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A PRELIMINARY INVESTIGATION OF THE RELATIONSHIP BETWEEN CIRCULATING TUMOR CELLS AND CANCER STEM CELLS IN PATIENTS WITH BREAST CANCER

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Abstract

In this study, we explored the relationship between the circulating tumor cells (CTC) and the CTC-cancer stem cells (CSC) in the patients with breast cancer. The magnetic-activated cell separation (MACS) method and flow cytometry (FCM) for selection of epithelial cells from the peripheral blood mononuclear cells (PBMC) were used to analyze the enriched epithelial cells that were labeled with anti-cytokeratin(CK)-fluorescein isothiocyanate, anti-CD44-phycoerythrin (PE) and anti-CD24-PE, respectively. The CK^+ cells were attributed to CTC and the $CK^+CD44^+CD24^{-/low}$ cells were thought as to CTC-CSC in 26 breast cancer patients, respectively. Our results showed the CK^+ tumor cells were detected in 19 of 26 patients, with the CK^+ tumor cells varying from 0.11% to 5.42 %. The CTC-CSC were identified in 18 of the 19 patients with CTC and the percentage of CTC-CSC in CTC was 19.01%. The results yet suggested the breast cancer patients with high-rate CK^+ tumor cells were at the advanced tumor node metastases (TNM) stage III, and the patients with low-rate CK^+ cells were at the modest TNM stage I. The difference between the two groups was statistically significant ($p<0.001$). We concluded that there is a significant relationship between CTC and CTC-CSC, but not among TNM stages, in breast cancer metastasis.

Key words: Breast cancer, circulating tumor cells, cancer stem cells, peripheral blood mononuclear cells.

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INTRODUCTION

Breast cancer is the most common type of cancer in women. Despite new treatment strategies of combining surgery, radiotherapy, chemotherapy and biotherapy, the percentage of patients developing metastases and advanced stages remains high (14). In patients with operable breast cancers, the number of axillary nodes with metastases is a key prognostic factor, and an early and unambiguous detection of these metastases tumor cells is of importance to evaluating tumor progress and to monitoring therapy responses (4, 32) Nevertheless, the current methods for sampling lymph nodes or bone marrow for detecting regions of metastatic tumor in patients with breast cancer are inaccurate, time consuming, and difficult to implement in routine screening in order to determine cancer recurrences or responses to the treatment(16). Recently, much special attention has been paid to the laboratory techniques that are used to identify and enumerate circulating tumor cells (CTC) in blood because viable tumor-derived epithelial cell CTC is probably the origin of intractable metastatic tumor cells (3,8,10,18). Detection and monitoring of CTC might be useful for screening, prognosis, prediction of response to therapy, or monitoring clinical course in patients with primary or metastatic cancer (20). However, CTC is rarely found in a cancer patient's blood and enrichment is necessary for increasing CTC detection sensitivity. Thus, great effort has been focused on enriching CTC from peripheral blood mononuclear cells(PBMC) in breast cancer patients (9,24). The overall detection rates for CTC now range from about 10% to 30% for patients with tumour node metastases (TNM) stages I to TNM III breast cancer or from 50% to 70% for patients with metastatic breast cancer. In both cases, the presence and elevation of CTC are associated with worse prognosis (21).

It is now evident that the hematopoietic origin and some solid tumors have provided proofs that cancers originate from cancer stem cells (CSC), or tumor stem cells, or tumor-initiating cells within a cancer through the detection of CTC progression (11,19,27). The discovery of CSC in solid tumors and in non-solid ones has changed our view of carcinogenesis and chemotherapy. The findings from some studies suggest that putative breast cancer stem cells may reside in the $CD44^+CD24^{-/low}$ population in patients (2,5). A significant effort is being made to identify both CSC-specific markers and the molecular mechanism that

underpins the tumorigenic potentials of these cells because this will have a critical impact on the understanding of the origins of malignant tumors and the discovery of new and more specific therapeutic approaches (15,23,29,33). In this study, we focused on the relationship between the CTC and the CTC-CSC in the patients with breast cancer in an effort to find the molecular characteristics of CTC subpopulations and CTC-CSC biomarkers, and to expand our understanding of the biology of breast cancer metastasis (31). Our investigation was aimed at determining the diagnostic importance as well as the clinical and laboratory feasibility of detecting putative CTC and CTC-CSC in the patients with breast cancer in routine clinical practice.

MATERIALS AND METHODS

Blood sample preparation and magnetic activated cell sorting

With informed consent, peripheral blood was obtained from 8 healthy female volunteers and 26 breast cancer patients with and without evidence of distant metastasis. The patients received surgery at Zhongda Hospital, Southeast University, Nanjing, China. The data about the patients' age, sex, the date of diagnosis, therapeutic interventions, and clinical status were retrieved from the patients' charts and the patients' disease stage groups were determined according to the 1987 and 2002 American Joint Committee on Cancer Staging TNM classifications (12). Ten milliliters of peripheral blood was sampled from each patient's antecubital vein prior to her surgery. PBMC was isolated from the fresh blood obtained by Ficoll gradient separation and washed in 10 ml phosphate- buffered saline (PBS). To increase the sensitivity of the detection of CTC in PBMC, a magnetic-activated cell separation(MACS) technique was used for magnetic enrichment of the cells of the epithelial origin; the procedure followed the manufacturer's protocols (Miltenyi Biotec, Bergisch Gladbach, Germany) (6, 25). Briefly, 10µl reagent for blocking immunoglobulin G fragment crystallizable receptor was put in the 1×10^6 cell suspensions in 0.2 ml PBS and incubated at 4°C for 30 minutes. After a centrifugation (600 *g* for 10 min), the supernatant was aspirated and discarded, and the cells were re-suspended in 0.2 ml PBS. Then, 10µl monoclonal antibody against the human epithelial cell adhesion molecule (EpCAM), which were ordered from Miltenyi Biotec, Germany, was added for 30 min incubation at 4°C. The cells were then rinsed twice in 1 ml PBS and the cells were re-suspended in 0.1 ml PBS for further MACS. According to the relevant protocols (6, 27, 28), the magnetically labeled cell population was applied to MACS CS1 columns that had been equilibrated with 3 ml PBS in the VarioMACS unit (Miltenyi Biotec) and was allowed to separate for 30 min. The non-magnetic negative cell population were washed out of the column and the column was taken out of the magnetic separator. The magnetic-positive cell population retained in column were collected as eluate after a PBS washing step with a total volume of 2 ml. These magnetically labeled cells were later used in a FCM (Becton Dickinson) analysis.

Detection of $CK^+/CD44^+/CD24^{-/low}$ cells by FCM

As was described above, the magnetic-positive cells were stained respectively with the following: 1) the FITC-conjugated mouse against human CK mAb CAM5.2 recognizing CK 8 and CK 18; 2) the phycoerythrin (PE)-conjugated mouse against human CD44 mAb, which were purchased from eBioscience Company, USA, and 3) the PE-conjugated mouse against human CD24 mAb, which was ordered from Bejing Sai Shi Biological Technology Company, China. Those cells with different mAb labels were incubated at 4°C in PBS containing 0.5µg/ml propidium iodide for 30 min in a volume of 1 ml. The cells were then washed twice in PBS, and immediately analyzed by FCM. Subsequent FCM analyses showed less than 5% contamination by relevant antigen expressing cells. The FCM analyses were performed according to the protocols found in some published reports (7,16) that resided in the Flow Cytometry Laboratory at the Center for Clinical Medicine, Southeast University. The data was acquired in list mode by using a threshold on the fluorescence of the $CD44^+/CD24^{-/low}$ cells. The criteria for multi-parameter data analysis included size defined by forward light scatter and granularity defined by orthogonal light scatter. A standard used in the present investigation was that CK^+ epithelial cells were considered CTC and (25) the $CK^+/CD44^+/CD24^{-/low}$ epithelial cells were considered CTC-CSC in the patients with breast cancer (2).

Statistical analysis

The data from the individual patient's experiment was maintained in a Paradox database. Statistical comparisons were performed using the Student's t-test method to test any statistically significant difference in the results between the different test groups. A p value less than 0.05 was considered statistically significant.

RESULTS

Detecting CK positive cells from PBMC in breast cancer patients

Twenty-six female patients with breast cancer aged from 49 to 71 (the average age was 59.5) from the Department of Surgery or the Department of Oncology at the Zhongda Hospital, Southeast University, Nanjing, China were enrolled into the study. Additionally, 8 blood samples from healthy female volunteers without malignant findings were obtained from the Department of Gynecology & Obstetrics for comparison. To increase the sensitivity of the detection of CTC, a new technology was used to enrich disseminated epithelial tumor cells from PBMC samples by MACS. The results showed that CK^+ tumor cells were detected in 19 of the 26 breast cancer patients (73.07%), and that the percentages of CK^+ tumor cells varied from 0.11% to 5.42 % in the patients with detected breast cancer. To our surprise, the CK^+ tumor cells were also detected in 1 of the 8 health female volunteers (0.07%), which was shown in Figure 1 B. It was believed that this detection of CK^+ tumor cells from the healthy sample was due to contamination in the blood sample collection process. According to the designed standard in the present investigation, the detecting rate of CTC (CK^+ cells) was 73.07% (19/26) in the patients with breast cancer, and the detection sensitivity was greatly increased with the immunomagnetic enrichment of the epithelial cells from PBMC by MACS.

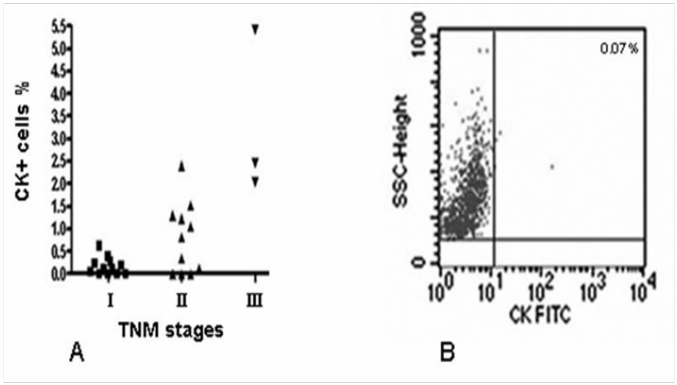

Figure 1. Occurrence of the CK^+ cells from PBMC in patients with breast cancer and healthy female volunteers.

Detecting $CK^+CD44^+CD24^{-/low}$ cells and analyzing rates of CTC-CSC in CTC from PBMC in breast cancer patients at different TNM stages

In order to investigate the CTC-CSC in breast cancer patients at different TNM stages, we designed the test that would indicate the rates of $CK+CD44^+CD24^{-/low}$ cells and CSC-CTC in CTC in breast cancer patients at different TNM stages. The results indicated that the CTC-CSC ($CK^+CD44^+CD24^{-/low}$ cells) were identified in 18 of the 19 (94.74%) breast cancer patients with CTC and that either CSC ($CD44^+CD24^{-/low}$ cells) or CTC was found in the other 7 breast cancer patients (data not shown). The detection results suggested that there was an obvious relationship between CTC and CTC-CSC in the patients with breast cancer. Since CTC-CSC being associated with occurrences of metastases, we further analyzed the percentages of positive CTC-CSC in CTC from PBMC in breast cancer patients at different TNM stages. Although the rate of CTC-CSC in CTC was 19.01%, no statistically significant difference ($p>0.05$) was found among TNM stage I (17.8%), TNM stage II (22.6%), and TNM stage III (16.9%).

Characteristic of breast cancer patients with CK^+ cells and $CK^+CD44^+CD24^{-/low}$ cells in PBMC

To analyze the clinical significance of the prevalence of CTC and CTC-CSC and to investigate the relationship between CTC and CTC-CSC from PBMCs in the patients with breast cancer, we detected the CK^+ cells and $CK^+CD44^+CD24^{-/low}$ tumor cells by both MACS and FCM methods. Table 1 shows the results of the detected CK^+ cells and $CK^+CD44^+CD24^{-/low}$cells from PBMCs in the breast cancer patients. The CK^+ cell rates (in percent) were 0.175±0.192 (TNM stage I), 0.802±0.788 (TNM stageII), and 3.307±1.842 (TNM stageIII), respectively. The differences in the rates were statistically significant (3.307±1.842 vs. 0.802±0.788, $p<0.05$; 3.307±1.842 vs. 0.175±0.192; $p<0.001$ and 0.802±0.788 vs. 0.175±0.192, $p<0.01$). The $CK^+CD44^+CD24^{-/low}$ cell rates (in percent) were 0.014±0.018 (TNM stage I), 0.166±0.271 (TNM stageII), and 0.483±0.121, (TNM stage III), respectively. The rate differences were also statistically significant (0.483±0.12 vs. 0.166±0.271, $p<0.05$; 0.483±0.121 vs. 0.014±0.018; $p<0.001$ and 0.166±0.271vs. 0.014±0.018, $p<0.05$) as is shown in Table 2.

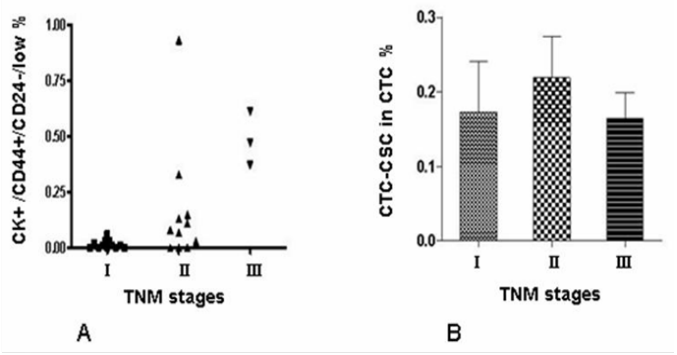

Figure 2. Rates of $CK^+CD44^+CD24^{-/low}$ cells and CSC-CTC in CTC in breast cancer patients at different TNM stages.

DISCUSSION

In breast cancer patients, cancer-related death is mainly caused by the occurrence of distant metastases (8, 30). Even though serum tumor markers have been used for early diagnosis of metastases, their systematic determination has not any effect on survival rates. More reliable methods are needed to detect metastases earlier than with the current common clinical methods. Because CTC can be detected in most cancer patients, detection of CTC may offer a solution to meet the existing medical need for monitoring patients during the course of treatments and for helping determine recurrent diseases. Accordingly, detection of CTC could be a useful tool in early detection of relapses and responses to systemic chemotherapy (14, 32). Some studies have shown that high levels of CTC were correlated with poor survival rates in the metastatic breast cancer patients, therefore, CTC was emerging as a powerful prognostic and predictive biomarker in breast cancer patients. However, CTC is rare in cancer patient's blood, counting for as few as one cell per 10^9 blood cells in patients with metastatic cancer. Hence isolating CTC presents a tremendous technical challenge (22, 24, 31).

To increase the sensitivity of the detection of CTC from PBMC in patients with breast cancer in the present study, an immunomagnetic enrichment of CTC from PBMC by MACS assay was firstly adopted, which means the magnetic microbeads coated with anti-epithelial cell adhesion molecular mAb was used to capture epithelial cells from PBMC. The magnetically captured epithelial tumor cell population were then sorted by the MACS assay. The sensitivity to detect and to isolate epithelial tumor cells was markedly increased by 100 times, with 1-2 epithelial cells having been detected from per 10^7 PBMC in the patients with breast cancer, which may provide a susceptible way for detection of epithelial tumor cells from PBMC in the breast cancer patients. Soon after the detection, the sorted epithelial cells labeled with the FITC-conjugated CK mAb were analyzed by FCM. In our current investigation, 19/26 breast cancer patients carried CK^+ cells (73.07%) and their CK^+ rates ranged from 0.11% to 5.42 %. An average recovery rate of 1.09% of CTC was determined by our modified detecting method. More importantly, the relationship between the CK^+ cells rates and the TNM stages was found in the patients with breast cancer: increased CK^+ cell rates (3.307±1.842 %) in the breast cancer patients with the advanced TNM stage III and the low CK^+ cell rates (0.175±0.192 %) in the breast cancer patients with modest TNM stage I. The difference was statistically significant

Table 1. Results of detecting CK^+ cells and $CK^+CD44^+CD24^{-/low}$ cells from PBMC in patients breast cancer.

Cases	TNM stages	CK+ cells rates (%)	CK+CD44+CD24-/lowcells rates (%)
1	II	0.35	0.15
2	III	5.42	0.61
3	II	0	0
4	I	0.11	0.02
5	I	0.27	0.05
6	II	0	0
7	I	0	0
8	II	2.41	0.93
9	II	1.3	0.13
10	II	1.06	0.07
11	II	1.23	0.08
12	III	2.46	0.37
13	I	0	0
14	I	0.24	0.03
15	II	0	0
16	II	0.82	0.11
17	I	0	0
18	III	2.04	0.47
19	I	0	0
20	I	0.05	0.01
21	II	1.53	0.33
22	I	0.19	0
23	I	0.39	0.01
24	I	0.62	0.01
25	II	0.12	0.03
26	I	0.11	0.06

Table 2. Relationship between the TNM stages and the rates of CK^+ cells and $CK^+CD44^+CD24^{-/low}$ cells from PBMC in patients breast cancer.

TNM stages	CK+ cells rates (%)	CK+CD44+CD24-/lowcells rates (%)
I	0.175 ±0.192	0.014 ±0.018
II	0.802 ±0.788*	0.166 ±0.271#
III	3.307 ±1.842**	0.483 ±0.121##

*p<0.01 and #p<0.05 , all compared with stage I; ** p<0.01 and ##p<0.05, all compared with stage II.

difference (p<0.001). We also found that two patients in TNM stage II have pretty high percentage measurement of CSC-CTC since they had metastasis in their lungs. This finding may have an important clinical significance for doctors to evaluate the breast cancer development status directly according to the original FACS sorting results for each TNM stage by detecting CTC from the PBMC.

In addition to an unambiguous detection and quantification of rare CTC in the background of PBMC, information about CTC's functional status would be helpful for accurately determining the clinical impact of detectable CTC (26). For example, some of the detected CTC may lack metastatic potential in breast cancer patients, such as apoptotic cells, which might occur spontaneously or could be induced by cytotoxic therapy, surgery, etc. These apoptotic cells might be one source of CTC in patients with breast cancer. On the other hand, loss of cell-matrix adherence in epithelial cells can directly trigger apoptosis cell formation (13, 26). Therefore, we think that the ability to identify, to isolate, to propagate, and to investigate the molecular characteristics of the CTC subpopulations could further assist in the discovery of CSC biomarkers and expand the understanding of the biology of metastatic breast cancer. Therefore, the detection of the CTC-CSC that have CSC markers is very valuable to the diagnosis, prognosis, and monitoring treatment of patients with breast cancer.

Because $CD44^+CD24^{-/low}$ breast cancer cells exhibit enhanced invasive properties and represent a population that relates to human breast CSC(1, 33), we designed the experiment for testing $CD44^+$ $CD24^{-/low}$ cells in the current study while detecting CK^+ cells. The results indicated that the $CD44^+$ $CD24^{-/low}$ cells were detected in 18 of the 19 (94.74%) patients with the CTC. Although this was extremely rare, it mostly represented a potential CSC as a source of the distant metastasis. It was also interesting to find that the patients with the elevated $CK^+CD44^+CD24^{-/low}$ cells (0.483± 0.121 %) were in the metastatic breast cancer (TNM stage III) and the patients with the low-level CK^+ $CD44^+CD24^{-/low}$ tumor cells (0.014±0.018 %) were in the modest breast cancer (TNM stage I), and that the difference was statistically significant (p<0.01) The data is shown in Table 2.

It has been reported that the prevalence of $CD44^+CD24^{-/low}$ tumor cells in breast cancer may not be associated with clinical outcomes and survival rates, but may favor distant metastasis (23). Reported findings are consistent with the results from our current investigation. Although the positive percentage of CTC-CSC in CTC reached up to 19.01%, there was no statistically significant difference between the positive percentage of CTC-CSC in CTC and the different TNM stages, p>0.05 (Figure 2B). However, the rates of CTC were consistent with those of CTC-CSC in the patients with breast cancer. Furthermore, the increased CTC and CTC-CSC were found in the breast cancer patients at the high-grade TNM stages (see Table 2). The data presented in the reported studies indicated

that the prevalence of CTC was closely related to the CTC-CSC in the patients with breast cancer. The association that we found between the CK^+ cells and the $CK^+CD44^+CD24^{-/low}$cells could be considered an important monitoring marker to be employed as an independent predictor of progression-free survival rates and overall survival rates in patients with metastatic breast cancer.

In conclusion, little information has been reported on the relationship between CSC and breast cancer's metastasis in peripheral blood. The detection method developed in our study offers an excellent tool for the enrichment of micrometastatic tumor cell clusters that are $CK^+CD44^+CD24^{-/low}$cells (CTC-CSC). The CTC-CSC may represent the initial stage of development from a single disseminated tumor cell towards an overt metastasis, and the presence of CTC-CSC in patients with breast cancer was found to be associated with clinical stages.

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