Original Article

Circulating tumor cells in whole process management of gastrointestinal stromal tumor in a real-life setting

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Abstract

Background/Aim: Liquid biopsy is changing the diagnosis and treatment strategies of various neoplasms. However, the circulating tumor cells (CTCs) of gastrointestinal stromal tumor (GIST) patients with different disease process are not clear. To better understand the dynamic change of CTCs in GIST patients, we conducted a real-life setting study.

Patients and Methods: One-hundred fifty GIST patients were included. The isolation by size of tumor cell (ISET) method was employed to detect the CTCs/circulating tumor microemboli (CTM). Imatinib (IM) plasma concentration was detected by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Multivariate and univariate analysis were used to analyze the effects of clinical characteristics on the positive rate of CTC and the number of CTCs/CTM.

Results: The positive rate of CTCs was 72%. The median number of CTCs and CTM was 4 and 0. Logistic multivariate regression analysis showed that tumor diameter was the only independent factor of the positive rate of CTCs ($P < 0.05$). The numbers of CTCs and CTM had intensive linear correlation ($P < 0.001$). Tumor diameter, Ki 67 expression and mitotic were related to the number of CTCs ($P < 0.05$). Patients with higher Ki 67 expression tend to have more CTM ($P < 0.05$). IM plasma concentration showed no influence to the CTCs/CTM ($P > 0.05$).

Conclusions: In the current study, we assessed the CTCs and CTM of GIST patients in various disease progressions and identified clinicopathological factors influencing the detection of CTCs and CTM. These results are instructive for clinicians to understand CTCs/CTM in GIST patients.

Keywords: Circulating tumor cells, circulating tumor microemboli, gastrointestinal stromal tumors, imatinib plasma concentration

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs), the most common mesenchymal tumors of digestive system, account for 80% of the digestive mesenchymal tumors.[1] After the "Patient Zero" treated with imatinib (IM) by Dr. Heikki, the management of GIST patients has entered a brand

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new era.[2] Since the IM was approved by the U.S. Food and Drug Administration (FDA) as an option for GIST patients, the median survival has been greatly prolonged compared with GIST patients before the IM era.[3] At the moment when precision treatment is prevalent, where 85% of GIST patients with KIT exon 11 mutations can benefit from the IM treatment, the management of GIST patients on IM is not completely satisfactory. During IM administration, especially for the elderly GIST patients, the dose change rate is about 16.7%.[4] The methods to assess the efficacy of GIST patients treated with IM are very limited, such as computed tomography (CT) or magnetic resonance imaging (MRI), and the validity of which have a hysteretic effect.[5] In several recent studies, we have focused on the effect of IM plasma concentration on the efficacy and adverse reaction of GISTs.[6,7] However, we urgently need timely efficacy evaluation methods to guide the whole process management of GIST patients.

Circulating tumor cells (CTCs), speculated in 1829 and confirmed in 1955, are tumor cells originating from a solid tumor and then released into the circulation.[8,9] Some CTCs can escape from the body's immune recognition or drug treatment, find a suitable microenvironment in the body, form seeds, grow in distant tissues or primary plants, causing tumor metastasis or recurrence.[10-12] CTCs have different forms in peripheral blood, both free single CTC and aggregated CTC cell mass, namely circulating tumor microemboli (CTM). As a vital component of liquid biopsy, the numbers of CTCs can be precisely enumerated in a certain amount of peripheral blood in a non-invasive way.[13] Recently, CTCs or CTM of many distinct tumors, such as breast cancer, colorectal cancer, prostate cancer, and pancreatic cancer, have been shown as a powerful biomarker to predict tumor metastasis, prognosis, and disease conditions.[14-17] Meanwhile, the exact cut-off numbers of CTCs in many kinds of cancers to predict prognosis have been described. Cornelis *et al.* found that for metastatic colorectal cancer, patients with CTCs ≥3 will have a shorter median progression-free survival (PFS) and overall survival (OS).[15] For patients with early or advanced breast cancer, CTCs ≥1 or ≥5 from peripheral blood indicate poor prognosis.[18,19] In the newest American Joint Committee on Cancer (AJCC) Tumor Staging Manual, CTCs are listed as a prognosis assessment tool.[20]

The values of CTCs/CTM in GIST patients have yet to be elucidated, especially the whole process management of disease outcomes. A previous study on CTCs of GIST patients employed anoctamin 1 (ANO1), previously called Discovered On Gastrointestinal tumor protein 1 (DOG1), as the biomarker and quantitative real-time polymerase chain reaction (qRT-PCR) to detect the expression of DOG1 in the blood of GIST patients. DOG1 expression in peripheral blood significantly correlated with poor disease-free survival (DFS) and DOG1 detection is of clinical potential for monitoring recurrence and IM treatment.[21] Others developed a monoclonal antibody 84-1, specific cell-surface vimentin (CSV) to capture and enumerate CTCs of GISTs. However, the classic CSV positive CTCs was abundant in metastatic GISTs but failed to predict metastasis.[22] The detection and value of CTCs/CTM in the whole process management of GIST patient treatment is unclear.

To explore the features of CTCs/CTM under the whole course of GIST treatment, we conducted a real-life study and enrolled 150 GIST patients confirmed by pathologic diagnosis and analyzed the characteristics of CTCs/CTM in the whole process management of GIST patients.

PATIENTS AND METHODS

Study patients

The first affiliated hospital of Nanjing Medical University offers physicians the possibility to detect the CTCs/CTM and drug plasma concentration of their patients from both outpatients and inpatients. During February 2018 to September 2019, a cohort of 150 patients with pathologically confirmed GIST in the First Affiliated Hospital of Nanjing Medical University were included in this real-life study. All patients had complete demographic, clinical, and pathological information. The study was approved by the First Affiliated Hospital of Nanjing Medical University ethics committee (ethical approval code: 2013-SR-142, date: 02-12-2013).

Data collection

All patients signed informed consent at the time of enrollment. Demographic and clinicopathological information were collected at enrollment and the subsequent follow up. After the enrollment, all patients were followed up every three months. The cohort was divided into five subgroups by the disease status: Group A, patients before surgery; Group B, patients after surgery and without IM; Group C, patients after surgery and treated with IM; Group D, recurrent GISTs; Group E, unresectable GISTs. Of these 150 GIST patients, 109 patients from group B, D, and E were treated with IM CTC detection and IM plasma concentration tests were performed each visit.

CTC detection

The isolation by size of epithelial tumor cell (ISET) assay was performed as described in an earlier study by Vona *et al*.[23] The samples were processed on an automated

testing platform as per the manufacturer's instructions. The filtration module was provided by Wuhan YZY Medical Science and Technology Co., Ltd. (Wuhan, China). A total of 5 ml of whole blood was diluted up to 8 mL with buffer containing 0.2% formaldehyde and filtered through a membrane having 8 mm pore size. Harvested CTCs/CTM were stained with Romanowsky stain, air-dried at room temperature, and mounted. Based on our own experience and the criteria proposed by other research groups, cells isolated in this study were assigned as tumor cells only if they had the following morphological characteristics: atypia of the nucleus (irregular shape, presence of a nodular, lobulated contour); nuclear– cytoplasmic ratio >0.8; nuclear diameter (the long diameter) >18 mm; hyperchromatic nuclei and nonhomogeneous staining; thickened, sunken, wrinkled, and jagged nuclear membrane; the presence of nuclear chromatin side-shift or large nucleoli or presence of abnormal mitotic figures; and presence of tumor cell aggregations, or CTM. If four features or more were met, they were considered as malignant tumor cells (CTCs). All candidate CTCs/CTM were blindly reviewed and identified independently by three senior cytopathologists. Immunofluorescence staining for cells of differentiation CD45, CSV, and DAPI was conducted for further confirmation. The detailed method of staining was recorded in the Supplementary materials.

Imatinib plasma concentration

For IM trough plasma concentration detection, 5 ml venous blood of GIST patients treated with IM was collected before taking the medicine. The IM concentration was tested by the liquid chromatography-tandem massspectrometry (LC-MS/MS) method, as previously described.[6] The lowest detectable blood concentration is 10 ng/ml. For the accuracy of plasma concentration, each sample was tested three times independently.

Statistical analysis

Statistical analysis was conducted with the Graphpad Prism 6.0 (GraphPad Software, California, USA) and Statistical Package for the Social Sciences (SPSS) 20.0 (Chicago, USA). Data are presented as mean ± standard deviation (SD). The association of clinicopathological factors with positive rate of CTCs was evaluated via Chi-square test. Multivariate analysis of the positive rate of CTCs using a binary logistic regression model was done. The correlation between the counting data was tested by linear correlation, and the variables of the two groups were analyzed by Student's *t*-test.

RESULTS

CTCs and CTM determination results

During the immunofluorescence staining, 4',6-diamidino-2-phenylindole (DAPI) was used to identify the nucleus, CD45 was employed to combine the leukocytes and the CSV was designed to distinguish the CTCs origin from GIST tumor. Figure 1a is the result of CTCs from two independent GIST patients. Figure 1b demonstrates the CTM from two other different GIST patients.

Clinicopathological characteristics of GIST patients

As demonstrated in Figure 2, 17 patients were drawn before surgery (Group A), 112 GIST patients were enrolled after surgery with 84 patients who were treated with IM (Group C), 25 were advanced GIST patients with 8 unresectable GISTs (Group D), and 17 recurrent GIST patients (Group E). The mean age was 56.81 ± 10.64 years. Of these, 87 (58%) cases had tumor located in the stomach, 54 (36%) in small intestine, 4 (2%) in the colorectum, and 5 (3%) in the other sites of the abdomen. According to the revised National Institutes of Health (NIH) 2009 risk classification,[3] 2 (1%) of the 150 patients were defined as very low risk, 14 (9%) patients were characterized as low risk, 47 (31%) patients as intermediate risk, and 87 (58%) as high risk. The detailed demographic and clinical features are summarized in Table 1.

Variables affecting the positive rate of CTCs

In terms of qualitative analysis, 72% exhibited positive CTCs. Table 1 shows the results of univariate analysis for positivity of CTCs. Tumor diameter, mitotic count, and Ki 67 may significantly influence the detection rate of CTCs ($P < 0.05$). Then, a logistic regression model analysis was used to explore the independent factors affecting the positive rate of CTCs. As shown in Table 2, only tumor diameter significantly correlated with the detection of CTCs ($P < 0.05$). Moreover, although patients' status at detection is not a variable affecting the positive rate of CTCs, patients with unresectable tumors have the highest positive rate, at 87.5%. After the tumor resection, the positive rate of CTCs decreased from 82.35% to 66.67% [Figure 3a].

Factors influencing the number of CTCs

For the quantitative analysis, we analyzed the clinicopathological factors influencing the exact number of CTCs. The average and median of the CTCs are 28 and 4, respectively, ranging from 0 to 301. Like positive rate of CTCs, we found that patients with unresectable tumors have the highest median CTC number [Figure 3a]. Tumor size and Ki 67 expression significantly correlated with CTC number [Figure 3b and c, $P < 0.001$]. Compared with patients with mitotic count fewer than 5/10 HPF, patients with mitotic count of more than 10/50 HPF have more CTCs [Figure 3d, $P < 0.01$]. However, patients

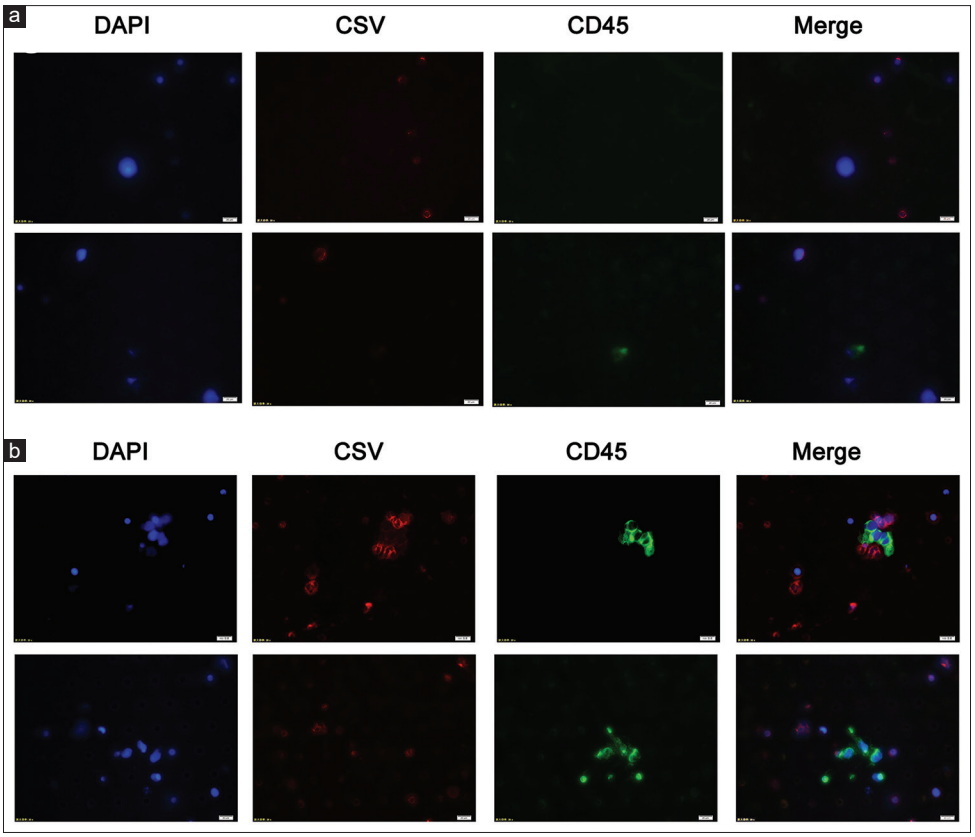

Figure 1: Isolation of CTCs and CTM in peripheral blood samples from GIST patients. (a) CTCs diagram of two different patients with GIST. (b) CTM diagram of two different patients with GIST. Nuclei were stained blue with DAPI, CSV and CD45 were dyed red and green

with disparate risk stratification showed no significant difference [Figure 3e, $P > 0.05$]. Besides, the number of CTCs in patients with unresectable tumor was higher than that in patients before surgery [Figure 3f, $P < 0.05$]. We also explored the effect of tumor location on the number of CTCs, but the result showed no difference. [Supplement Figure 1a].

Variables effecting the number of CTM

The number of CTM is strongly linearly correlated with CTCs with R-square value of 0.708 [Figure 4a, $P < 0.001$]. Unlike the CTCs, the number of CTM did not correspond to tumor diameter [Figure 4b, $P > 0.05$], while the CTM was linearly bound up with Ki 67 expression [Figure 4c, $P < 0.001$]. In addition, patients with a distinct mitotic count, risk stratification, status, and tumor location showed no differences [Figure 4d-f, Supplement Figure 1b, $P > 0.05$].

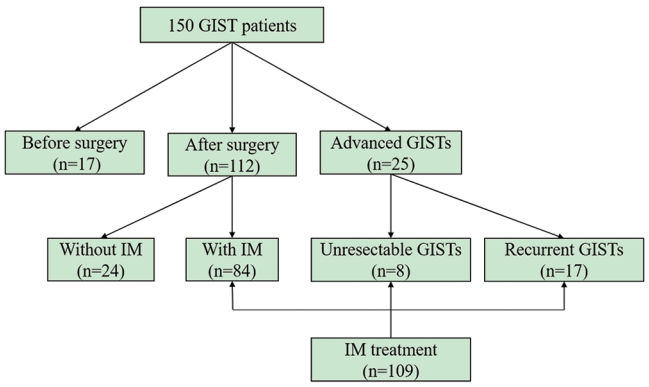

Figure 2: The Flow chart of the study

IM plasma concentration may not affect the CTCs/CTM of GIST patients

The average IM plasma concentration was 1173 ± 425 ng/ml, ranging from 425 ng/ml to 2660 ng/ml. In Figure 3f and 4f, we found that taking IM is not the significant influencing factor of CTC and CTM detection. To further explore whether IM blood concentration could affect the CTCs/CTM, the IM trough plasma concentration of 109 GIST patients was determined by LC-MS/MS method. We divided the 130 patients into 2 groups by the plasma concentration 1100 ng/ml.[24] As shown in Figure 5a and b, there was no difference in the number of CTCs/CTM in patients with different IM blood concentration ($P > 0.05$).

DISCUSSION

Recently, new developments have brought us more ways to diagnose, treat, and evaluate GIST patients, such as liquid biopsy, circulating tumor DNA (ctDNA),

Table 1: Association between clinicopathological variables of GIST patients and positive rate of CTCs

Characteristics	Number	CTC positive (*n*=120)		CTC negative (*n*=48)		*P*
		n	%	*n*	%	
Gender						0.735
Male	86	61	70.93	25	29.07	
Female	64	47	73.44	17	26.56	
Age						0.165
<65	108	80	74.07	28	25.93	
≥65	42	28	66.67	14	33.33	
Location						0.385
Stomach	87	65	74.71	22	25.29	
Extra-stomach	63	43	68.25	20	31.75	
Diameter						0.049*
≤5 cm	48	30	62.50	18	37.50	
5-10 cm	65	46	70.77	19	29.23	
>10 cm	37	32	86.49	5	13.51	
Risk						0.318
Very low/low	16	9	56.25	7	43.75	
Intermediate	47	34	72.34	13	27.66	
High	87	65	74.71	22	25.29	
Condition						0.736
Group A	17	14	82.35	3	17.65	
Group B	24	16	66.67	8	33.33	
Group C	84	59	70.24	25	29.76	
Group D	17	12	70.59	5	29.41	
Group E	8	7	87.50	1	12.50	
Mitotic count						
<5/50 HPF	63	39	61.90	24	38.10	0.042*
≤10/50 HPF	51	41	80.39	10	19.61	
>10/50 HPF	36	39	61.90	24	38.10	
KIT						0.553
EXO 11	77	58	75.32	19	24.68	
Non-EXO 11	17	11	64.71	6	28.57	
WT		3		3		
WT	7	5	71.43	2	28.57	
NON-WT	90	64	71.11	26	28.89	
Ki67						0.032*
≤5%	60	38	0.6667	22	0.3860	
≤10%	46	32	0.6957	14	0.3043	
>10%	44	38	0.8085	6	0.1277	

*$P<0.05$

CTCs, next-generation sequencing (NGS), and artificial intelligence (AI).[25-27] Of these new methods, liquid biopsy is more attractive to clinicians and researchers because of its non-invasive or minimally invasive, accurate, and direct characteristics.[25] CTCs of many solid tumors have been well studied, while CTCs of GIST patients are rarely reported.[14-17] The research of CTCs in varied tumors can be divided into clinical studies and fundamental researches. From a clinical perspective, efforts have been made to summarize the characteristic of CTCs and use it to diagnose the disease early, evaluate prognoses, and even predict relapse.[15,18,19,21] As regard to fundamental researches, investigators explored the mechanisms of CTCs derivation in neoplasm, movement in peripheral blood, communication with other cells or tissues, and formation of metastases.[28-30]

Table 2: Multivariate analysis of clinicopathological factors affecting positive rate of CTC

Variables	*P*	HR (95%CI)	95%CI
Diameter	0.049*	1.145	1.001-1.309
Mitotic count	0.075	1.744	0.946-3.214
Ki67	0.620	1.008	0.977-1.040
Risk	0.492	1.287	0.627-2.643
Location	0.719	1.114	0.619-2.002
Status	0.419	0.828	0.525-1.308
Constant	0.085	0.202	

*$P<0.05$

In this study, we enrolled 150 GIST patients in different disease processes and drew their peripheral blood to isolate the CTCs and CTM. The CTCs and CTM were detected via the ISET method. After detection, we used a variety of statistical methods to analyze the influencing factors of CTCs and CTM. The positive rate of CTCs in our study is 72%, which is similar to previous reports (54-73%).[21,22] While some investigators employed DOG1 to recognize GIST CTCs via qRT-PCR method, we used CSV to isolate the single CTC and counted the exact number of CTCs under the fluorescence microscope.[22] Meanwhile, unlike the metastatic GIST patients enrolled by others, we included GIST patients at various stages of the disease process including before surgery, after surgery with/without IM, recurrent GIST and unresectable GIST.

Here, we included patients from the whole process management of GIST for the first time in a real-life setting. For patients after surgery, the positive rate of CTCs is decreased compared with patients before surgery, but without significant difference. Tumor diameter is the independent factor affecting the positive rate of CTCs. Our results are different from Li *et al*., which found that the tumor size, mitotic count, and risk levels are related to the positive rate of CTC merely by univariate analysis.[21] Regarding CTCs and CTM, we found a number of them have an intensive linear correlation. Moreover, we demonstrated that GIST patients with bigger tumor size, higher Ki 67 expression, and the mitotic count have more CTCs. However, of all the clinicopathological variables, only Ki 67 expression is significantly related to the number of CTM. Certainly, the current study still has some limitations. In a real-life setting study, the design is not as strict as the randomized control trial, and the results may be less reliable. While the sample size could be larger, but the number is not insignificant considering the very low incidence of GIST. We also intended to explore the relationship between IM plasma trough concentration and CTCs/CTM, but the results showed the number of CTCs/CTM would not be affected by the plasma concentration. We speculate that the phenomenon may be due to the fact that IM is a classic kind of molecular targeted drug rather than a cytotoxic drug.

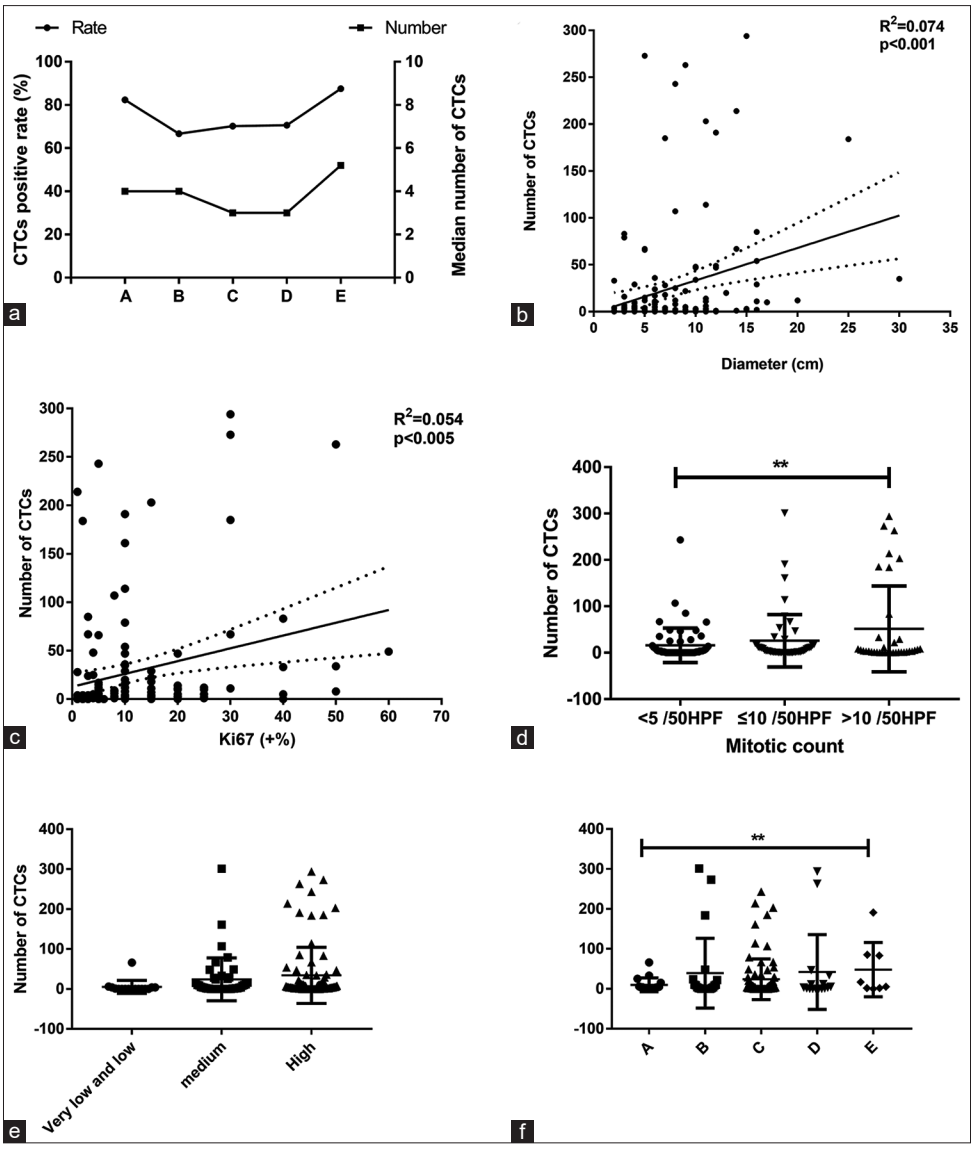

Figure 3: Correlation between clinical-pathological features and the number of CTCs in GIST patients. (a) CTCs positive rate and median CTCs number of GIST patients in different status. The left axis is CTCs positive rate and the right axis is median CTCs number. The abscissa is different state of GIST patients (A, patients before surgery; B, patients after surgery and without IM; C, patients after surgery and treated with IM; D, Recurrent GIST; E, Unresectable GIST). (b) Tumor diameter is correlated with the number of CTCs (R^2 = 0.074; P < 0.001). (c) Ki 67 expression is correlated with the number of CTCs (R^2 = 0.054; P < 0.005). (d) The association of mitotic count and CTCs numbers (** P < 0.01). (e) The association of risk stratification and CTCs numbers. (f) The relationship between patients' status and the number of CTCs (** P < 0.01).

In conclusion, we included GIST patients from different disease processes in the real-life setting study and illustrated that tumor diameter predicted positivity of positive rate of CTCs. Furthermore, we found that the numbers of CTCs and CTM have an intensive linear correlation. Tumor diameter, Ki 67 expression, and mitotic count are related to the number of CTCs. Patients with higher Ki 67 expression tend to have more CTM. These results are instructive for clinicians to understand CTC or CTM in patients with GIST.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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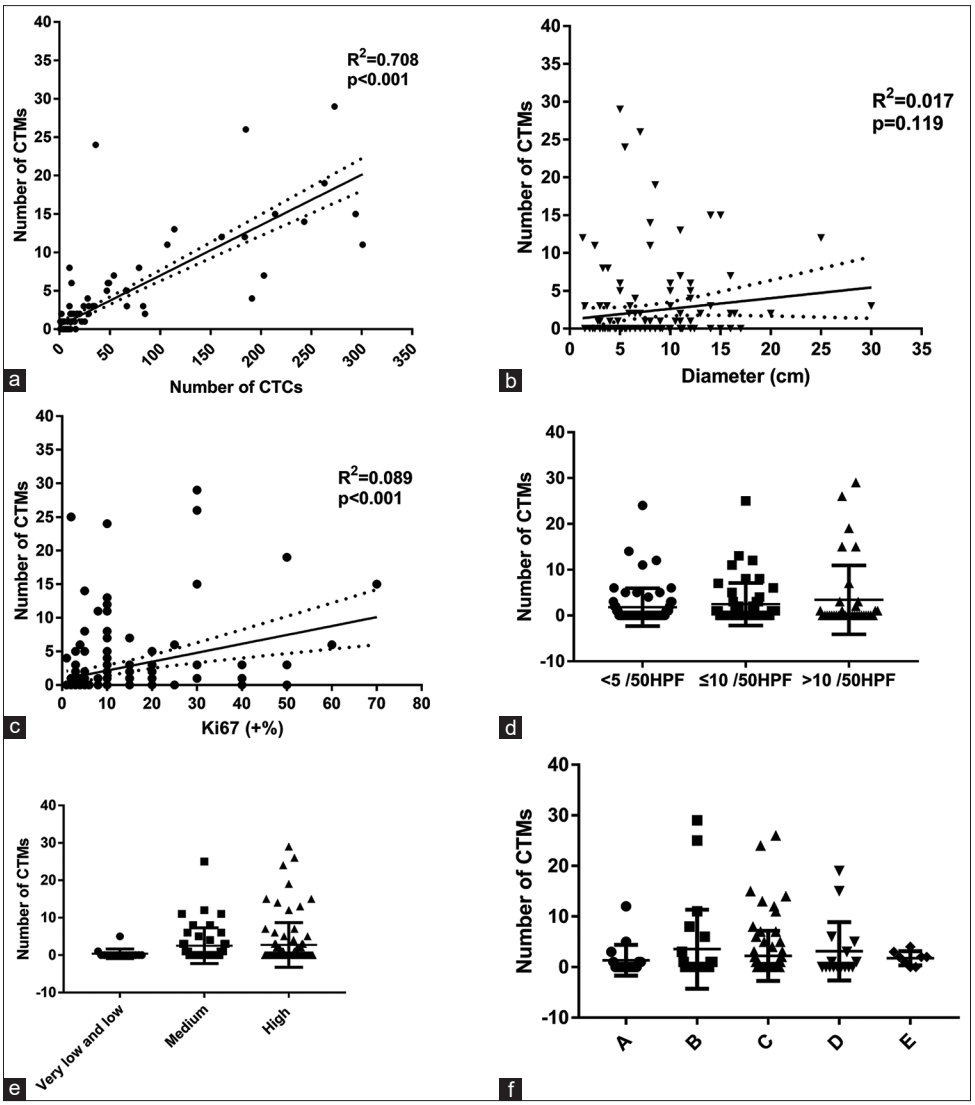

Figure 4: Association of CTM number and clinicopathological variables of GIST patients. (a) The CTM number is significantly correlated with CTCs number ($R^2 = 0.708$; $P < 0.001$). (b) Tumor diameter is not correlated with the number of CTM ($R^2 = 0.017$; $P = 0.119$). (c) Ki 67 expression is correlated with the number of CTM ($R^2 = 0.089$; $P < 0.001$). (d) The association of mitotic count and CTM numbers. (e) The association of risk stratification and CTM numbers. (f) The relationship between patients' status and the number of CTCs

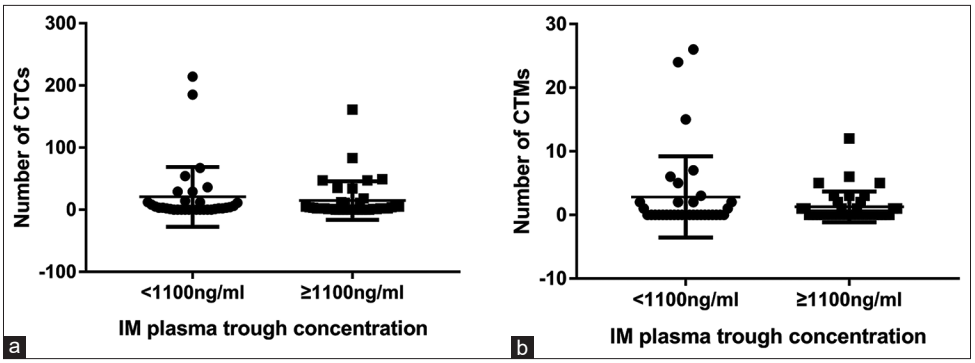

Figure 5: Effect of IM plasma trough concentration on CTCs/CTM. (a) There is no difference in the number of CTCs in patients with different IM blood concentration ($P > 0.05$). (b) There is no difference in the number of CTM in patients with different IM blood concentration ($P > 0.05$)

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Conflicts of interest

There are no conflicts of interest.

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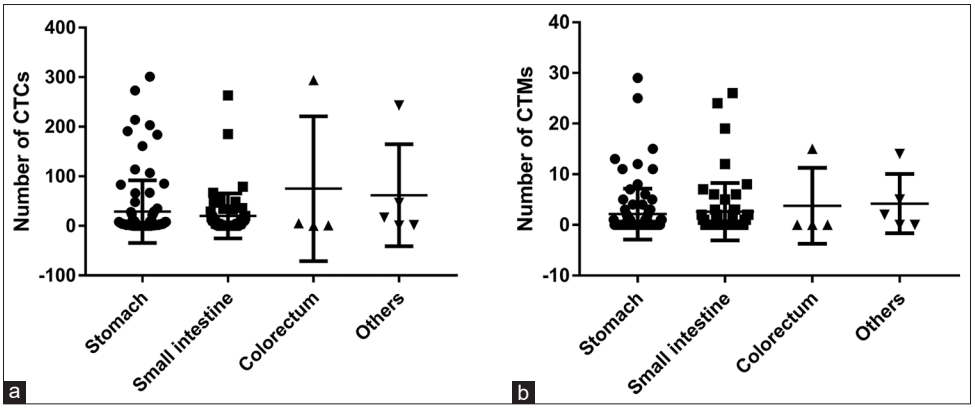
SUPPLEMENTARY FILES

Supplementary Methods: Immunofluorescence staining

The sample was subsequently treated with 200 µl 0.5% Triton X 100 for 5 min and rinsed with PBS for 3 × 2 min. Subsequently, 100 µl 10% goat serum (Jackson ImmunoResearch Europe, Ltd., Newmarket, UK) in PBS was added to the filter membrane and allowed to stand for 30 min at room temperature; the excess serum was removed. The samples were incubated at 4˚C overnight with 100 µl primary antibody (anti CD45; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat no. sc 70699; and anti CSV; Abnova, Taiwan, CN; cat no. H00007431-M08), diluted 1:500 and 1:200, respectively, with 10% goat serum. Thee samples were rinsed with PBS for 3 × 3 min, 100 µl secondary antibody (Alexa Fluor 488 conjugated goat anti rat; cat no. A11006; or Alexa Fluor 647 conjugated goat anti rabbit; cat no. A21245; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) diluted 1:500 with 10% goat serum was added, and the slides were incubated for 50 min at room temperature. Following washing with PBS 3 × 2 min, the films were sealed with DAPI and observed by fluorescence microscopy (magnification, x40). When images of the slides had been captured, Wright Giemsa staining was performed (10,17) for comparison with the immunofluorescence results. The slides were stained with 100 µl diff A (Eosin; YZY Medical Science and Technology Co., Ltd., Wuhan, China; catalog no. YZY CTC P100) for 1 min at room temperature. Following rinsing with PBS for 1 min, 100 µl diffB (Methylthioninium Chloride; YZY Medical Science and Technology Co., Ltd.; catalog no. YZY CTC P100) was added for 90 sec at room temperature. The slides were then rinsed with deionized water three times for 30 sec each time and dried for 30 min at 50˚C. Following mounting with permanent mounting medium (Baso Ultra Clear Advanced Mounting Resin; Baso Biotechnology Co., Ltd.; catalog no. BASE BA 7004), the slides were dried for 1 h at 50°C. Finally, the cells were observed using an optical microscope (magnification, x40).



Supplementary Figure 1: The association of tumor location and CTCs (a) numbers and CTM (b)