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Original Article

Phenotypes of circulating tumor cells predict lymph node involvement in patients with early breast cancer

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Abstract: *Background:* Base on the "seed and soil" theory, the presence of circulating tumor cells (CTC) in breast cancer has been suggested to be the active source of metastatic spread in primary tumors. During metastasis formation, epithelial cells undergo massive changes in their characteristics and increase their motility in order to migrate, a process termed epithelial-to-mesenchymal transition (EMT). Here, we applied a quantifiable, dual-colorimetric RNA-in situ hybridization assay in exploring whether CTC or its subtypes in peripheral blood could be suitable biomarkers in early breast cancer. *Methods:* A total of 5 ml of blood was analyzed for CTC with the CanPatrol™ system (SurExam, Guangzhou, China) for the detection and classification of CTCs. *Results:* CTCs consist of different epithelial and mesenchymal compositions were observed in 115/142 (81.0%) patients. Sixty-seven of 142 patients investigated were positive for lymph nodes, and 52.8% (75/142) were negative, respectively. The presence of CTC at baseline was correlated to lymph node metastases (P=0.042), but no association was found with tumor size, grading, histological types, hormone receptor status or molecular subtypes. Strikingly, nodal involvement is positively correlated with the exclusively epithelial marker positive CTC (defined as EpCAM+ and/or CK8+ and/or CK18+ and/or CK19+ by a multi-marker probe, P=0.010). *Conclusions:* Our finding suggest that CTC and its subpopulations were observed visually by the currently used detection methods and evaluation of CTCs in early stage breast cancer patients provides potential clinical information. A subset of CTCs in patients with early BC shows EMT and the clinical relevance on the prognosis of CTCs has to be further validated in a prospective trial.

Keywords: Circulating tumor cells, phenotypes, early breast cancer

Introduction

Breast cancer is most common carcinoma in women. Formation of metastasis is ultimately responsible for the majority of cancer-related deaths. Two-hundred years ago, Paget (1889) proposed that tumor cells are seeds which grow more readily in certain types of soil (organs) [1, 2]. With the increasing understanding of metastasis as a multistep cascades, cancer disseminated cell in the blood is responsible to one of the first step of the metastatic process, while micrometastasis in a distant organ, like the bone marrow, reflects a more advanced stage [3]. Circulating tumor cells (CTCs) are rare tumor cells that survive in peripheral blood of cancer patients. In recent decades, researches report the detection of CTCs in different types of tumors and suggest evaluating CTCs has clinical relevance in the monitoring and the outcomes of metastatic tumors [4, 5]. Some studies have confirmed that CTCs can be enriched and identified using a wide variety of different strategies [6], which are not only based on physical methods but also focus on biological properties [7], endowing their ability to invade, proliferate, and cause a metastasis.

The presence of epithelial-mesenchymal transition (EMT) features on CTCs has been investigated in several studies. EMT is thought to be induced by the tumor microenvironment or in response to hypoxia [3], and to be involved in CTCs generation and survival [8]. In the actuation of the EMT program, epithelial cells lose epithelial characteristics and mesenchymal traits such as N-cadherin, vimentin, twist and fibronectin are overexpressed, resulting in enhanced their ability of aggressive potential [9]. By the study of Yu indicates that CTCs consist of epithelial and mesenchymal compositions and exhibit dynamic changes during a new line

of systemic treatment [10]. Therefore, compared with conventional clinical tools for cancer management, CTCs can be used as an early predictor of metastatic potential in breast cancer patients [11-13], although the underlying nature of these cells and their biological features are still poorly to be further confirmed. Most large prospective trials concentrate CTC detection on metastatic breast cancer [14-16], and conduct evaluation of CTC to clinical relevance. But only a few studies devoted to CTCs in early breast cancer patients.

We postulated that the CTCs may lose their epithelial characteristics and acquire mesenchymal phenotypes at the time of initial process of metastasis. Thus, our study is to test the presence of CTCs and evaluate the CTC subpopulations at baseline using quantifiable, mRNA-in situ hybridization in early breast cancer patients prior to a systemic treatment.

Materials and methods

Patients

This study was conducted at the First Affiliated Hospital of Guangxi Medical University from July 2015 to December 2015. One hundred and forty-two patients with invasive breast carcinoma, staging I-III, have been studied. Patients with distant metastasis at the time of initial diagnosis were excluded. For eligible patients, peripheral blood samples for CTC status analysis were collected at the time of primary surgery or prior to neoadjuvant chemotherapy. This study was approved by our institutional review board. Informed and written consent was obtained from all the patients involved in this retrospective study.

Collection of CTCs and classification of CTCs by mRNA in situ hybridization (ISH) assay

For the recruited patients, 5 mL peripheral blood samples were collected pre-surgery or prior to neoadjuvant chemotherapy, and analyzed with The CanPatrol CTC enrichment technique. Red blood cell lysis buffer was used to lyse erythrocytes, and then PBS containing 4% formaldehyde (Sigma, St. Louis, MO, United States) was used to resuspend the remaining cells for 5 min. After that, a filtration system consisted of a calibrated membrane (SurExam, Guangzhou, China) with 8μm pores filters, a manifold vacuum plate with valve settings (SurExam, Guangzhou, China), an E-Z 96 vacuum manifold (Omega, Norcross, USA), and a vacuum pump (Auto Science, Tianjin, China) were applied, which enable to separate small leukocytes from the large epithelial cells.

We identified and examined the expression levels of epithelial and mesenchymal markers in CTCs using a multiplex RNA-in situ hybridization (RNA-ISH) assay combined with a branched DNA signal amplification technology. The detail procedure was performed as previously described [17]. Briefly, the cells retained on membrane were treated with a protease (Qiagen, Hilden, Germany). In situ hybridization was carried out with a cocktail probe which was specific for the epithelial biomarkers (EpCAM and CK8/18/19), the mesenchymal biomarkers (vimentin and twist), and the leukocyte biomarker CD45. Hybridizations were performed at 42°C for 2 hours. The membranes subjected a serial of hybridization reactions were washed and incubated in preamplifier solu-tion (30% horse serum (Sigma, St. Louis, USA), 1.5% sodium dodecyl sulfate (Sigma, St. Louis, USA), and the signal amplification step were then performed. Subsequently, Three types of fluorescently labeled probes had been conjugated with 3 kinds of fluorescent dyes , and incubated at 42°C for 20 minutes. After washing with 0.1× SSC, the cell nucl-eus was stained with 4', 6-diamidino-2-phenylindole (DAPI) [17].

Evaluation of ISH staining and Immunohistochemical analysis of the primary tumor

The samples were analyzed with a fluorescence microscope using a 100× oil objective (Olympus BX53, Tokyo, Japan). The red and green dots of fluorescent signal observed in the cells represented the epithelial and mesenchymal gene expression levels, respectively. The purple fluorescent dots represented the CD45 gene level (the markers of leukocytes). For the patients enrolled in this study, the tumor type, TNM staging and grading were assessed according to the WHO Classification of tumors of the breast and the 7th edition of the TNM Classification System. Tumors were defined as hormone receptor-positive if 1 percentage or higher of cells with immunohistochemical nuclear staining for estrogen, progesterone, or both. HER-2/neu (also knew as ErbB-2 oncogene) status was determined using IHC analysis or FISH analysis, samples that had a strong (3+) stain intensity on IHC analysis or gene amplification by FISH were considered HER-2 positive.

Table 1. Data of patient characteristics

Variable	Number of Cases	%
Total	142	-
Age Median (range)	47 (25-77)	-
Histological type		
Ductal	130	91.5
Lobular	6	4.2
Others	6	4.2
Tumor sizes		
T1	58	40.8
T2	80	56.3
T3	4	2.8
Grading		
G1	5	3.5
G2	82	57.7
G3	27	19.0
Gx	28	19.7
Nodal status		
Negative	75	52.8
Positive	67	47.2
ER status		
Negative	44	31.0
Positive	98	69.0
PR status		
Negative	46	32.4
Positive	96	67.6
HER-2 status		
Undefined	18	12.7
Negative	97	68.3
Positive	27	19.0
Molecular subtypes		
Luminal A	32	22.5
Luminal B	70	49.3
HER2-overexpression	16	11.3
Triple-negative	24	16.9

HER-2 status: samples that had a strong (3+) stain intensity on IHC analysis or gene amplification by FISH were defined as HER-2 positive.

Statistical analysis

Statistical analyses were performed using IBM SPSS statistics, Version 21. All patients data were displayed as median and range (or mean and SD) for continuous variables or as frequencies and proportions for categorical variables. Associations between CTC count and clinicopathological parameters were assessed by chi-squared test or Fisher's Exact Test. A *p*-values less than 0.05 were considered to be statistically significant.

Results

Patient characteristics

A total of 142 patients with early breast cancer (median age 47 years, range 25-77 years, staging I-III) were enrolled in our study. Detailed clinical characteristic is presented in **Table 1**.

Prevalence of CTCs and correlations to clinical relevance

Patients were assessed as CTC-positive if at least one CTC was detected. CTCs were defined as exclusively epithelial biomarkers-positive (E+) cells or exclusively mesenchymal biomarkers-positive (M+) cells if at least one red dot or green dot fluorescent signal was observed with the fluorescence microscope, respectively. Notably, we have found that a portion of CTCs those emitting red and green dots fluorescent signal, which defined as biphenotypic (E+ and M+) cells (**Figure 1**).

CTCs enrichment and classification with mRNA in situ hybridization (ISH) assay was successful in 81.0% (115/142) of cases before the start of systemic treatment (median=2.0 cells, range 0-21 per 5 ml of peripheral blood). The frequency of CTC positive patients was 74.7% (n=56 of 75) of node-negative and 88.1% (n=59 of 67) of node-positive patients showing CTCs (P=0.042, **Table 2**), whereas no any statistically significant correlation between presence of CTC and other clinicopathelogical characteristics including tumor size, TNM staging, histological types, hormone receptor status, HER-2 status or molecular subtypes (data not shown).

Strikingly, nodal involvement is statistically significant correlated with the presence of exclusively epithelial marker positive CTC (defined as EpCAM+ and/or CK8+ and/or CK18+ and/or CK19+ in a multi-marker probe, P=0.010, **Table 3**), but no statistically significant correlations with the presence of biphenotypic (E+ and M+) cells or exclusively mesenchymal marker positive CTC.

EMT-related phenomenon in early breast cancer

One-hundred fifteen patients (81%) were CTC positive. The frequency of epithelial and mesenchymal compositions was heterogeneous in

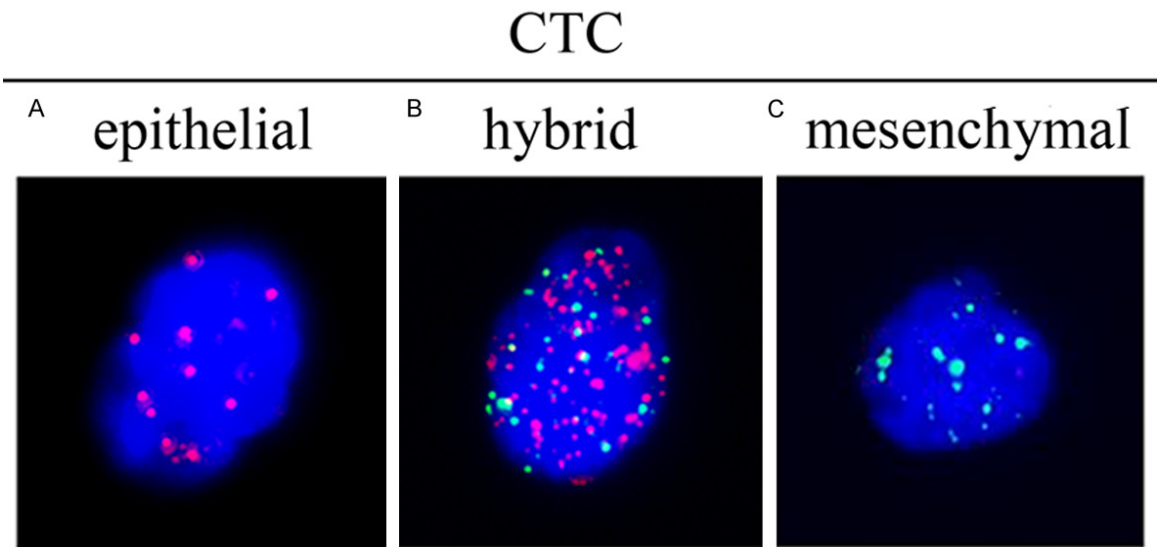

Figure 1. EpCAM, CK8/18/19, vimentin and twist expression in CTCs and 3 types of CTCs from patient with breast cancer. A: Epithelial CTCs stained for EpCAM, CK8/18/19 expression (red fluorescence); B: Hybrid CTCs stained for EpCAM, CK8/18/19 (red fluorescence), vimentin and twist expression (green fluorescence); C: Mesenchymal CTCs stained for vimentin and twist expression (green fluorescence).

Table 2. Patient characteristics at baseline for CTC count (CTC=0 per 5 mL vs CTC ≥1 per 5 mL) before start a systemic treatment (n=142)

Variable	*P*-value
Histological type Ductal vs Lobular vs others	0.615*
Tumor sizes T1 vs T2 vs T3	0.114*
Grading G1 vs G2-3	0.327*
Nodal status Negative vs Positive	0.042Δ
ER status Negative vs Positive	0.866Δ
PR status Negative vs Positive	0.908Δ
HER-2 status Negative vs Positive	0.434Δ
Molecular subtypes Luminal A vs Luminal B vs HER-2 overexpression vs Triple-negative	0.432*

ΔTwo-sided x^2 Test. *Two-sided Fisher's Exact Test.

Table 3. Association between CTC presence at baseline and lymph node metastases

CTC		Lymph node metastases		*P*-value
		Negative (%) n=75	Positive (%) n=67	
Total CTCs	0	19 (25.3)	8 (11.9)	0.042
	≥1	56 (74.7)	59 (88.1)	
Number of samples containing exclusively E+ CTC	0	36 (48.0)	18 (26.9)	0.010
	≥1	39 (52.0)	49 (73.1)	
Number of samples containing Biphenotypic E+ and M+ CTC	0	36 (48.0)	30 (44.8)	0.701
	≥1	39 (52.0)	37 (55.2)	
Number of samples containing exclusively M+ CTC	0	55 (73.3)	50 (74.6)	0.861
	≥1	20 (26.7)	17 (25.4)	

individual patients. A intermediate state which coexpression of epithelial markers (EpCAM, CK8/18/19) and mesenchymal markers (vimentin and twist) was also observed as men-

tioned above. Hence, we defined eight types of combination of three different CTC phenotypes, including: 1) none of three subtypes were detected, 2) only purely E+ CTC was detected but not biphenotypic or exclusively M+, 3) only biphenotypic E+ CTC was detected but not exclusively E+ or exclusively M+, 4) only purely M+ CTC was detected but not biphenotypic or exclusively E+, 5) both of biphenotypic CTC and exclusively E+ CTC were detected but not exclusively M+, 6) both of biphenotypic CTC and exclusively M+ CTC were detected but not exclusively E+, 7) both of exclusively E+ CTC and exclusively M+ CTC were detected but not biphenotypic, 8) three of subtypes were observed.

Subsequently, we found that exclusively M+ CTC but not biphenotypic CTC or exclusively E+ CTC was not observed. The CTCs from patients predominantly compose of exclusively E+ CTC and biphenotypic (E+/M+) phenotypes. The occurrence of exclusively E+ cells in patients with nodal involvement was more frequent than patient with negative lymph nodes (**Figure 1**). Although there was an increase in the number of mesenchymal CTCs in patients without lymph nodes metastases, these data did not show a statistically significant difference in our analysis, which might be due to the relative small sample size.

Discussion

Numerous studies have confirmed that a negative correlation between the evaluation of CTC levels, assessed by CellSearchTM system, and the prognosis in patients with metastatic cancers of the breast, prostate, and colon [18]. CTC detection rate is varying in different researches. Raimondi and co-workers showed that EpCAM-positive, CK-negative cells can be found in blood circulation. Since epithelial biomarkers on cell surface were downregulated induced by EMT process in cancer dissemination, using the standard definition of CTCs as EpCAM+, CK+, and CD45-cells, proximately 30% patients failed to find them in peripheral blood [19]. They also reported about a subset of CTCs characterized by EpCAM+, CK-, and EMT maker+ (vimentin/fibronectin+), while the prognosis significance of these cells is unclear. A previous study shows that a correlation was observed between mesenchymal CTCs and disease progression [10] and these cells being frequently detected in metastatic breast cancer compared to early stage [9]. Therefore, it is necessary for CTC detection using a multimarker-based approach. Taking all these considerations into account, we applied a quantifiable, multi-marker ISH assay combine with physical isolation for the identification of CTC-associated transcripts in early breast cancer patients. This method has been successfully applied for the detection and characterization of CTC in patients with liver, nasopharyngeal, breast, colon, gastric cancer, and non-small-cell lung cancer (NSCLC) in a limited patient cohort [17, 20].

In this study, CTCs were detected in 81.0% (115/142) of cases. We confirmed that a significantly different CTCs (define as EpCAM+ and/or CK8, 18, 19+ and/or vimentin+ and/or twist+) detection rate between node-positive and node-negative patients (88.1% vs 74.7%, P=0.042). Some studies have revealed that CTCs might be independent of lymph node status [21, 22]. Controversies still persist regarding whether CTCs presence is related to nodal involvement or other clinicopathological characteristics, especially in early breast cancer patients. The SUCCESS trial indicates that the presence of CTCs was positively correlated with lymph node metastases and their presence predicted decreased progression-free survival and overall survival both before and after adjuvant chemotherapy [23].

Hara Polioudaki et al. demonstrated that variable expression levels of keratin and vimentin of circulating tumor cells revealing differential EMT status [24]. Yu and colleagues also showed that epithelial and mesenchymal patterns exist dynamic changes during therapy, and mesenchymal CTC occurred frequently at the time of tumor progression [10]. Strikingly, in our analysis, we observed a positive correlation between purely E+ phenotype presence and nodal metastasis (73.1% for LN-positive patients vs 52.0% for LN-negative patients, P=0.010). Our findings also show that in addition to cells coexpressing epithelial and mesenchymal markers, there exists a subset of CTCs that are EpCAM-negative, CK-negative and more mesenchymal-like. At least one of the mesenchymal markers was expressed in 54.9% (78/142) of the patients (data no shown), but lack of relationship between this CTC subpopulation and clinical characteristics.

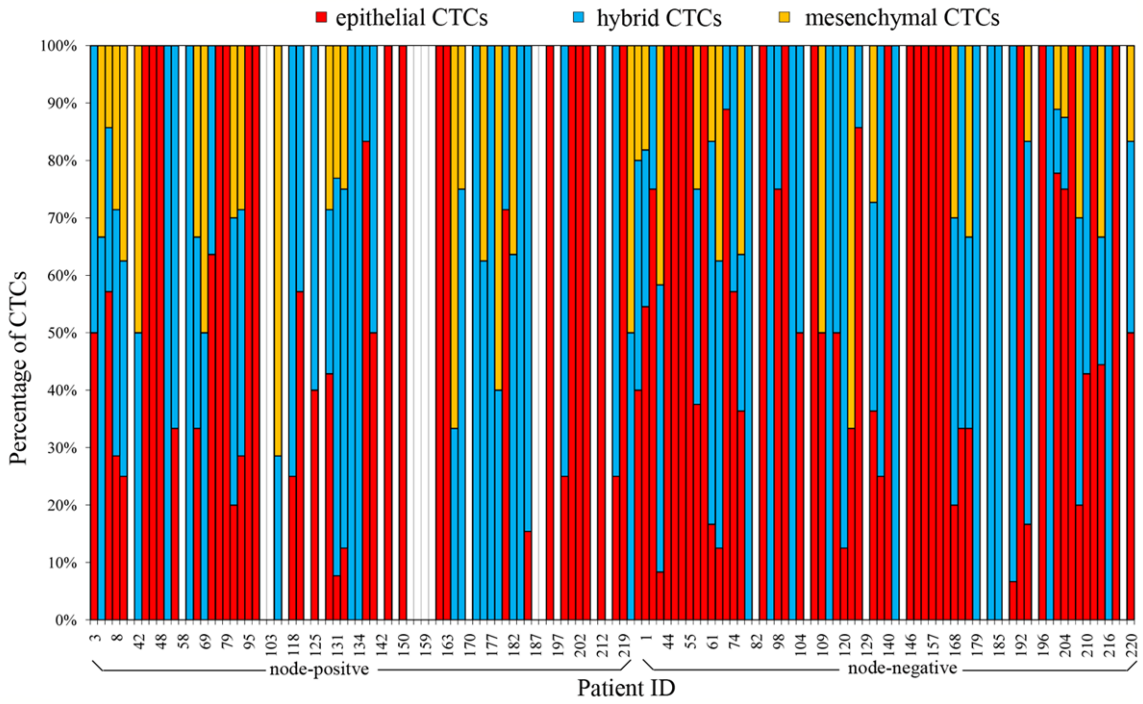

Figure 2. Distribution of Epithelial CTCs, Hybrid CTCs, Mesenchymal CTCs of 142 patient with breast cancer (Node-negative n=75, Node-positive n=67).

Unfortunately, we have not found only purely M+ CTC phenotypes in individual patients (**Figure 2**). Several studies also reported mesenchymal CTC from breast cancer patients were isolated, but the clinical impact and basis mechanism of these cells have to be further confirmed yet [25, 26]. It is still unclear whether no clinical relevance of these mesenchymal CTCs at present are due to either the limitation of single cell characterization techniques, leading to false negative results, or to a real major intrinsic dissemination properties that we ignored at the very first. To form a micrometastasis, disseminated tumors cells might be subject to a complex selection, and the outcome of the process is dependent on both the intrinsic properties of tumor cells and the response of the host [27]. Although the enumeration of mesenchymal marker-positive CTCs did not relate to lymph node status in current study, there is great heterogeneity to the number of CTCs isolated from individual patients, and further studies have to be done to determine functionality of these CTC subpopulations.

A limitation of our study is that due to the relatively small sample size and shorter follow-up time, assessment of the impact of the analyzed markers on patients' survival is not allowed. We have to point out that we do not evaluate EMT-related dynamic changes on circulating tumor cells because lack of sequential blood samples post-surgery or post-chemotherapy. However, patients in this study must be under observation all the way and survival analysis will be done at the proper time.

Conclusion

Many studies indicate that CTC is an independent factor associated with prognosis in metastatic breast cancer. Very few studies have been performed for CTCs in early breast cancer patients. We suggested that CTCs detected in early stage breast cancer patients provides potential clinical information. Notably, we visually observed CTCs undergoing EMT using a quantifiable, multi-marker RNA-in situ hybridization assay, indicating that CTCs is a subset of tumor cells which has heterogeneous biological properties. Furthermore, it could be shown that classify CTCs into the subtypes characterized by epithelial-to-mesenchymal marker might help identify patients at higher risk of an unfavorable outcome.

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Disclosure of conflict of interest

None.

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References

[1] Paget S. The distribution of secondary growths in cancer of the breast. 1889. Cancer Metastasis Rev 1989; 8: 98-101.

[2] Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer 2003; 3: 453-8.

[3] Bidard FC, Pierga JY, Vincent-Salomon A, Poupon MF. A "class action" against the microenvironment: do cancer cells cooperate in metastasis? Cancer Metastasis Rev 2008; 27: 5-10.

[4] Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW, Hayes DF. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004; 351: 781-91.

[5] Krebs MG, Sloane R, Priest L, Lancashire L, Hou JM, Greystoke A, Ward TH, Ferraldeschi R, Hughes A, Clack G, Ranson M, Dive C, Blackhall FH. Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer. J Clin Oncol 2011; 29: 1556-63.

[6] Gorges TM, Pantel K. Pantel, Circulating tumor cells as therapy-related biomarkers in cancer patients. Cancer Immunol Immunother 2013; 62: 931-9.

[7] Wicha MS, Hayes DF. Circulating tumor cells: not all detected cells are bad and not all bad cells are detected. J Clin Oncol 2011; 29: 1508-11.

[8] Banyard J, Bielenberg DR. Bielenberg, The role of EMT and MET in cancer dissemination. Connect Tissue Res 2015; 56: 403-13.

[9] Kallergi G, Papadaki MA, Politaki E, Mavroudis D, Georgoulias V, Agelaki S. Epithelial to mesenchymal transition markers expressed in circulating tumour cells of early and metastatic breast cancer patients. Breast Cancer Res 2011; 13: R59.

[10] Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, Isakoff SJ, Ciciliano JC, Wells MN, Shah AM, Concannon KF, Donaldson MC, Sequist LV, Brachtel E, Sgroi D, Baselga J, Ramaswamy S, Toner M, Haber DA, Maheswaran S. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science 2013; 339: 580-4.

[11] Budd GT, Cristofanilli M, Ellis MJ, Stopeck A, Borden E, Miller MC, Matera J, Repollet M, Doyle GV, Terstappen LW, Hayes DF. Circulating tumor cells versus imaging-predicting overall survival in metastatic breast cancer. Clin Cancer Res 2006; 12: 6403-9.

[12] De Giorgi U, Valero V, Rohren E, Dawood S, Ueno NT, Miller MC, Doyle GV, Jackson S, Andreopoulou E, Handy BC, Reuben JM, Fritsche HA, Macapinlac HA, Hortobagyi GN, Cristofanilli M. Circulating tumor cells and [18F] fluorodeoxyglucose positron emission tomography/computed tomography for outcome prediction in metastatic breast cancer. J Clin Oncol 2009; 27: 3303-11.

[13] Pierga JY, Hajage D, Bachelot T, Delaloge S, Brain E, Campone M, Diéras V, Rolland E, Mignot L, Mathiot C, Bidard FC. High independent prognostic and predictive value of circulating tumor cells compared with serum tumor markers in a large prospective trial in first-line chemotherapy for metastatic breast cancer patients. Ann Oncol 2012; 23: 618-24.

[14] Smerage JB, Barlow WE, Hortobagyi GN, Winer EP, Leyland-Jones B, Srkalovic G, Tejwani S, Schott AF, O'Rourke MA, Lew DL, Doyle GV, Gralow JR, Livingston RB, Hayes DF. Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. J Clin Oncol 2014; 32: 3483-9.

[15] Jiang ZF, Cristofanilli M, Shao ZM, Tong ZS, Song EW, Wang XJ, Liao N, Hu XC, Liu Y, Wang Y, Zeng L, Zhang M. Circulating tumor cells predict progression-free and overall survival in Chinese patients with metastatic breast cancer, HER2-positive or triple-negative (CBCSG004): a multicenter, double-blind, prospective trial. Ann Oncol 2013; 24: 2766-72.

[16] Helissey C, Berger F, Cottu P, Diéras V, Mignot L, Servois V, Bouleuc C, Asselain B, Pelissier S, Vaucher I, Pierga JY, Bidard FC. Circulating tumor cell thresholds and survival scores in advanced metastatic breast cancer: the observational step of the CirCe01 phase III trial. Cancer Lett 2015; 360: 213-8.

[17] Wu S, Liu S, Liu Z, Huang J, Pu X, Li J, Yang D, Deng H, Yang N, Xu J. Classification of circulating tumor cells by epithelial-mesenchymal transition markers. PLoS One 2015; 10: 0123976.
[18] Miller MC, Doyle GV, Terstappen LW. Terstappen, Significance of Circulating Tumor Cells Detected by the CellSearch System in Patients with Metastatic Breast Colorectal and Prostate Cancer. J Oncol 2010; 2010: 617421.
[19] Raimondi C, Gradilone A, Naso G, Vincenzi B, Petracca A, Nicolazzo C, Palazzo A, Saltarelli R, Spremberg F, Cortesi E, Gazzaniga P. Epithelial-mesenchymal transition and stemness features in circulating tumor cells from breast cancer patients. Breast Cancer Res Treat 2011; 130: 449-55.
[20] Li TT, Liu H, Li FP, Hu YF, Mou TY, Lin T, Yu J, Zheng L, Li GX. Evaluation of epithelial-mesenchymal transitioned circulating tumor cells in patients with resectable gastric cancer: Relevance to therapy response. World J Gastroenterol 2015; 21: 13259-67.
[21] Kasimir-Bauer S, Hoffmann O, Wallwiener D, Kimmig R, Fehm T. Expression of stem cell and epithelial-mesenchymal transition markers in primary breast cancer patients with circulating tumor cells. Breast Cancer Res 2012; 14: R15.
[22] Lucci A, Hall CS, Lodhi AK, Bhattacharyya A, Anderson AE, Xiao L, Bedrosian I, Kuerer HM, Krishnamurthy S. Circulating tumour cells in non-metastatic breast cancer: a prospective study. Lancet Oncol 2012; 13: 688-95.
[23] Rack B, Schindlbeck C, Jückstock J, Andergassen U, Hepp P, Zwingers T, Friedl TW, Lorenz R, Tesch H, Fasching PA, Fehm T, Schneeweiss A, Lichtenegger W, Beckmann MW, Friese K, Pantel K, Janni W; SUCCESS Study Group. Circulating tumor cells predict survival in early average-to-high risk breast cancer patients. J Natl Cancer Inst 2014; 15: 106.
[24] Polioudaki H, Agelaki S, Chiotaki R, Politaki E, Mavroudis D, Matikas A, Georgoulias V, Theodoropoulos PA. Variable expression levels of keratin and vimentin reveal differential EMT status of circulating tumor cells and correlation with clinical characteristics and outcome of patients with metastatic breast cancer. BMC Cancer 2015; 15: 399.
[25] Mego M, Cierna Z, Janega P, Karaba M, Minarik G, Benca J, Sedlácková T, Sieberova G, Gronesova P, Manasova D, Pindak D, Sufliarsky J, Danihel L, Reuben JM, Mardiak J. Relationship between circulating tumor cells and epithelial to mesenchymal transition in early breast cancer. BMC Cancer 2015; 15: 533.
[26] Papadaki MA, Kallergi G, Zafeiriou Z, Manouras L, Theodoropoulos PA, Mavroudis D, Georgoulias V, Agelaki S. Co-expression of putative stemness and epithelial-to-mesenchymal transition markers on single circulating tumour cells from patients with early and metastatic breast cancer. BMC Cancer 2014; 14: 651.
[27] Gao D, Vahdat LT, Wong S, Chang JC, Mittal V. Microenvironmental regulation of epithelial-mesenchymal transitions in cancer. Cancer Res 2012; 72: 4883-9.