

Distribution of circulating tumor cell phenotype in early cervical cancer

This article was published in the following Dove Press journal:
Cancer Management and Research

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Background and objective: Circulating tumor cells (CTCs) can be classified into three phenotypes based on epithelial-to-mesenchymal transition (EMT) markers, including epithelial CTCs, mesenchymal CTCs, and mixed phenotypic CTCs. This study is aimed to analyze the correlation between CTC phenotypes and the clinicopathological features of patients with early cervical cancer.

Methods: Peripheral blood samples were obtained from 90 patients with early cervical cancer. CTCs were isolated and classified. The correlations of CTC counts and CTC phenotypes with clinicopathological features of patients were analyzed.

Results: The positivity rate for CTCs in patients with stage I-IIA cervical cancer was 90%. An increased CTC number was observed in patients with FIGO stage II, pelvic lymph node metastasis, and lymphovascular involvement. There were 38.89% epithelial CTCs, 23.33% mesenchymal CTCs, and 14.44% mixed phenotypic CTCs. Mesenchymal CTCs were more common in patients with FIGO stage II, pelvic lymph node metastasis, lymphovascular involvement, and deep stromal invasion.

Conclusion: CTCs with mesenchymal phenotypes are closely related to pelvic lymph node metastasis and lymphatic vascular invasion in stage I-IIA cervical cancer. Detection of circulating tumor cell phenotypes is helpful for the early diagnosis of cervical cancer micro-metastasis and for the assessment of disease status.

Keywords: circulating tumor cell, phenotype, cervical cancer

Introduction

Cervical cancer is a common malignant tumor in women. Its incidence is second only to breast cancer, and it has a tendency to occur in young women, thereby seriously threatening their reproductive health and quality of life.^{1,2} The early detection of cervical cancer is remarkably significant for achieving radical cure. The treatment of patients with advanced cervical cancer is poor, and the recurrence and metastasis rates are high.³ Therefore, the diagnosis, treatment, curative effect, and prognosis of cervical cancer have attracted worldwide attention. The recurrence and metastasis of cervical cancer can seriously perplex clinicians.

Recently, several researchers have reported that circulating tumor cells (CTCs) play an important role in metastasis.⁴⁻⁶ They penetrate the basement membrane into the peripheral circulatory system and eventually grow in distant organs to form metastases.⁷ CTCs are an important hub between primary and metastatic tumors in the process of tumor metastasis and function in the induction of metastatic tumor and preservation of biological characteristics similar to those of primary tumors.⁸ CTCs can further clarify the characteristics of the primary lesion to develop a more

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effective individualized treatment.⁹ There have been significant achievements in the treatment of various malignant tumors, especially breast cancer. It is now clear that the number of CTCs in breast cancer patients is related to the prognosis of patients. Cristofanilli et al¹⁰ reported that more than 5 CTCs for every 7.5 milliliter blood is a prognostic factor for metastatic breast cancer. In addition, CTCs are associated with the progression and poor prognosis of many other solid tumors including lung cancer,^{11,12} colorectal cancer,^{13,14} and pancreatic cancer.^{15,16} The detection of CTCs is non-invasive and more acceptable to patients. Moreover, CTC detection may facilitate an earlier and more effective diagnosis of tumor recurrence and metastasis than computed tomography and positron emission tomography.^{17–20} Therefore, the study of cervical cancer CTCs may play a significant role in clinical diagnosis and treatment.

There are different phenotypes of CTC in peripheral blood. The main phenotypes include epithelial CTCs, mesenchymal CTCs, and mixed epithelial–mesenchymal CTCs.²¹ Epithelial-to-mesenchymal transition (EMT) is an important characteristic of cancer with high metastatic ability.^{22,23} It is believed that EMT activation facilitates the generation of CTCs and improves their survival in the bloodstream.^{21,24,25} During EMT, the epithelial tumor cells can become more aggressive and migratory; these cells can easily enter distant organs by invading blood vessels or lymphatic vessels.

The purpose of this study was to analyze the correlation between CTC phenotypes and the clinicopathological features of patients with stage I-II A (International Federation of gynecology and obstetrics, FIGO) cervical cancer.

Material and methods

Patients

A total of 90 primary cervical cancer patients with stage I-IIA who underwent radical surgical resection in the Sichuan Cancer Hospital & Institute & School of Medicine, University of Electronic Science and Technology of China, between 2015 and 2017 were selected. All patients were confirmed by preoperative cervical biopsy pathology and post-operative pathological diagnosis. The clinicopathological features of patients are listed in Table 1. Twenty healthy controls were enrolled. The study is approved by the University of Electronic Science and Technology of China according to the Declaration of Helsinki (6th revision, 2008), and all subjects were asked to sign an informed consent form.

Table 1 CTC number and clinicopathological features in early cervix cancer patients

Features	n	CTC number (Canpatrol™)		t	P
		Mean	SD		
Age (years)				-0.324	0.746
≤45	18	10.31	13.25		
>45	72	11.25	12.28		
Pathology type				1.531	0.130
Squamous cell carcinoma	75	10.23	10.48		
Adenocarcinoma	11	9.87	13.16		
Others	4	11.13	12.93		
FIGO stage				-3.253	0.002
I	34	6.22	10.43		
II	56	13.25	14.76		
Pelvic lymph node metastasis				2.885	0.005
With	67	12.58	11.68		
Without	23	7.91	13.41		
Lymphovascular involvement				3.497	<0.001
With	73	15.32	13.65		
Without	17	8.26	12.83		
Deepstromal invasion				1.232	0.224
With	52	10.24	11.82		
Without	38	8.99	10.68		
Parametrial extension				1.340	0.184
With	76	12.51	12.26		
Without	14	9.15	10.40		

Abbreviations: CTC, circulating tumor cell; FIGO, International Federation of Gynecology and Obstetrics.

CTC detection

A total of 5 mL of peripheral blood was collected from each patient in the morning before surgery. CTCs were isolated via optimized CanPatrol™ CTC enrichment technique implemented using CD45 antibody.^{26,27} First, erythrocytes were removed by carrying out red blood cell lysis and CD45+ leukocytes were depleted using magnetic bead separation method. CTCs were enriched by passing them through calibrated membrane filters having 8-μm-diameter pores; CTCs were identified and characterized by RNA-in situ hybridization (ISH). Based on branched DNA (bDNA) signal amplification technology, CTCs were classified using EMT markers into three subpopulations including epithelial CTCs (CTCs with the epithelial marker EpCAM or CK8), mesenchymal

CTCs (CTCs with the mesenchymal marker vimentin or TWIST), and mixed phenotypic CTCs (CTCs with both epithelial and mesenchymal markers) as previously described.²⁸ Cellsearch[®] detection was also performed.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (IBM, USA). The comparison between CTCs and clinicopathological features were analyzed using Student's *t*-test or one-way ANOVA test. The correlation between CTCs and clinicopathological features was examined using Pearson's chi-square test. A *P*-value of <0.05 was considered to be statistically significant.

Results

CTCs in cervical cancer patients

The positive rate of CTCs in patients with stage I-IIA cervical cancer was 90% (80 out of 90 samples). The CTC count per 5 mL of peripheral blood in patients with positive CTCs ranged from 1 to 39 (median 10).

Correlation between CTCs and clinicopathological features of cervical cancer

We assessed the correlation between clinicopathological features and CTC count (Table 1). There were no significant differences in CTC count between patients of different ages or those with different pathological types ($P>0.05$). An increased CTC count was observed in patients with FIGO stage II, pelvic lymph node metastasis and lymphovascular involvement ($P<0.01$). There was no relationship between CTC count and patients with deep stromal invasion or parametrial extension ($P>0.05$). CTCs could not be detected in healthy controls. The rate of CTC detection using Cellsearch[®] was lower than that of optimized CanPatrol[™] CTC enrichment technique (Figure 1).

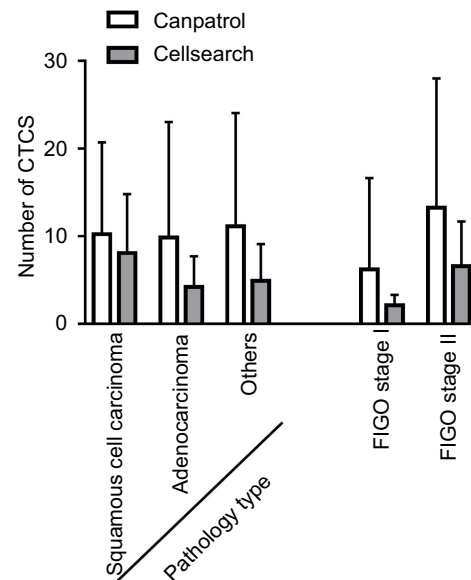


Figure 1 The comparison on CTC number detection between Cellsearch[®] and optimized CanPatrol[™] CTC enrichment techniques.

Abbreviations: CTCs, circulating tumor cells; FIGO, International Federation of Gynecology and Obstetrics.

Correlation between CTC phenotypes and clinicopathological features of cervical cancer

RNA-in situ hybridization led to the identification of EMT markers in an epithelial CTC (red dots), a mesenchymal CTC (green dots), and a mixed phenotypic CTC (red and green dots) (Figure 2). Thus, we classified CTC phenotypes of patients into three subpopulations based on EMT markers, including epithelial CTCs, mesenchymal CTCs, and mixed phenotypic CTCs. As shown in Figure 3, there were 38.89% epithelial CTCs, 23.33% mesenchymal CTCs, 14.44% mixed phenotypic CTCs, and 23.33% CTCs without detectable phenotypes. The correlation between CTC phenotypes and clinicopathological features was also analyzed (Table 2). There was no significant relationship between CTC phenotypes and age, pathology type, or parametrial extension ($P>0.05$).

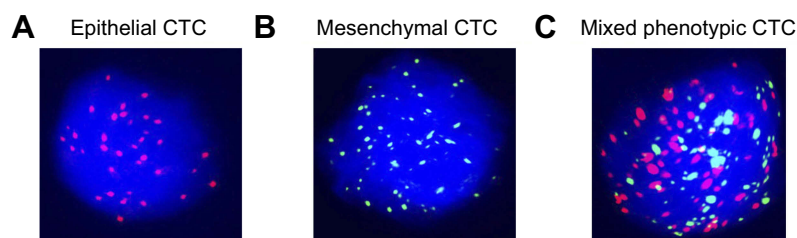


Figure 2 Identified EMT markers by RNA-in situ hybridization. (A) an epithelial CTC (red dots); (B) a mesenchymal CTC (green dots); (C) a mixed phenotypic CTC (red and green dots).

Abbreviations: EMT, epithelial-to-mesenchymal transition; CTC, circulating tumor cell.

■ 23.33% Not detected
 ■ 38.89% Epithelial CTCs
 ■ 23.33% Mesenchymal CTCs
 ■ 14.44% Mixed phenotypic CTCs

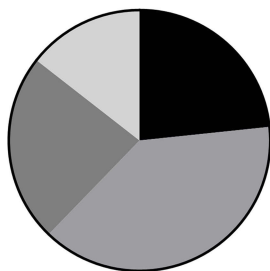


Figure 3 CTC phenotype distribution in peripheral blood from early cervix cancer patients (percentage of CTC phenotype).

Abbreviations: CTCs, circulating tumor cells; FIGO, International Federation of Gynecology and Obstetrics.

However, mesenchymal CTCs were more common in patients with FIGO stage II, pelvic lymph node metastasis, lymphovascular involvement, and deep stromal invasion.

Discussion

At present, the diagnosis of recurrence and metastasis of cervical cancer is dependent on pathological tissues. As an emerging molecular marker, CTCs facilitate individualized treatment of tumor and act as guiding tools for early clinical diagnosis as well as the evaluation of treatment efficacy and prognosis.²⁹ Moreover, the presence of peripheral blood CTCs, as a noninvasive indicator, will enable clinicians to design an individualized postoperative adjuvant therapy for patients with cervical cancer for reducing recurrence and metastasis in and improving the over survival and quality of life of cancer patients.³⁰ A poor prognosis has been suggested in patients with early breast cancer who have ≥ 5 CTCs per 30 mL of blood.¹⁰ When the CTC count in the peripheral blood of patients with nonmetastatic colorectal cancer is $\geq 1/7.5$ mL, it greater risk of recurrence and metastasis and worse prognosis have been indicated.³¹ This study revealed that patients with early cervix cancer who had higher CTC

Table 2 CTC phenotypes and clinicopathological features in early cervix cancer patients

Features	n	Not detected	Epithelial CTCs	Mesenchymal CTCs	Mixed phenotypic CTCs	χ^2	P
Age (years)		21	35	21	13	2.637	0.451
≤45	18	3 (16.7%)	10 (55.6%)	3 (16.7%)	2 (11.1%)		
>45	72	18 (25.0%)	25 (34.7%)	18 (25.0%)	11 (15.3%)		
Pathology type						5.987	0.425
Squamous cell carcinoma	75	14 (18.7%)	31 (41.3%)	18 (24.0%)	12 (16.0%)		
Adenocarcinoma	11	5 (45.5%)	3 (27.3%)	2 (18.2%)	1 (9.1%)		
Others	4	2 (50.0%)	1 (25.0%)	1 (25.0%)	0 (0.0%)		
FIGO stage						20.705	<0.001
I	34	6 (17.6%)	23 (67.6%)	2 (5.9%)	3 (8.8%)		
II	56	15 (26.8%)	12 (21.4%)	19 (33.9%)	10 (17.9%)		
Pelviclymphnode metastasis						9.018	0.029
With	67	18 (26.9%)	20 (29.9%)	18 (26.9%)	11 (16.4%)		
Without	23	3 (13.0%)	15 (65.2%)	3 (13.0%)	2 (8.7%)		
Lymphovascular involvement						13.187	0.004
With	73	20 (27.4%)	22 (30.1%)	20 (27.4%)	11 (15.1%)		
Without	17	1 (5.9%)	13 (76.5%)	1 (5.9%)	2 (11.8%)		
Deepstromal invasion						11.968	0.007
With	52	8 (15.4%)	17 (32.7%)	18 (34.6%)	9 (17.3%)		
Without	38	13 (34.2%)	18 (47.4%)	3 (7.9%)	4 (10.5%)		
Parametrial extension						2.640	0.451
With	76	19 (25.0%)	27 (35.5%)	18 (23.7%)	12 (15.8%)		
Without	14	2 (14.3%)	8 (57.1%)	3 (21.4%)	1 (7.1%)		

Abbreviations: CTCs, circulating tumor cells; FIGO, International Federation of Gynecology and Obstetrics.

count in their peripheral blood also were at a higher risk of reaching FIGO stage II or experiencing pelvic lymph node metastasis, lymphovascular invasion, and deep stromal invasion.

Lecharpentier³² found that none of the CTCs extracted from six patients with non-small cell lung cancer metastasis expressed only epithelial markers but presented with both mesenchymal and epithelial markers. Moreover, in three other cases, only mesenchymal CTCs were expressed; although this study confirmed the existence of EMT, it did not primarily focus on tumor tissues that express only epithelial markers. Mego et al³³ examined the expression of EMT markers such as TWIST1, SNAIL1, SLUG, ZEB1, and FOXC2 in breast cancer CTCs and showed that the chemotherapy did not change the EMT markers present in CTCs. With the development of CTC detection, RNA-in situ hybridization technology is gradually applied in the clinic. This technique makes use of both epithelial and mesenchymal markers to specifically identify the EMT phenotypes of CTCs. In 2013, Yu et al³⁴ used RNA-in situ hybridization to study the effect of epithelial–mesenchymal ratio in patients with breast cancer and found that higher the proportion of epithelial phenotype, higher is the effectiveness of this technique. On the contrary, poor prognosis was suggested in associated with a high proportion of mesenchymal phenotype. We classified the CTC phenotypes of patients into three subpopulations based on EMT markers, including epithelial CTCs, mesenchymal CTCs, and mixed phenotypic CTCs. There were 38.89% epithelial CTCs, 23.33% mesenchymal CTCs, and 14.44% mixed phenotypic CTCs. There were 23.33% CTCs without detectable phenotypes, which might be owing to the concealing of markers. Other markers should be considered in the future. The mesenchymal CTCs were more common in patients with FIGO stage II, pelvic lymph node metastasis, lymphovascular involvement, and deep stromal invasion. We speculated that tumor cells may enter peripheral blood by invading lymphatic vessels, blood vessels, and lymphatic vessels as positive CTCs. The infiltration of the uterus may penetrate the deep muscle layer and result in the infiltration of surrounding interstitial tissues, but the tumor cells do not invade the lymphatic and blood vessels and do not enter the peripheral blood circulation. Postoperative pathology suggests that pelvic lymph node metastasis and lymphatic vessel infarction are associated with a high risk of recurrence and metastasis. It is recommended to perform adjuvant radiotherapy and chemotherapy in clinical practice, and CTCs are associated with pelvic lymph node metastasis and lymphatic vascular

invasion. Cervical cancer patients with CTCs are at a higher risk of distant metastasis. For these patients, it is recommended to further improve the implementation of relevant examinations to exclude metastasis and consider strengthening the intensity of chemotherapy. Thus, monitoring of CTCs is helpful for the diagnosis and evaluation of the prognosis of cervical cancer. Owing to the nature of cervical cancer that is usually accompanied by HPV infection, a definitive study should be performed to understand the HPV genome, RNA, or protein sequences in the CTCs of selected HPV16/18-positive patients in the future.

In summary, CTCs with mesenchymal phenotypes are closely related to pelvic lymph node metastasis and lymphatic vascular invasion in stage I-IIA cervical cancer. Detection of circulating tumor cell phenotypes is helpful for the early diagnosis of cervical cancer micro-metastasis and assessment of disease status. In the future, the clinical value of CTC detection in cervical cancer needs to be estimated via a large-scale prospective randomized study.

Disclosure

The authors report no conflicts of interest in this work.

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