Detection of circulating stage III–IV gastric cancer tumor cells based on isolation by size of epithelial tumor: using the circulating tumor cell biopsy technology

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Background: To explore the correlations between circulating tumor cells (CTCs) and the patients' clinical characteristics and other tumor markers in stage III–IV gastric cancer patients, and assess the value of CTCs as a predictor of the efficacy of chemotherapy.

Methods: CTCs were measured in 42 patients with stage III–IV gastric cancer using the isolation by size of epithelial tumor (ISET) method. We analyzed the correlations between the number of CTCs, patients' pathological characteristics and peripheral blood tumor markers. Then divided the 42 patients into two groups, the progressive disease group (PD group) and the disease control rate group (DCR group), according to the efficacy of chemotherapy, and analyzed the differences in the CTC expression between the two groups.

Results: The threshold number of CTCs was closely related to the clinical stage (P=0.044), and was positively correlated with the value in U/mL of CA724 (P<0.05). The treatment response to cytotoxic chemotherapy in the high threshold number group was significantly poorer.

Conclusions: CTCs technology based on ISET method has a high detection rate. CTCs are promising predictor for the evaluation and prediction of treatment responses in stage III–IV gastric cancer.

Keywords: Circulating tumor cells (CTCs); gastric cancer; chemotherapy; CA724; isolation by size of epithelial tumor cell

Introduction

Gastric cancer is one of the most common malignant tumors, and in China the morbidity and mortality from GC rank second among all tumor types (1). According to the 7th Edition of the AJCC Cancer Staging Manual, the prognosis for stage III–IV gastric cancer patients is poor, with the 5-year survival rate ranging from 26.9% to 8.4% (2),

and adjuvant chemotherapy or palliative chemotherapy are the main treatments for these patients. Currently, the evaluation of chemotherapy efficacy mainly relies on end points such as progress free survival (PFS), time to progression (TTP), and overall survival (OS), which cannot monitor the efficacy in real time. Studies have shown that in patients with advanced gastric cancer the prognosis of

the patients with a good chemotherapy response is better than those with a poor response (3). Even patients with metastasis may also achieve longer-term survival through effective chemotherapy (4,5). But more than 40% of the patients show primary or secondary resistance to it (6). Studies have revealed that pre-treatment detection of the biomarkers related to treatment sensitivity can help screen tumor chemosensitivity (7-9). For patients with advanced gastric cancer the impossibility of repeating tumor tissue harvesting limits the capability of tumor tissue biomarkers being a tool for continuous real-time monitoring of clinical efficacy (10,11). Consequently, the detection of circulating tumor cells (CTCs) could serve as a marker to predict prognosis and efficacy.

The term CTCs refers to tumor cells that are shed from primary or metastatic lesions and released into the peripheral blood circulation during the formation and development of solid tumors (12,13). In recent years, CTCs have attracted attention and have been investigated in various tumor studies (14-16). From the year 2004 to 2008, the United States FDA approved the usage of Johnson & Johnson's CellSearch technology for the clinical diagnosis of metastatic breast cancer, prostate cancer, and colorectal cancer (16-18), but for gastric cancer, this technology was not further developed because of a high false negative rate and low detection rate (19-21).

In 2000, Vona *et al.* (22) proposed a membrane isolation method to isolate tumor cells based on size and morphological differences between tumor cells and normal cells, namely, the isolation by size of epithelial tumor cells (ISET) technology. This technology preserves biological characteristics of the cells, allows direct and routine pathological staining, and the observation of CTCs under a light microscope. It can avoid the false negatives caused by the epithelial to mesenchymal transition (EMT) process and can improve the detection rate of CTCs, and it is applicable to all types of tumor cells.

CTCBIOPSY® is a novel one-stop ISET device developed by Wuhan Youzhiyou Medical Technology Co. Ltd, which can automatically isolate and identify CTCs. And it had been approved by the CFDA for clinical application in cancer management. It mainly utilizes the physical differences between CTCs and normal blood cells in cell size (the vast majority of CTCs have a larger diameter than normal blood cells), cell deformability (CTCs are hard to deform but easily trapped on the microporous membranes due to the complex structure formed by keratin and other substances), and cell

chromatophilia. CTCBIOPSY® uses a micro-sieve nano-microporous membrane filter to effectively separate the CTCs from the vast majority of blood cells; retaining most of the CTCs. CTCs do not depend on specific surface markers, and preserves the morphological integrity of the cells.

In this study, we detect CTCs from 42 stage III–IV gastric cancer patients using the ISET technology and analyze the clinical significance of CTCs and its relationship with peripheral blood tumor markers associated with gastric cancer. We then discuss the significance of the CTC threshold number predicting response to treatment in a cohort of III–IV gastric cancer patients receiving chemotherapy.

Methods

Research design

Forty two patients with gastric cancer that had been admitted for treatment to the Union Hospital affiliated to Fujian Medical University from September, 2015 to April, 2016, were incorporated into this study. The study was approved by the hospital's ethics committee and informed consent was obtained from patients before they entered the study. The inclusion criteria included: diagnosed stage III–IV gastric cancer (according to the 7th Edition of the AJCC Cancer TNM Staging Manual, 2010), adenocarcinoma as the pathological type, ECOG PS ≤ score 2, all patients receive routine courses of postoperative adjuvant chemotherapy or palliative chemotherapy. Patients with other malignant tumors at the time of admission or in the past were excluded.

One to three days before starting chemotherapy 5ml of peripheral venous blood was extracted and processed by the CTCBIOPSY® cell staining and isolation apparatus, and CTCs were observed and enumerated under a light microscope. Meanwhile, CA724, CA199, and CEA were detected, and information, including patients' basic clinical features, their chemotherapy regimens, and imaging examinations after chemotherapy, were collected in baseline and every 2 to 3 chemotherapy cycles. The evaluation of the efficacy of therapy was fulfilled according to the Response Evaluation Criteria in Solid Tumor 1.1 (RECIST1.1).

CTCs isolation and staining
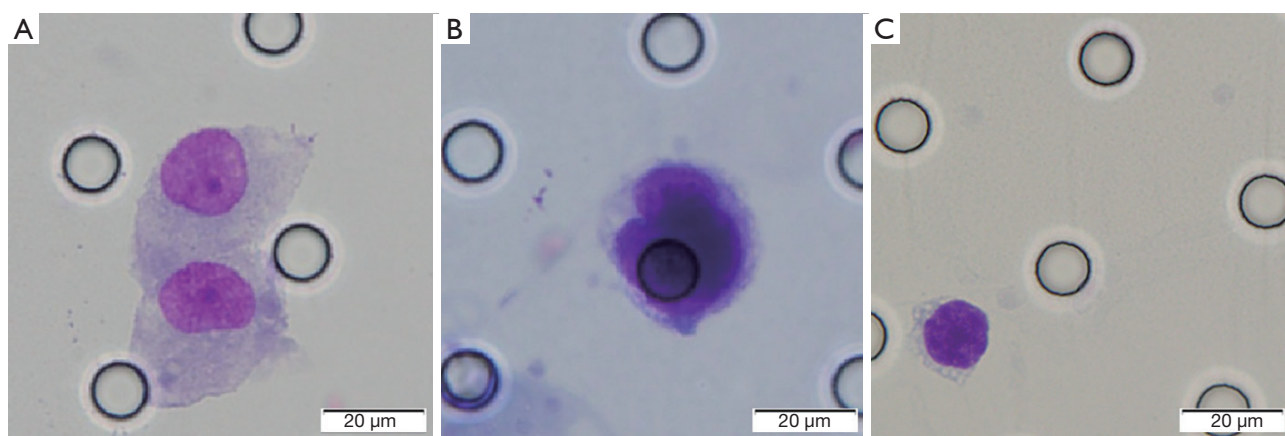
Samples were transferred onto CTCBIOPSY® cell isolation

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Figure 1 CTC morphology with Diff-Quick staining after ISET filtering. (A) Cell diameter >20 µm, nucleo-cytoplasmic ratio >0.8, obvious nuclear heteromorphism. (B) Cell diameter <15 µm, some nuclear heteromorphism, folded nucleus, marginal nuclear chromatin and unevenly stained cell, thus cells in A and B both considered CTCs. (C) Normal blood cells isolated on the membrane. The scale bar represents 20 µm. CTC, circulating tumor cell; ISET, isolation by size of epithelial tumor.

filters, followed by immediately adding 200µl of 4% paraformaldehyde (PFA) solution. The sample and the solution were fully mixed by gentle pipetting with a Pasteur pipette. The sample was then fixed for 10 min at room temperature before CTCs isolation and automatic Diff-Quick staining with the CTCBIOPSY® apparatus. When isolation and staining was complete the filter was removed. First the upper plug of the filter was opened then the lower one was opened. The filter membrane was taken out using a membrane extractor and placed face up on the slide, which was then dried in the air-dry oven at 50 to 60 ℃ for 30 to 60 min. Mounting medium was added dropwise onto the membrane surface, which was then sealed under a cover glass and solidified in an air-dry oven for 30 to 60 min. When completely prepared, the slide was checked for staining and observed under a light microscope.

Result reading

In order to reduce errors, two experienced pathologists reviewed all the counting results. The morphological evaluation parameters for CTCs should meet at least four of the following criterion: (I) nuclear heteromorphism; (II) nucleo-cytoplasmic ratio greater than 0.8; (III) cell length greater than 15 µm; (IV) hyperchromatic and unevenly stained nucleus; (V) thickened nuclear membrane with pits or folds, making it serrated; (VI) nuclear marginal chromatin, or large nucleoli, or abnormal nuclear division (*Figure 1*).

Statistical analysis

All data were analyzed using IBM SPSS version 19.0. Patients' clinical data descriptions are presented as percentages or median values, and the measurement data expressed as mean values ± standard deviations. A *t*-test, variance analysis, or a chi-square (χ^2) test was used to compare the differences between the groups and a Mann-Whitney U-test was used to compare the differences in the tumor marker values between the two groups. GraphPad Prism 7 was used for graph generation analysis. All the data tests were two-tailed, and it was considered statistically significant with a P value is less than 0.05 (P<0.05).

Results

CTC threshold number

A CTCs count of ≥4 per 5 mL was defined as CTC high threshold numbers (23). In 15 out of the 42 cases the patients were positive for CTCs, with the detection rate being 35.7% (*Figure 2*). The number of CTCs detected ranged from 0 to 49 per 5 mL of blood, with a median value of 1 per 5 mL, and the total number of CTCs detected was 173, with a mean value of 4.12±8.60 per 5 mL.

The correlation between CTCs detection and clinical features

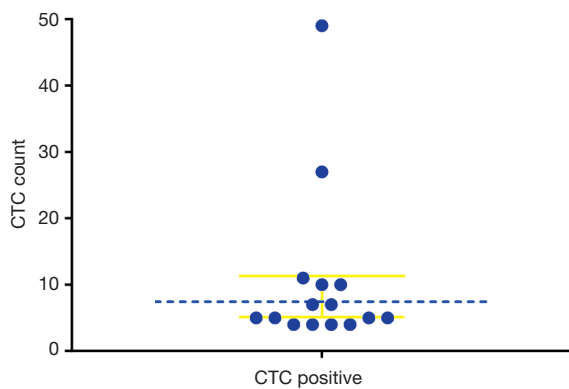The correlations between CTCs detection and clinical

Figure 2 Overview of the CTC positive samples. CTC, circulating tumor cell.

factors are shown in *Table 1*. Of the 25 cases of stage IV gastric cancer patients, 12 presented with CTCs ≥4, accounting for 48%, while in the 17 stage III patients CTCs were found in only 17.6%. The threshold number of CTCs is significantly associated with different clinical stags (P=0.044).

The correlation between CTC rate and the tumor markers in patients with advanced gastric cancer

For the 42 patients with advanced gastric cancer, there is a statistically significant difference in the expression of the tumor marker CA724 between the patients with CTCs ≥4 and those with CTCs <4 (P=0.004), while no differences are observed in the expression of either CEA or CA199 (*Table 2*).

The correlation between CTC rate in peripheral blood of patients with advanced gastric cancer and their chemotherapy regimens and efficacy

The 42 patients were divided into two groups according to the chemotherapy efficacy evaluation standard, namely the disease control rate (DCR) group and the progressive disease (PD) group. DCR was defined as complete response (CR), partial response (PR) and stable disease (SD) of those with prior progression. There were 22 cases in the DCR group, and 4 of them had CTCs ≥4 (18.2%). A total of 20 cases were in the PD group, and 11 of them presented with CTCs ≥4 (55.0%). The difference in the CTCs threshold number between the two groups was statistically significant (P=0.023) (*Table 3*).

Discussion

In our study, CTCs were detected in stage III–IV gastric cancer by using ISET technology, and were found to be associated with Clinical stage, and was positively correlated with the value in U/mL of CA724. The high threshold number of CTCs in peripheral blood indicates poor tumor response to chemotherapy in patients with gastric cancer. CTC counts can serve as an indicator in predicting efficacy of chemotherapy for stage III–IV gastric cancer.

In this study, CTCs were enriched and identified by ISET combined with cytology. The CTCs were detected in 17 out of 42 patients with stage III–IV gastric cancer (38.6%), which was more sensitive than other approaches previously reported. Okabe *et al.* (24) used the CellSearch system to evaluate 140 patients with advanced gastric cancer in 2016, with CTCs ≥2 per 7.5 mL being their positive standard, and only 25 (18.4%) were positive for CTC. Matsusaka *et al.* (23) assayed for CTCs in the peripheral blood of 52 patients with advanced gastric cancer, and the CTC positive rates in the PD and the non-PD groups were 13.4% and 19.2% respectively, when CTCs ≥4 per 7.5 mL was considered positive. Khoja *et al.* (25) was also observed that compared with the Celltracks AutoPrep system, ISET has the greater sensitivity. The ISET rules out the interference of the false negative results caused by tumor cells that show no expression or incomplete expression of the EpCAM antigen, especially the ones undergoing EMT, and it detects more CTCs with a smaller amount of blood (5 mL), greatly boosting the detection sensitivity.

This study has also found that the incidence of CTC positive was closely correlated to the clinical staging of the tumor. This may be due to the fact that stage IV gastric cancer patients possess more aggressive tumor cells that more easily break through their surrounding blood vessels and enter the peripheral blood circulation. There have been several meta-analyses pointing out that stage III–IV gastric cancer patients compared with stage I–II gastric cancer patients have a markedly increased incidence of CTC positive results (26,27). Zheng *et al.* (28) used the ISET method to detect CTCs and have shown that stage IV gastric cancer patients have much higher numbers of CTCs detected than do stage I, II, III patients. Therefore, detection of the CTCs in patients' peripheral blood may be a supplement to the TNM staging system, and the number of CTCs may be an important prognostic indicator. Although in this study, between the CTC positive group and the CTC negative group, the number of cases and

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Table 1 Relationship between CTC and basic clinical features

Basic clinical feature	CTC threshold numbers		χ^2 or t	P value
	≥4 CTC/mL (N=15) (%)	<4 CTC/mL (N=27) (%)		
Gender			1.310	0.252
Male	12 (41.4)	17 (58.6)		
Female	3 (23.1)	10 (76.9)		
Tumor site			0.036	0.982
Fundus/cardia	3 (37.5)	5 (62.5)		
Antrum pylorus	9 (34.6)	17 (65.4)		
Body	3 (37.5)	5 (62.5)		
Age	59.33±12.40	59.41±15.61	0.016	0.987
Grade of differentiation			3.389	0.066
Moderate or well differentiated	2 (15.4)	11 (84.6)		
Poorly differentiated	13 (44. 8)	16 (55.2)		
Clinical stage			4.061	0.044
Stage III	3 (17.6)	14 (82.4)		
Stage IV	12 (48.0)	13 (52.0)		
Tumor depth			0.084	0.359
T3	3 (25.0)	9 (75.0)		
T4	12 (40.0)	18 (60.0)		
Lymph node metastasis			0.104	0.747
N1–N2	8 (38.1)	13 (61.9)		
N3	7 (33.3)	14 (66.7)		
Distant metastasis			1. 847	0.174
No	4 (23.5)	13 (76.5)		
Yes	11 (44.0)	14 (56.0)		
Vessel carcinoma embolus			0.089	0.956
Negative	5 (38.5)	8 (61.5)		
Not detected	4 (36.4)	7 (63.6)		
Positive	6 (33.3)	12 (66.7)		
Surgery			2.586	0.274
Radical surgery	5 (25.0)	15 (75.0)		
Palliative surgery	5 (55.6)	4 (44.4)		
No surgery	5 (38.5)	8 (61.5)		

CTC, circulating tumor cell.

Table 2 Relationship between positivity or negativity of CTC and the tumor markers of gastric cancer

Gastric cancer related tumor markers	CTC threshold numbers		Z	P value
	≥4 CTC/mL	<4 CTC/mL		
CA724 (U/mL)	28.72±37.14	10.63±16.22	2.849	0.004
CEA (ng/mL)	13.11±18.24	34.06±70.86	0.578	0.563
CA199 (U/mL)	126.42±282.79	130.19±259.13	0.433	0.665

CTC, circulating tumor cell.

Table 3 Relationship between positivity or negativity of CTC and chemotherapy sensitivity

Variable	CTC threshold numbers		χ^2	P value
	≥4 CTC/mL (N=15) (%)	<4 CTC/mL (N=27) (%)		
Chemotherapy response				
DCR	4 (18.2)	18 (81.8)	6.185	0.023
PD	11 (55.0)	9 (45.0)		

CTC, circulating tumor cell; DCR, disease control rate; PD, progressive disease.

percentage differences in the degree of differentiation, depth of invasion, and distant metastasis were relatively large, no statistically significant difference was found (P>0.05). This may be associated with the small number of cases and further research is needed to confirm our findings.

In this study, it was found that there was a statistically significant difference in the presence of the tumor marker CA724 between the CTC positive and the CTC negative group. At present, CA724 detection is attracting increasing attention due to its high sensitivity and specificity in the diagnosis of gastric cancer (29). A meta-analysis has demonstrated that serum CA724 is the most relevant gastric cancer tumor marker, and its correlation with gastric cancer is significantly superior to CEA, CA199, CA242, CA125, or CA153 (30). Moreover, CA724 is also related to the prognosis of gastric cancer patients and the efficacy of chemotherapy. The probability of metastasis of diffuse gastric cancer is as high as 36%, and some studies have shown that CA724 can be used to detect lymph node metastasis of gastric cancer (31). Zou *et al.* (32) also found that the serum CA724 level was related to the chemotherapy efficacy on advanced gastric cancer. The number of patients with PD + stable disease chemotherapy results in their CA724 >8.2 U/mL group was significantly higher than that in the CA724 <8.2 U/mL group. Our study found that the presence of CTCs is very well correlated with CA724 and, to a certain extent, to the

chemotherapy efficacy of patients with advanced gastric cancer. It can potentially be part of a combined index to evaluate the tumor sensitivity of chemotherapy drugs and the efficacy of future chemotherapy, and whether a patient has recurrence or metastasis. However, the value of this combination still needs to be confirmed by large-sample clinical trials and in-depth and standardized long-term clinical follow-ups.

It can be perceived from the statistical analyses in this experiment that the PD group had a significantly higher CTC threshold number than the DCR group. As for advanced gastric cancer, there are very few articles on clinical detection of CTCs to evaluate the efficacy of chemotherapy in patients. In one study, CellSearch was used to detect the number of CTCs in blood from 95 patients with metastatic gastric cancer before chemotherapy, and it was found that the probability that patients were evaluated as PD after chemotherapy in the CTCs ≥5 per 7.5 mL group was much higher than in the CTCs <5 per 7.5 mL group (60% *vs.* 23.4%) (33). Another study in Japan that involved 52 patients with advanced gastric cancer found that two weeks and four weeks after chemotherapy, the number of OS and PFS patients with a high number of CTCs is significantly lower than for patients with a low number of CTCs. This suggests a patient with a high CTCs count may have stronger drug resistance to chemotherapy and poorer efficacy (23). The above results

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are consistent with the conclusions of our study. Tumor cells in primary and metastatic lesions in cancer patients are heterogeneous (34). Some chemotherapy regimens or drugs are effective for primary lesions but ineffective for metastatic lesions, making it difficult to achieve an accurate judgment with conventional examinations. CTCs is closely related to cancer metastasis, so detection of CTCs can indirectly reflect the response of tumor cells in metastatic lesions to chemotherapy drugs, so as to evaluate chemotherapy regimens or drug efficacy (35). The CTCs test may also imply efficacy of chemotherapy earlier than imaging examinations, and evaluate in real time without the interference of radiation side effects (36). Studies have found that in the process of EMT the CTCs can acquire stem cell properties, which can result in drug resistance in tumor patients and resistance to chemotherapy (37). The more mesenchymal cells there are among the tumor cells in patients, the stronger the drug resistance may be (38). In our study we detected CTCs by means of ISET, excluding the interference from EMT, so the reliability and the sensitivity were well guaranteed.

Since the sample size was small in the study, and the CTCs tests were only run before the treatment, enlarging the sample size and dynamic monitoring of patients will be warranted to further study the significance of the CTCs detected by ISET technology in the treatment of gastric cancer.

Conclusions

This study utilized the ISET technology to monitor CTCs in peripheral blood with high sensitivity, and found that patients with higher CTCs counts might be more likely to be prone to drug resistance and have a poor prognosis, and that the detection of the number of CTCs could predict the chemotherapy efficacy. Our study also revealed that the level of CA724 was positively correlated with CTCs expression in the peripheral blood of advanced gastric cancer patients. Combined detection of these two indicators has the potential to be a predictive index for chemotherapy efficacy in patients with advanced gastric cancer.

Acknowledgments

Footnote

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