Original Article

Circulating Tumor Cells in Metastatic Breast Cancer: Monitoring Response to Chemotherapy and Predicting Progression-Free Survival

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ABSTRACT

Objective: The purpose of this study is to explore RT-PCR method to set up the examination platform for detecting circulating tumor cells (CTC) in peripheral blood from metastatic breast cancer patients. The primary endpoint is to find out the correlation of existence of CTC with clinical responses and progression-free survival (PFS).

Methods: The breast cancer cell line MCF-7 was serially diluted into the peripheral blood from 45 healthy donors to set up the sensitivity of RT-PCR assay. The expression of CK19 mRNA was amplified from both 49 patients and 45 healthy donors respectively. The CK19 protein quantity from plasma was measured by competitive inhibition ELISA assay.

Results: The sensitivity of RT-PCR could reach $1/10^6$–10^7 white blood cells with specificity of 95.6%. The objective response rate (ORR) of patients with CK19 mRNA-negative undertaken one cycle chemotherapy was significantly higher than those with positive (P<0.0001). PFS among CK19 mRNA-negative patients was also increased, although there was no significance (P=0.098). The results of ELISA assay showed that CK19 protein was decreased significantly after one cycle chemotherapy, which gave rise to a little higher ORR (P=0.015) and increased PFS (P=0.016).

Conclusion: Patients with unamplified CK19 mRNA after one cycle chemotherapy could achieve better radiographic evaluation and increased PFS, which was showed to be of consistency with the CK19 protein assay among the patients treated.

Key words: Breast cancer; Circulating tumor cells; CK19; RT-PCR; ELISA

INTRODUCTION

Despite early diagnosis, accurate surgical and adjuvant chemotherapy after surgery are conducted and the mortality of breast cancer is decreased, most of patients eventually die from relapse and metastasis. After the metastasis of breast cancer appears, the treatments are more troublesome. With some effective drugs as well as combination therapy, patients with breast cancer achieve longer overall survival to some extent. However, if there is no timely and efficient tool to monitor the therapy efficacy for metastatic breast cancer patients, there may be a long-term invalid treatment or interruption of a potentially effective therapy.

A simple and efficient tool is required to guide physician to choose therapeutic regimen and monitor treatment precisely. Based on the theory of tumor cell dissemination, there were many

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documents reported[1, 2] that CTC in breast cancer patients was an independent prognostic factor. Some researcher concluded that CTC was also correlated with PFS[3]. In order to further clarify the relationship between CTC of metastatic breast cancer and chemotherapy efficacy and PFS, in this study CTC was detected in peripheral blood of newly diagnosed metastatic breast cancer patients before and after first cycle of chemotherapy by RT-PCR method with detecting CK19 mRNA. CK19 mRNA is a common and reliable marker of CTC. CK19 protein was detected by ELISA. We analyzed whether the expression of CK19 mRNA before and after the first cycle of chemotherapy can predict imaging evaluation of two cycles of chemotherapy efficacy. The relationship between CK19 mRNA and PFS was also investigated. We further analyzed the link between CK19 protein before and after the first cycle of chemotherapy and imaging evaluation of two cycles of chemotherapy efficacy and PFS.

MATERIALS AND METHODS

Patients

A total of 58 patients were collected from May 2009 to March 2010 in Beijing Cancer Hospital. All the Patients were female. Median age was 50.5 (spanning 27–72 years old), nine patients lacked paired results, including four had no initial CTC assessments, and five had no subsequent clinical and/or radiographic documentation of disease status. Thus, 49 patients were evaluable for the correlation between CTC detection and radiographic imaging (they had at least two pairs of results after the baseline visit). All patients signed an informed consent to participate in the study. Clinical and pathological features are shown in Table 1.

Eligibility criteria included: (1) newly diagnosed breast carcinoma patients with distant metastasis; (2) planed to first-line chemotherapy; (3) radiographically measurable disease per Response Evaluation Criteria in Solid Tumors (RECIST).

Treatment and Follow-up

Eligible patients received treatment of docetaxel-based chemotherapy regimen, which was docetaxel 75 mg/m^2 (d1, d8) plus capecitabine 1250 mg/m^2 (d1–14) or thiotepa 60 mg/m^2 (d1) repeated every 3 weeks. All the patients completed a baseline imaging evaluation and blood collection for detecting CTC before starting the first cycle of chemotherapy. After the first cycle of chemotherapy, blood samples were collected on the day before the date starting the second cycle of chemotherapy, and imaging evaluation were performed again after two cycles of chemotherapy. The results of imaging evaluation were assessed by RECIST (Response Evaluation Criteria in Solid Tumors), and divided into: complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD). CR or PR means effective therapy, SD or PD means invalid. The patients were followed up for 12 months and information was collected from study entry until the day of the first evidence of progressive disease. The relationship between CTC and PFS was investigated. We also analyzed the link among CK19 protein level, imaging evaluation and PFS. The route of study was showed in Figure 1.

Table 1. *Clinical data of 49 patients with metastatic breast cancer*

Features	No (%)
Menopausal status	
Postmenopausal	37(75.5%)
Premenopausal	12 (24.5%)
ECOG	
0	32 (65.3%)
1	17 (34.7%)
ER	
Positive	27 (55.1%)
Negative	22 (44.9%)
PR	
Positive	33 (67.3%)
Negative	16 (32.7%)
HER2	
Positive	19 (38.8%)
Negative	24 (49.0%)
Unknown	6 (12.2%)
Metastatic sites	
Liver	23 (46.9%)
Bone	22 (44.9%)
Lymph nodes	30 (61.2%)
Chest wall	13 (26.5%)
Pleura	11 (22.4%)
Lung	23 (53.1%)

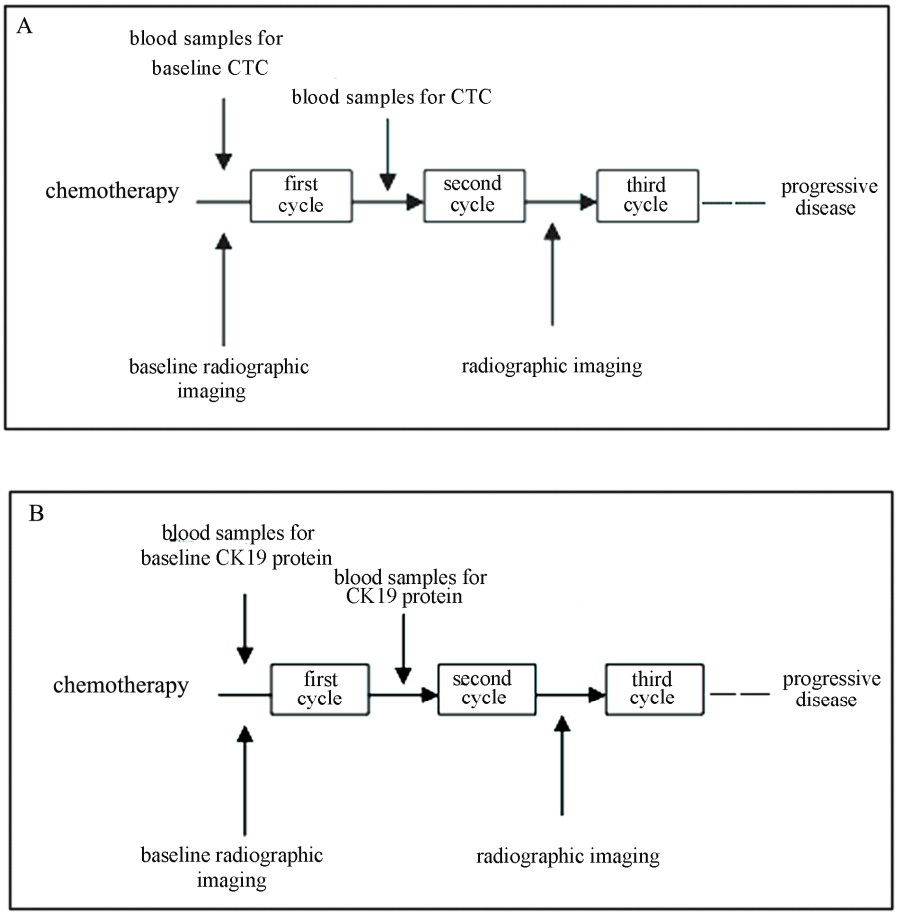

Figure 1. Study design. A: the timing and strategy of detection of CTC and imaging evaluation; B: the timing and the strategy of detection of CK19 protein and imaging evaluation.

Sensitivity and Specificity of RT-PCR for Detecting CTC

MCF7 cells were purchased from Cell Center of Chinese Academy of Medical Sciences, and were cultured in Dulbecco's modified Eagle's medium with 10% calf serum at 37°C in 5% CO_2 incubator. MCF-7 cells were diluted into the peripheral blood of healthy volunteers to evaluate the sensitivity of this method. Different numbers of MCF-7 cells (10^4, 10^3, 10^2, 10^1, 10^0, 0) were mixed in 4 ml peripheral blood of healthy volunteers respectively, which simulated directly CTC of patients with breast cancer. Then we evaluated the sensitivity by detecting CK19 mRNA by RT-PCR. CK19 mRNA was detected in 45 healthy volunteers to determine the specificity.

Detection of Circulating Tumor Cells with RT-PCR

The first sample was collected immediately before the first cycle of chemotherapy; the second was collected after the first cycle (the day before the date starting the second cycle of chemotherapy). Blood samples were mixed with anti-coagulant EDTA. The blood of first tube was susceptible to be contaminated by epithelial cells and discarded. To prevent RNA degradation, the blood was processed within 30 min after collected. Mononuclear cells were enriched by density gradient. Peripheral blood RNA was extracted according to Trizol method (Trizol reagent, Invitrogen Inc. USA). RNA reverse transcription experiment was completed according to the instructions of reverse transcriptase kit (Promega Inc. USA). cDNA solution obtained from reverse transcription experiment was diluted to 100 μl with nucleic acid free water, and 2 μl cDNA was taken for polymerase chain reaction according to the PCR kit. mRNA free sample was used as negative control. Plasmid containing the full length CK19 sequence was used as positive control.

The primers and product size were as follows,

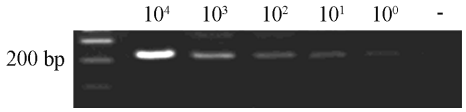

Figure 2. The breast cancer cell line MCF-7 was serially diluted into the peripheral blood from 45 healthy donors to detect CK19 mRNA, the number of MCF-7 cells was 10^4, 10^3, 10^2, 10^1, 10^0, 0.

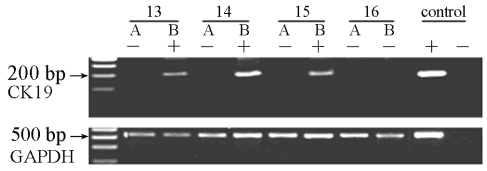

Figure 3. The expression of CK19 mRNA and GAPDH of No.13–16 patients was showed. A was before the first cycle; B was after the first cycle. "-": negative, "+": positive.

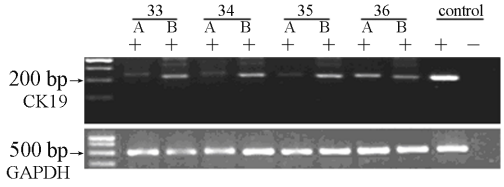

Figure 4. The expression of CK19 mRNA and GAPDH of No. 33–36 patients was showed. A was before the first cycle; B was after the first cycle. "-": negative, "+": positive.

for CK19: Forward: 5'-ATGCGAAGCCAATAT-GAGG-3', Reverse: 5'-TCCGTTTCTGCCAGTG-TGT-3', 230 bp; for GAPDH: Forward: 5'-GTC-AACGGATTTGGTCGTATT-3', Reverse: 5'-AGT-CCTCTGGGTGGCAGTGAT-3', 540 bp. Cycle parameters were as follows: for GAPDH: 95°C for 5 min; 26 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min; 72°C for 7 min, for CK19: 95°C for 5 min; 35 cycles of 94°C for 15 s, 60°C for 15 s, 72°C for 15 s; 72°C for 7 min. Finally the PCR products were identified.

To ensure comparability of the experiments, all the reagents, laboratory conditions, experimental and operating procedures were kept strictly consistent.

ELISA (Competitive Inhibition Assay)

CK19 protein in peripheral blood plasma was detected by CK19 ELISA kit (GBD Inc. Canada). Experimental procedures were performed strictly in accordance with the operation instructions of ELISA kit. According to kit instructions, data were processed using CurveExpert1.4 to draw the standard curve.

Statistical Analysis

All the statistical results of data analysis were obtained using SPSS version14.0. The results of CK19 mRNA expression are divided into two categories: negative and positive. The positive result of CK19 mRNA means the discovery of CTC, the contrary result means negative. The relationship between CTC and the efficacy of chemotherapy was conduct by the chi-square analysis. Using paired T test analyzed the changes of CK19 protein before and after chemotherapy. The relationship between CK19 protein with elevated or reduced level and chemotherapy efficacy was obtained by the chi-square analysis. Survival curves were draw by Kaplan-Meier survival analysis, log-rank test was used to compare the difference between groups. P value <0.05 is considered that there is a statistical significance.

RESULTS

Sensitivity and Specificity of RT-PCR Test

The sensitivity of RT-PCR method for detecting CK19 mRNA could detect one tumor cell in 4 ml peripheral blood. In other words, it could detect $1/10^6$–10^7. The result was shown in Figure 2.

In order to clarify the specificity of the method, CK19 mRNA was detected in peripheral blood of 45 healthy volunteers by this method. It was detected only in two cases of 45 healthy volunteers. The method had a high specificity of 95.6%.

Detection of CK19 mRNA in Metastatic Breast Cancer

We collected blood sample from 49 patients with newly diagnosed metastatic breast cancer before the first and second cycle of chemotherapy, and detected CK19 mRNA. We selected No. 13-16 and 33–36 patients as representative of all the patients (Figure 3, 4). 73.5% of the patients were CK19 mRNA-positive before the first cycle of

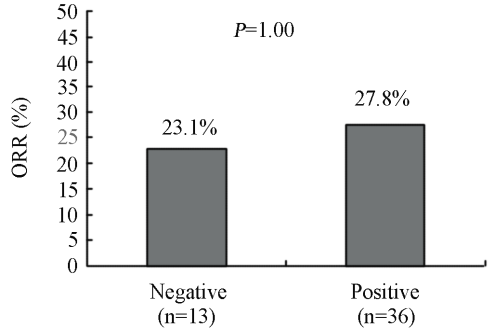

Figure 5. The ORR of patients with CK19 mRNA-negative before the first cycle was 23.1%, while that of ones with CK19 mRNA-positive was 27.8%. There was no significant difference. ORR: objective response rate.

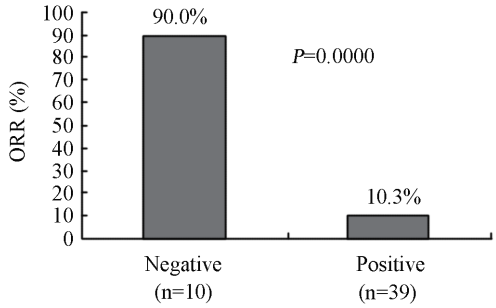

Figure 6. The ORR of patients with CK19 mRNA-negative after the first cycle was 90.0%, which was significantly higher than that of CK19 mRNA-positive ones with 10.3%. ORR: objective response rate.

chemotherapy, and the CK19 mRNA-positive rate increased to 79.6% after one cycle chemotherapy. The change had no significant difference (P=0.902).

The Relationship between CK19 mRNA and Chemotherapy Efficacy

The Relationship between CK19 mRNA before the First Cycle of Chemotherapy and Chemotherapy Efficacy

Based on the results of imaging evaluation after two cycles of chemotherapy, the ORR for patients with negative and positive CK19 mRNA expression before the first chemotherapy cycle was 23.1% and 27.8% respectively. The expression of CK19 mRNA before the first chemotherapy cycle had nothing to do with chemotherapy efficacy (P=1.000, Figure 5).

The Relationship between CK19 mRNA after the First Chemotherapy Cycle and Chemotherapy Efficacy

After the first cycle of chemotherapy, the ORR of patients with CK19 mRNA-negative was 90.0%, CK19 mRNA-positive patients' ORR was 10.3%, the ORR was significantly different (P<0.0001, Figure 6), which indicated that after the first chemotherapy cycle, those with CK19 mRNA-negative had better results of imaging evaluation than CK19 mRNA-positive ones. The results above showed CK19 mRNA-negative patients after the first chemotherapy cycle achieved better results of clinical imaging evaluation than positive ones, suggesting the detection of CK19 mRNA after the first chemotherapy cycle can predict the results of clinical imaging evaluation.

The Correlation between CK19 mRNA and PFS

In order to further clarify the relationship between CK19 mRNA and PFS, we followed up patients for 12 months. The results showed that CK19 mRNA before the first chemotherapy cycle had no correlation with PFS (P=0.304, Figure7); PFS of patients with CK19-negative after the first chemotherapy cycle was better than CK19-positive ones, but had no significant difference (P=0.098, Figure 8).

The Relationship between CK19 Protein in the Peripheral Blood and Chemotherapy Efficacy and PFS

The average concentration of CK19 protein before the first chemotherapy cycle was 3.85 ng/ml, and significantly reduced to 3.16 ng/ml after one cycle chemotherapy (P=0.001).

The ORR of the patients with reduced CK19 protein was 38.2% and that of patients with CK19 protein increased was 0%. The patients with decreased CK19 protein had significantly better results of imaging evaluation than patients with increased ones (P=0.015, Figure 9).

The relationship between CK19 protein expression and PFS showed that PFS of patients with decreased CK19 after one cycle of chemotherapy was better than patients with increased level (P=0.016), as shown in Figure 10. The data indicated that the detection of CK19 protein before and after one chemotherapy cycle could predict the results of clinical imaging

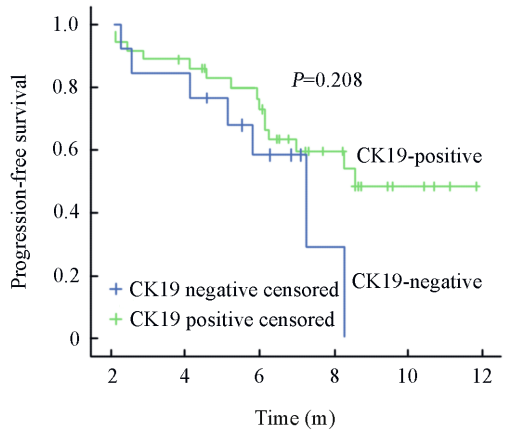

Figure 7. PFS of patients with CK19mRNA-negative before the first cycle had no difference from that of ones with CK19 mRNA-positive. PFS: progression-free survival.

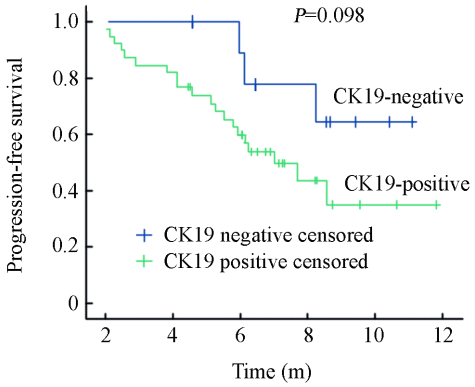

Figure 8. PFS of patients with CK19mRNA-negative after the first cycle was better than that of CK19 mRNA-positive ones. PFS: progression-free survival.

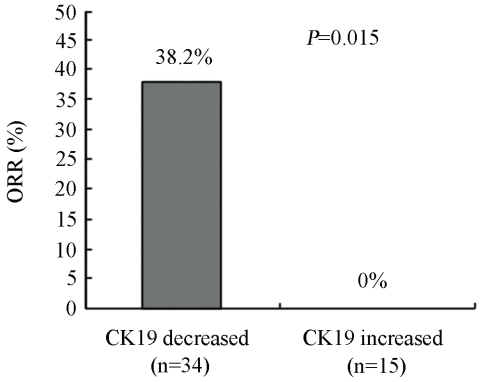

Figure 9. The ORR of patients with CK19 protein decreased after the first cycle was 38.2%, which was significantly higher than that of ones with CK19 protein increased with 0%. ORR: objective response rate.

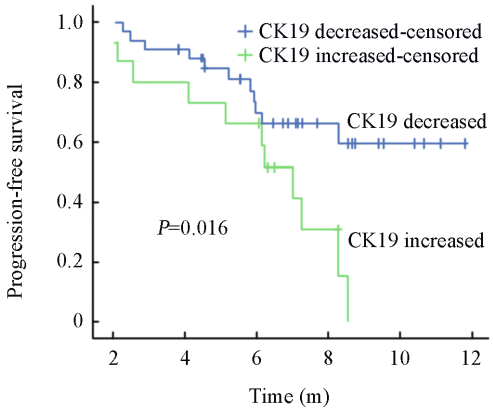

Figure 10. PFS of patients with CK19 protein decreased after the first cycle was better than that of ones with CK19 protein increased. PFS: progression-free survival.

evaluation after two cycles of chemotherapy and PFS.

The Relation of Conventional Clinical Factors with ORR and PFS

There are many conventional clinic pathologic factors known to be associated with prognosis, suchas menopausal status (premenopausal vs post-menopausal), ECOG, ER status (negative vs positive), PR status (negative vs positive), HER-2/neu status (positive vs negative), tumor burden, and so on. We analyzed the relation of these factors with ORR. We found that these factors had no relation with ORR and PFS except PR. The ORR of PR-negative patients was 36.4%, much higher than PR-positive patients 6.7% ($P<0.05$). We further analyzed the relation of these factors with PFS. There was no relationship between these factors and PFS.

DISCUSSION

The therapy efficacy of metastatic breast cancer (MBC) is relatively poor and the objective response rate is low[4]. Palliation of metastatic disease could be achieved by considering

pretreatment prognostic and predictive factors, which could assist in choosing a therapy with the greatest likelihood of patient benefit[5]. Factors that may accurately predict prognosis and treatment efficacy in patients with MBC are very important. Therefore, we need a timely and efficient tool to predict accurately the efficacy. CTC in peripheral blood of patients could monitor the treatment of metastatic breast cancer and would be a simple and efficient tool. Yagata et al.[6] used the Cell Search System to detect tumor cells in peripheral blood, found that PFS of patients with >5CTC/7.5 ml was significantly shorter than patients with <5CTC/7.5 ml (P=0.0036). This showed CTC-positive patients might reflect less effective therapy. Other researchers obtained the same results[7, 8]. Therefore, the detection of CTC in peripheral blood could evaluate the therapeutic effect of patients with metastatic breast cancer accurately and efficiently.

In order to further clarify the relationship between the CTC of metastatic breast cancer and chemotherapy efficacy and PFS, our study detected CTC in peripheral blood of newly diagnosed metastatic breast cancer patients before and after first chemotherapy cycle with RT-PCR method by detecting CK19 mRNA. CK19 is a common and reliable marker of CTC[9−11]. We analyzed whether the level of CK19 mRNA before and after first cycle of chemotherapy could predict the results of the imaging evaluation.

There are many methods for detecting CTC in patients with metastatic breast cancer. The sensitivity of different methods are different. RT-PCR has the highest sensitivity[12]. The positive rates of CTC are different with regard to breast cancers of different stages[13]. The heavier the tumor burden was, the higher the positive rate of CTC was in metastatic breast cancer, which had been reported in many documents[1, 14]. The positive rate of CTC in metastatic breast cancer is about 50%−70%. In our study, using RT-PCR method, CK19 mRNA was tested in 49 patients with newly diagnosed metastasis breast cancer before and after the first cycle of chemotherapy. The positive rates of CK19 mRNA were 73.5% and 79.6% respectively. Comparing with the reported data, the positive rate of this study was higher, the reasons may be that the tumor burden of the patients enrolled in this study was very heavy, and the sensitivity of this method was also higher.

A number of studies have reported that the existence of CTC to some extent reflected the effect of chemotherapy[15]. Bidard et al.[16] reported that CTC positivity was a significant prognostic marker for PFS at a threshold of 3 CTC/7.5 ml (P< 0.05), but not at 5 CTC/7.5 ml. These studies indicated that the detection of CTC can predict the effect of chemotherapy. We detected CTC nucleic acid marker CK19 mRNA before and after the first cycle of chemotherapy and found that CTC before the first chemotherapy cycle had no relation with ORR or PFS. The ORR of CTC-negative patients after the first cycle of chemotherapy was significantly higher than CTC-positive patients (P<0.0001). We found that CTC could predict radiographic evaluation of chemotherapy efficacy and other common conventional factors had no relation with ORR and PFS, such as ER, HER2 and tumor burden. The study manifested that CTC seemed to be superior or additiv to other conventional factors, which conformed to the results of other studies. De Giorgi, et al.[17] considered that CTC was superior to radiographic assessment, including computed tomograms. It was concluded that CTC was a better indicator of therapeutic effect than common predictive factors. Cristofanilli et al.[18] reported that metastatic breast cancer patients with >5CTC/7.5 ml blood had worse PFS and OS, which had a higher predictive value than tumor burden and tumor subtype. These results conformed to our studies.

If CTC could still be found after one cycle chemotherapy, it indicated that the results of radiographic evaluation would be worse. This suggested that the chemotherapeutics might not be sensitive or resistant to patients, and need change the chemotherapy regimen. If the patients were CTC-negative before chemotherapy and became positive after one cycle chemotherapy, it could predict that the patients would have worse chemotherapy efficacy after two cycles of chemotherapy. We proposed to change chemotherapy regimen for this type of patients, rather than wait until progressive disease appearing after imaging evaluation of two cycles of chemotherapy.

Molecular characterization of primary tumor tissue by gene expression profiling had shown to yield prognostic and predictive models in breast cancer[19]. However it was likely that in the tumor metastatic setting, molecular characterization of CTC represents tumor genetics than that of primary tumors. Studies had shown that expressions of clinically relevant markers such as ER, PR and HER2 could be different between the primary tumor and its metastases[20]. Prospective studies should clarify whether it was indeed of benefit, for example, to start trastuzumab therapy when the CTC of a prior HER2-negative primary tumor do

expressed HER2. A predictive and prognostic gene expression model for CTC could be of great help for the oncologist to make treatment decisions as the disease progressed. Therefore, the detection of CTC would play an important role in individual treatment of metastatic breast cancer.

Detection of CTC to predict chemotherapy efficacy for patients with no measurement of lesions may be more useful, such as patients with only bone metastasis. Imaging evaluation for these types of patients may be not helpful. The evaluation of chemotherapy efficacy can only be based on clinical symptoms observed. CTC can be detected for these patients to assess whether the treatment is effective or not. In addition, CTC in peripheral blood can be used to estimate adjuvant chemotherapy effect for early breast cancer patients, because the tumor has been removed and chemotherapy effect is generally not evaluated. Analyzing the changes of CTC before and after chemotherapy may be good for evaluation of chemotherapy effect. Pierga and colleagues[21] monitored CTC in 118 patients with large operable or locally advanced breast cancer before and after neoadjuvant chemotherapy, and showed that the presence of CTC after a short follow-up time of 18 months was an independent prognostic factor for reduced metastasis-free survival.

In our study, the detection of CTC by RT-PCR could predict chemotherapy efficacy and PFS, but there were some problems, such as false positives[22] and the relationship between CTC and PFS was not very clear. Mehes, et al.[23] found that 3 of 8 patients with CTC displayed apoptotic features and concluded that apoptotic cells significantly contributed to the CTC fraction in breast cancer patients. As the predictive value of such cells for the outcome of the disease was unclear, they should be considered separately when using CTC to predict chemotherapy efficacy and PFS. Therefore, we should pay attention to the pollution of apoptotic cells, and find a solution. Recently, Alix-Panabieres, et al.[24] screened for secretion of cytokeratin-19 (CK19), an intermediate filament of epithelial cells, and provided the first evidence that CK19 could be secreted. They[25] showed that full-length CK19 protein was released by viable epithelial tumor cells in breast cancer patients, and could be detected by ELISA. These CK19-releasing cells (RCs) might constitute a biologically active subset of breast cancer cells with high metastatic properties, and be relevant to the transfer and progression of breast cancer cells. As shown above, to some extent, CK19 protein might be in behalf of tumor cells with activity. In order to avoid the pollution of apoptotic cells, we further tested CK19 protein. There were many studies on the relationship between CK19 protein and circulating tumor cells[26].

We detected CK19 protein by ELISA. The results suggested that patients with decreased CK19 protein had significantly better results of imaging evaluation than patients with increased CK19 protein (P=0.015). PFS of patients with decreased CK19 protein was better than patients with increased level (P=0.002). These data indicated that the detection of CK19 protein before and after the first chemotherapy cycle could predict the results of clinical imaging evaluation of two chemotherapy cycles and PFS. These results conformed to those obtained by using RT-PCR and could further optimize the results of RT-PCR method for detecting of CTC to analyze PFS.

Through the detection of CTC and CK19 protein, we concluded that they both were better indicators for predicting the ORR and PFS, and had superiority over imaging assessment and other conventional measures, such as hormone receptor status and tumor burden. The CTC assay was more reproducible than radiographic evaluation, showed useful results at an earlier time point than do radiologic studies, and seemed to be a more robust predictor of PFS. The ability to serially quantitate and interrogate CTC in patients with breast cancer made possible new ways of managing and investigating this disease. The detection of CK19 protein could compensate for lacking of CTC analysis to predict PFS.

Recent studies showed that what played an important role in tumor recurrence and metastasis and affected treatment effect were not CTC in the peripheral blood, but stem cell-like tumor cells in CTC, which were considered as cancer stem cells with self-renewing ability[27]. Abraham et al.[28] reported that disseminated tumor cells detected in bone marrow of breast cancer patients with CD_{44}^{+}/CD_{24}^{-} phenotype had high incidence of bone metastasis. A stem cell subpopulation within CTC had also been recently reported in patients with metastatic breast cancer, Aktas[29] reported that a major proportion of CTC of metastatic breast cancer patients showed EMT and tumor stem cell characteristics. So further studies are needed to prove whether circulating tumor stem cells exist and to clarify the characteristics of these tumor cells, which may be an accurate indicator for therapy efficacy and PFS. Lu J, et al.[30] demonstrated the existence of distinct populations of CTC included these of epithelial lineage and

stem or progenitor cells. These data showed that the lineage and stem or progenitor cells in CTC should be further studied in future.

In summary, this study proved that the detection of CTC could provide an important biomarker for real-time monitoring of the efficacy of systemic therapies and PFS in individual cancer patients in advance, and gene expression profiles of CTC would provide an opportunity for specific and individualized treatment planning.

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