 patients with KIT exon9 mutation, 14 patients with KIT exon11 mutation, 1 patient with PDGFRA exon18 mutation and 3 patients with normal genotype. The concordance rate was 87.0% (20/23), and the kappa value was 0.754 (P<0.001), indicating high concordance. (Supplementary table 2)

Among the 19 patients with Ki-67>5%, concordant outcomes between ctDNA and tissue DNA detection were obtained for 17, including 2 patients with KIT exon9 mutation, 12 patients with KIT exon11 mutation, 1 patient with PDGFRA exon18 mutation and 2 patients with normal genotype. The concordance rate was 89.5% (17/19), and the kappa value was 0.800 (P<0.001), indicating strong concordance. (Supplementary table 3)

Univariate analysis of the influencing factors of prognosis

At last follow-up, 11 patients were progressed (including 2 patients died); progression did not occur in 21 patients (including 8 patients underwent surgery). The average progression-free survival (PFS) was 12.2 months. The log-rank test showed that

tumor size (P=0.023), mitotic figures (P=0.025), Ki-67 (P=0.039) and mutation type (P=0.045) were significant influencing factors of prognosis in advanced GIST patients. (Supplementary table 4)

Figure 3 shows the PFS curves for the significant influencing factors. The prognosis of patients with tumor size≤10cm was significantly superior to that of patients with tumor size>10cm (mean PFS: 14.1 months vs. 10.6 months, Figure 3a). The prognosis of patients with mitotic figures≤5/50HPF was significantly better than that of patients with mitotic figures>5/50HPF (mean PFS:12.9 months vs. 10.9 months, Figure 3b). The prognosis of patients with Ki-67≤5% was significantly better than that of patients with Ki-67>5% (mean PFS: 14.3 months vs. 11.2 months, Figure 3c). The prognosis of patients with KIT exon11 mutation of ctDNA was significantly superior to that of patients with other mutation types (mean PFS: 13.9 months vs. 10.8 months, Figure 3d).

Multivariate Cox regression analysis of significant influencing factors of prognosis

The four significant influencing factors were imported into the Cox regression model. The results indicated that tumor size (95%CI of hazard ratio, 1.299–602.921, P=0.033) and type of ctDNA mutation (95%CI of hazard ratio, 0.026–0.971, P=0.046) were independent prognostic factors for advanced GIST patients. (Table 3)

Discussion

The incidence of GIST, the most common mesenchymal tumor of the digestive tract, has increased greatly in recent years with the development of biotechnological detection (1). Despite improvements in the prognosis of GIST patients due to the introduction of TKIs and improved surgical strategies, clinical efficiency and PFS remain primarily dependent on the types of KIT and PDGFRA mutations (2). Therefore, genetic analysis is necessary to guide targeted therapy in GIST patients.

ctDNA detection is a promising method for genetic analysis and has been used in the genetic diagnosis and prognosis evaluation of diverse cancer types, including GIST (15-17). ctDNA can be easily obtained from the bloodstream, theoretically overcoming limitations of tissue biopsy such as invasiveness, the inability to characterize molecular heterogeneity, and unsuitability of formalin-fixed paraffin-embedded (FFPE) blocks for wider genome analysis (15). In the present study, ctDNA and tissue DNA detection were performed to assess the reliability of ctDNA in the diagnosis of GISTs and the concordance between ctDNA and tissue DNA detection in advanced GIST patients.

NGS, a high-throughput sequencing technology, was employed to detect ctDNA. Similar to the positive ctDNA mutation rate (52%) reported by Kang et al. (13), our results revealed a higher positive rate of ctDNA mutation (56.3%) than those detected by technologies such as BEAMing and PCR (8, 9). Repeated positive results or increased ctDNA mutations have been observed in GIST patients with relapse or progressive disease, indicating that ctDNA can be used as a tumor-specific biomarker (9). Similarly, we demonstrated a positive correlation between the number of ctDNA

mutations and tumor size; the number of ctDNA mutations was much higher in patients with tumor size＞10cm than in those with tumor size≤10cm.

In one patient, KIT exon14 mutation was identified by ctDNA detection but not tissue DNA detection, indicating that ctDNA detection can reveal gene mutations or even rare mutations in the case of a negative result by tissue DNA detection. We hypothesize that cell subsets with different mutations exist in tumor tissues, and thus tissue biopsy may only partly reflect the cell types in the tumor. By contrast, ctDNA detection can identify alloplasmic cell subsets or rare mutations and thus more comprehensively reflects tumor abnormalities. ctDNA detection will therefore be more beneficial in guiding treatment strategies.

A few studies have examined influencing factors of ctDNA detection in GIST patients. Brychta et al. (18) reported a positive correlation between ctDNA detection and the total number of tumor cells in the primary tumor in the early stage of pancreatic cancer. Gao et al. (19) showed that the presence of ctDNA was associated with larger tumor size, TNM stage and *Helicobacter pylori* infection in gastric cancer. Similarly, we demonstrated that the positive rate of ctDNA detection was positively correlated with tumor size and Ki-67 in advanced GIST patients. The positive rates of ctDNA detection were obviously higher in patients with tumor size>10cm or Ki-67>5%.

The concordance of gene mutations detected by ctDNA and tissue DNA tests has been analyzed in several studies. Yao et al. (20) showed that the overall concordance rate of gene mutations between tissue DNA and ctDNA detection was 78.21% in

patients with advanced non-small cell lung cancer. Another study indicated that the concordance of genomic alterations between tissue DNA and ctDNA was 91.0–94.2% in breast cancer (21). Consistent with these previous studies, we obtained an overall concordance rate between ctDNA and tissue DNA detection of 71.9%, with moderate concordance. In addition to influencing the positive rate of ctDNA detection, tumor size and Ki-67 were significant influencing factors of the concordance rate for mutations detected by ctDNA and tissue DNA. The concordance of genetic mutations between ctDNA and tissue DNA detection was significantly higher in patients with tumor size>10cm or Ki-67>5% than in all patients with GISTs. These results support the potential of ctDNA detection for tumor-related mutation profiling.

Several studies have examined prognostic factors for GIST patients. Tumor mitotic rate, Ki-67, tumor size, tumor site and tumor rupture are considered independent prognostic factors for GIST recurrence (2). Resistant mutations identified by ctDNA have been potentially associated with poor prognosis in patients with GIST (8). Feng et al. (22) showed that mitotic index and tumor size are also independent prognostic factors in patients with mesenteric GISTs. Furthermore, positive expression of Ki-67 is associated with poor prognosis in GIST patients (23). Similarly, we identified tumor size, mitotic figures, Ki-67 and ctDNA mutation type as significant influencing factors of prognosis, but only tumor size and ctDNA mutation type were identified as independent prognostic factors for advanced GIST patients. The prognoses of patients with tumor size>10cm or KIT exon11 mutation detected by ctDNA were significantly superior to those of patients with tumor size$\leqslant$10cm or

other mutations. Mitotic figures, Ki-67 and tumor site were not independent prognostic factors for GIST patients in our study, possibly due to the small sample size. Our results await further validation by additional clinical data.

In conclusion, as an alternative to tissue biopsy, ctDNA detection by NGS is a feasible method for the genetic analysis of advanced GIST patients. The concordance between ctDNA and tissue DNA detection was sufficiently high in patients with tumor size>10cm or Ki-67>5%. Tumor size and ctDNA mutation type were independent prognostic factors for advanced GIST patients. These findings support the potential role of ctDNA as a tumor-specific biomarker in the diagnosis and prognosis of patients with advanced GIST. Furthermore, with improvements in biotechnological detection, the tumor size cutoff of 10 cm in GIST patients may be reduced because of the higher sensitivity of ctDNA detection.

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Table 1 Univariate analysis of influence factors of positive rate of ctDNA detection

Factors	Patients	ctDNA		P value
		Positive	Negative	
Gender				0.341
Male	19	12	7	
Female	13	6	7	
Age				0.712
>70	10	5	5	
≤70	22	13	9	
Primary tumor site				0.178
Stomach	18	12	6	
Non-stomach	14	6	8	
Tumor size				0.004*
>10cm	23	17	6	
≤10cm	9	1	8	
mitotic figure				0.358
>5 /50HPF	12	8	4	
≤5 /50HPF	20	10	10	
Risk level				0.182
Very low/Low	5	1	4	
Intermediate	6	3	3	
High	21	14	7	
History of medicine				0.631
Taking imatinib	5	2	3	
None	27	16	11	
Ki-67				0.002*
≤ 5%	13	3	10	
> 5%	19	15	4	
Morphology				0.609
Spindle	27	16	11	
Epithelioid	1	0	1	
Mixed	4	2	2	
Total	32	18	14	

Table 1. Tumor size and Ki-67 were the significant influence factors of positive rate of ctDNA detection. *P<0.05.

Table 2 Concordance analysis between ctDNA and tissue DNA detections in genetic analysis (Kappa concordance test, n=32)

ctDNA mutation	Tissue DNA mutation					Total
	KIT exon 9	KIT exon 11	KIT exon 14	PDGFRA 18	WT	
KIT exon 9	2	0	0	0	0	2
KIT exon 11	0	14	0	0	0	14
KIT exon 14	0	0	0	0	1	1
PDGFRA 18	0	0	0	1	0	1
WT	3	4	0	1	6	14
Total	5	18	0	2	7	32

Table 2. In all patients, the concordance was moderate. (Weighted Kappa=0.489, P<0.001)

Table 3 Multivariate Cox regression analysis between significant influence factors and prognosis

Variables	PFS	
	HR (95% CI)	P value
Tumor size (>10cm Vs ≤10cm)	1.299～602.921	0.033*
Mitotic figure (>5/50HPF Vs ≤5/50HPF)	0.796～850.880	0.067
Ki-67 (>5% vs ≤ 5%)	0.007～1.604	0.106
Types of mutation (exon11 Vs others)	0.026～0.971	0.046*

Table 3. Tumor size and ctDNA mutation type were the independent prognostic factors of advanced GIST patients. *P<0.05.

Figure legends

Figure 1. Positive rate and number of ctDNA mutation.

(a) Compared with other technologies such as BEAMing and PCR reported previously, NGS obviously increased the positive rate of ctDNA mutation detection. **(b)** It almost couldn't detect any mutation when tumor size≤10cm, while one or more mutation types had been detected when tumor size>10cm. The number of ctDNA mutation is positively correlated with tumor size.

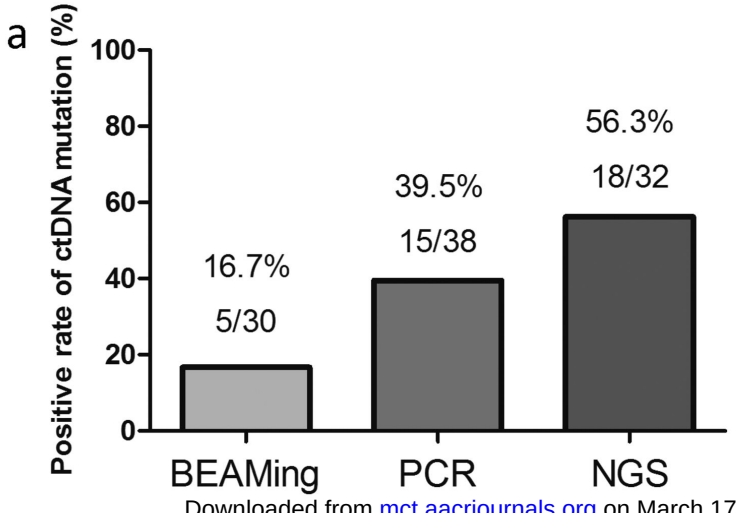
Figure 2. Correlation between tumor size, Ki-67 and the concordance rate of ctDNA detection.

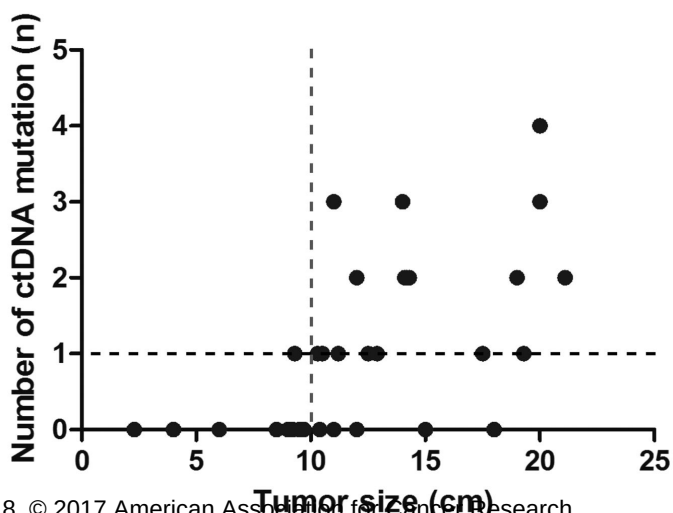
The concordance rates between ctDNA and tissue DNA detections in patients with tumor size>10cm (87.0%) or Ki-67>5% (89.5%) were significantly higher than that in patients with tumor size≤10cm (33.3%) or Ki-67≤5% (46.2%), respectively.

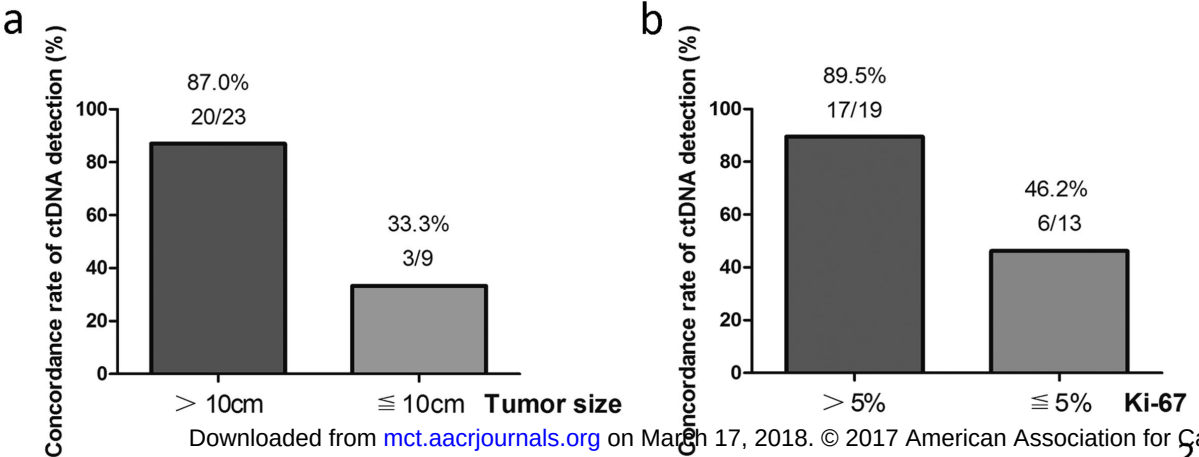
Figure 3. PFS curves of significant prognostic factors.

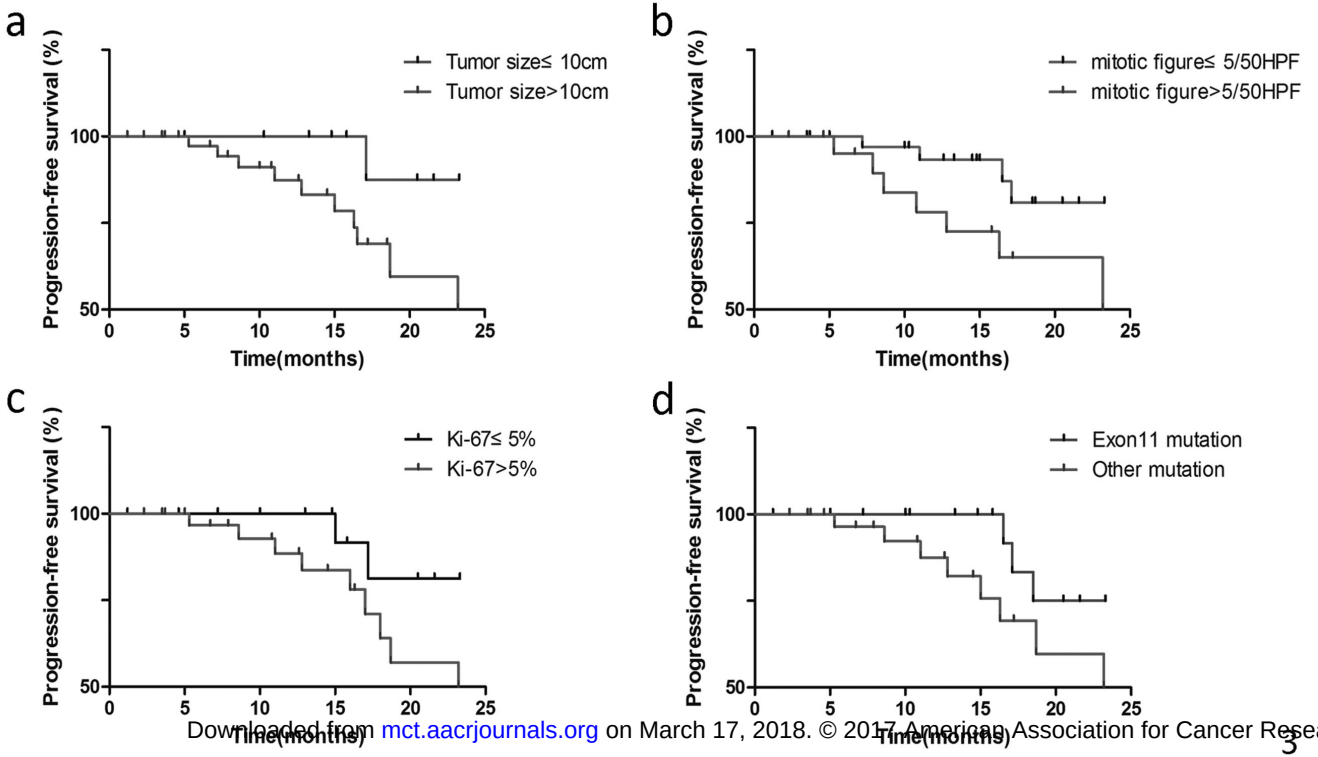
PFS was defined from the date of being selected for this study to the date of disease progression or death. At last follow-up, 11 patients had been with progression and 21 patients without progression. Average time of PFS was 12.2 months. Tumor size, mitotic figure, Ki-67 and ctDNA mutation type were indicated by log-rank test as the significant prognostic factors of patients. **(a)** Tumor size, mean time of PFS: 14.1 months Vs 10.6 months. P=0.023. **(b)** Mitotic figure, mean time of PFS: 12.9 months Vs 10.9 months. P=0.025. **(c)** Ki-67, mean time of PFS: 14.3 months Vs 11.2 months.

P=0.039. **(d)** Type of ctDNA mutation, mean time of PFS: 13.9 months Vs 10.8 months. P=0.045.









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Molecular Cancer Therapeutics

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