

Original Article

Circulating tumor cells can indicate the spread of prostate cancer through local invasion in addition to hematogenous metastasis

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Abstract: Circulating tumor cells (CTC) have been shown to be correlated with metastasis and poor prognosis in prostate cancer, but there are few studies describing the relationship between circulating tumor cells and local invasion in prostate cancer. Our aim was to determine whether circulating tumor cells predicted local invasion before distant metastasis occurs in prostate cancer. Blood samples from 77 treatment-naïve patients with prostate cancer were analyzed for CTCs. The CTCs were enriched with 7.5 mL blood using an EpCAM-independent assay and identified using immunostaining-fluorescence in situ hybridization. The number of CTCs was significantly different between the patients with and without local invasion ($P < 0.001$). The patients with distant metastasis had more CTCs than the patients without distant metastasis ($P < 0.001$). There was no significant difference in the number of CTCs in the patients with and without lymph node metastasis ($P = 0.0079$). The patients with 3 CTCs or more per 7.5 mL blood had poorer survival rates and higher hazards ratios (HR). In this study, we demonstrate that the presence of CTCs is associated with not only distant metastasis but also with local invasion.

Keywords: Circulating tumor cells (CTC), prostate cancer, tumor spread, local invasion, distance metastasis

Introduction

Prostate cancer is commonly diagnosed in Chinese men and is one of the leading causes of cancer-related deaths in men worldwide [1, 2]. The 5-year survival rate is more than 99% in non-metastatic patients but only 30% in metastatic prostate cancer [2]. Tumor metastasis results in the vast majority of cancer-related deaths in many types of cancer, including prostate cancer whether it preexists or develops after treatment [3, 4]. Prostate cancer metastasis is usually defined as tumor cells spreading through the lymph or blood into other distant sites [5, 6]. Local invasion is the stage before metastasis, characterized by cancer cells spreading into the nearby sites such as the seminal vesicles and the bladder [7]. Cancer with local invasion may still respond to treatment, but typically it is much more difficult to cure. It has been estimated that 15-25% of patients with localized prostate cancer develop a biochemical recurrence after radical

prostatectomy [8], suggesting that the tumor cells may have spread to vessels before the clinical diagnosis. Therefore, the prediction of tumor spread is very crucial for determining the prognosis and treatment of patients with prostate cancer.

Although computed tomography (CT) and magnetic resonance imaging (MRI) are widely used in the detection of cancer metastasis, such medical imaging is mainly used for cancer detection and not for prediction and prognosis [9-12]. Prostate-specific antigen (PSA) plays an important role in the noninvasive detection of prostate cancer metastasis [13] but with a low specificity during the early detection period of tumor spread [14, 15]. The accurate detection of progressive lesions and any response to treatment are prerequisites for the successful prediction of the survival of cancer patients.

Circulating tumor cells (CTCs) are shed from solid tumors and metastasis through the blood

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Table 1. Clinical characteristic of the patients

	Total	CTC $\geq 3/7.5$ ml	CTC $< 3/7.5$ ml
Total	77	28	49
Age, years (range)	73 (56-88)		
Gleason score			
< 8	34	16	8
≥ 8	43	20	23
PSA			
≤ 10 ng/ml	24	9	15
> 10 ng/ml	53	19	34
lymph node metastasis			
Positive	13	8	5
Negative	64	20	44
Local invasion			
Positive	20	19	1
Negative	57	9	48
Distant metastasis			
Positive	11	9	2
Negative	66	19	47

[6, 16, 17]. The number of CTCs in the peripheral blood before the start of systemic treatment has been shown to be an effective predictor of tumor progression, long-term prognosis, and overall survival in metastatic cancers [18, 19]. In fact, 5%-24% of nonmetastatic patients with prostate cancer were found to have CTCs in their peripheral blood [19]. So far, it is still not clear if the CTC number can be used to predict spreading into nearby site.

We designed this study to explore whether CTCs can be used to predict local invasions of prostate cancer, as well as to evaluate the value of CTCs in the prediction of local invasion in patients before treatment. This information might be useful for the prediction of tumor invasion and helpful for patients benefiting from in time treatment.

Material and methods

Patients

A total of 77 males ranging in age from 56 to 88 (median = 73) years with prostate cancer were enrolled at their first diagnosis. The inclusion criteria were: 1) patients who were histologically proven to have prostate adenocarcinoma, 2) patients who were able to attend clinical follow-ups, 3) patients with no history of tumors, and 4) patients who stopped antico-

agulant drugs for one week. The patients' Gleason scores, serum PSA levels, and blood samples were determined and collected before the start of any systemic treatment. However, any patients who previously had been treated with local therapy, radiation, or hormonal therapy were excluded. The clinical characteristics of the enrolled patients are listed in **Table 1**. This study was performed in accordance with established ethical standards. The study was approved by the Research Ethics Committee of Hubei Medical College (approval number 201702170b). Written informed consents were obtained from all the patients before the study began. Their blood was collected before any medical treatment. The results for the individual patients

were masked using a random number system as the unique patient identifier.

Enrichment and identification of CTC

Blood samples were drawn into ACD tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and processed within 48 h after collection. Cytelligen CTC enrichment kits (Cytelligen, San Diego, CA, USA) were used for the subtraction enrichment of the CTCs following procedures established in previous studies [20, 21]. Briefly, 7.5 ml of blood were thoroughly mixed with 3 mL of hCTC separation matrix (Cytelligen). After centrifugation, the collected supernatants were incubated with immunomagnetic beads coated with specific anti-leukocyte antigen CD45 antibodies for 10 minutes with gentle agitation. Then the leukocytes were removed from the mixture using magnetic separation and using a magnetic stand (Promega Madison, WI, USA). The cell pellets were mixed with cell fixative from Cytelligen, Inc. and used to coat the CTC slides. The enriched samples were processed for immunostaining-fluorescence in situ hybridization (iFISH). Hematopoietic white blood cells (WBCs) were excluded with monoclonal anti-CD45 antibody conjugated to Alexa Flora 594 (Invitrogen, Carlsbad, CA, USA). The FISH analysis was performed with Centromere Probe 8 (CEP8) SpectrumOrange DNA Probe

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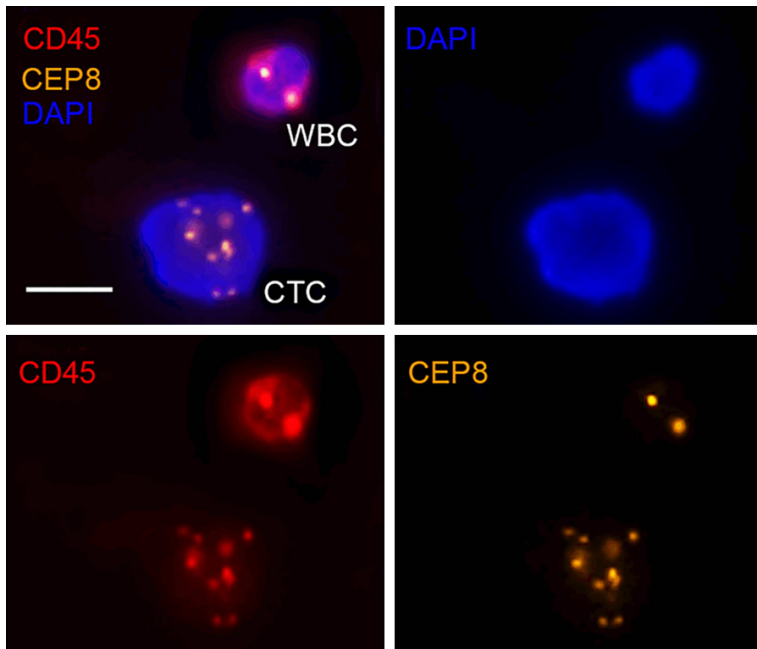


Figure 1. CTCs in patients with prostate cancer. CTC is defined as CD45-/DAPI+/CEP8 \geq 3. WBC is defined as CD45+/DAPI+/CEP8 = 2. DAPI, blue; CEP8, orange; CD45, red. The scale bar is 10 μ m.

Kits (Vysis, Abbott Laboratories, Abbott Park, IL, USA) and the nucleic acids were stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). The stained cells were analyzed using a fluorescence microscope (Nikon, Tokyo, Japan).

Statistical analysis

Survival was defined as the time elapsed between the date of diagnosis and the 30 months follow-up. Log-rank tests were used to detect the significant differences between the groups with different CTC numbers. We used the Cox proportional hazards regression model to establish univariate HRs for progression-free survival [19]. The *P* values were two-tailed, and values less than 0.05 were judged to be statistically significant. SPSS 17.0 software was used for the statistical analysis.

Results

Enumeration of CTC

We enrolled 77 patients into our study, and their mean age was 73 years (Table 1). No adverse events or complications were reported from the blood collections. The presence of a

nucleus, cellular morphology, a lack of CD45 expression, and more than 2 signals of Centromere Probe 8 (CEP8) were the required CTC characteristics (Figure 1). A Total of 180 CTCs were identified in 47 patients (61.0%) in our cohort, and 0 to 12 CTCs (median = 2) per 7.5 mL of blood were observed in each patient. In our study, 49 patients (49/77, 63.6%) were found harboring fewer than 3 CTCs/7.5 ml blood, while the rest of the patients (28/77, 36.4%) had three or more circulating tumor cells. The CTC number was found to have no significant association with age, PSA or the Gleason score.

CTCs and spreading

For the 13 patients with lymph node metastasis, the median number of CTCs was 4, and they ranged from 0 to 7 per 7.5 mL blood. For the 64 patients without lymph node metastasis, the median number of CTCs was 1.5, and they ranged from 0 to 12 per 7.5 mL blood. A detailed analysis found that the CTCs were not associated with lymph node metastasis (*P* = 0.078, left of Figure 2; Table 1). Twenty patients with local invasion harbored 5 CTCs per 7.5 mL of blood in median (1-8 CTCs), while 57 patients without local invasion harbored 0 CTCs per 7.5 mL of blood in (0-12 CTCs). The CTCs were significantly associated with local invasion (*P* < 0.001, middle of Figure 2; Table 1). The median number of CTCs in the 11 patients with distant metastasis was 5 per 7.5 mL blood (4-12 CTCs), while the median number of CTCs in the 66 patients without distant metastasis was 1 per 7.5 mL blood (0-8 CTCs). It was shown that CTCs are associated with distant metastasis (*P* < 0.001, right of Figure 2; Table 1).

To verify the relationship between the CTC number and tumor spreading (both local invasion and distant metastasis), we used ROC curves for a further analysis. The results showed that the AUC for CTCs in the patients

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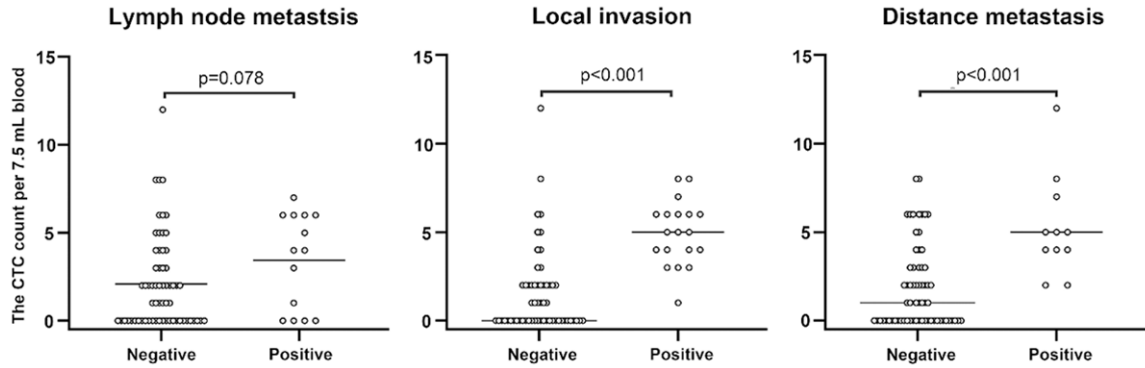


Figure 2. The correlation between the counts of CTCs and spreading. Left: a comparison of the CTCs in patients with and without lymph node metastasis; middle: a comparison of the CTCs in patients with and without local invasion; right: a comparison of the CTCs in patients with and without distant metastasis.

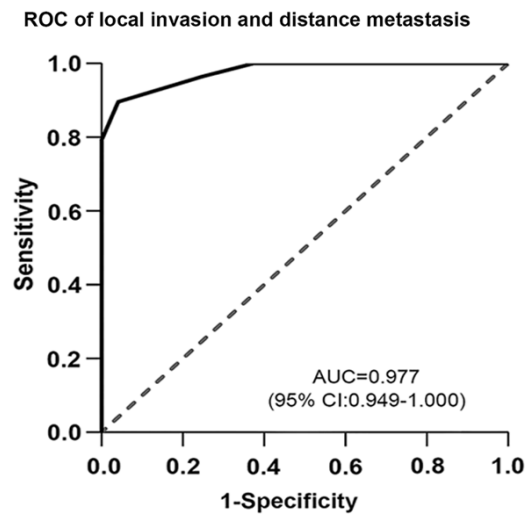


Figure 3. ROC of the local invasion and distant metastasis. The CTC ROC curve for discriminating between patients with and without invasion and distance metastasis. The area under the ROC curve was 0.977.

with local invasion and distant metastasis was 0.977 [95% confidence interval (CI), 0.949-1.000; **Figure 3**]. These data demonstrate that CTCs are associated with local invasion and hematogenous metastasis.

The number of CTCs in most patients with neither invasion nor hematogenous metastasis are generally no more than 3 with one exception. Six CTCs were observed in a special case without invasion or metastasis, of which a cancer thrombus was found in the vessel.

Prognosis by CTCs

When we use 3 CTCs/7.5 ml blood as a cutoff to divide these patients into 2 groups, 49

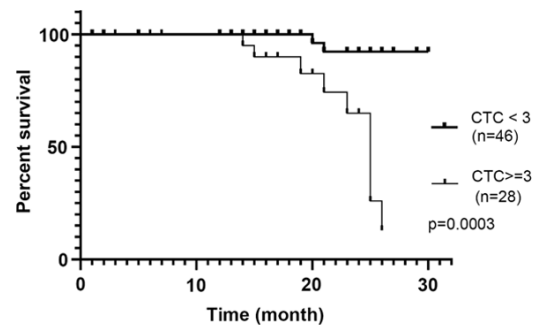


Figure 4. A Kaplan-Meier curve analysis of survival. The survival analysis curve between the patients with CTC counts ≥ 3 per 7.5 mL of blood and those with CTC counts < 3 per 7.5 mL of blood were significantly different ($P = 0.0003$, log-rank test).

patients with less than 3 CTCs/7.5 ml showed a high survival percentage at 30 months, which was much higher than it was in the other 28 patients with 3 or more CTCs/7.5 ml (**Figure 4**). The survival rate was nearly the same during the first 14 months in the patients with CTC < 3 group and CTC ≥ 3 group. However, from 14 to 30 months, the patients with three or more CTCs showed a lower survival rate than the patients with less than 3 CTCs ($P < 0.05$).

The Cox regression analyses showed that CTC ≥ 3 had an HR of 12.81 for disease progression ($P = 0.0003$, **Table 2**), which was much higher than the HR of the Gleason score ≥ 8 (HR = 1.983, $P = 0.3396$) and PAS level > 10 ng/L (HR = 2.257, $P = 0.0798$). Therefore, considering the results of HR and the progression-free survival analysis, the number of CTCs is more useful for the early detection and prognosis of local invasion in prostate cancer patients.

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Table 2. Cox regression analyses

Parameter	P value	HR	95% CI
CTC \geq 3/7.5 ml blood	0.0003	12.81	3.330-49.290
Gleason score \geq 8	0.3396	1.983	0.509-7.724
PSA > 10 ng/ml	0.0798	2.257	0.407-12.51

Discussion

CTC can be identified in 20-49% of patients with high-risk localized prostate cancer (> T3a disease) with CTC counts ranging from 1 to 5 cells/7.5 ml blood using the CellSearch system [22, 23]. The relatively low rate and count of CTC might be caused by the different criteria for the patients enrolled and the different methods used for the CTC quantification. It was demonstrated that the subtraction enrichment combined with the FISH analysis presents a relatively high sensitivity for CTC enumeration [20, 21]. In our cohort, for example, the CTCs were observed in a higher ratio of patients compared with previous studies.

Our findings showed that the number of CTCs per 7.5 ml blood was associated with local invasion and hematogenous metastasis, but not associated with lymph node metastasis. Although the lymph node involvement has been regarded as being associated with systemic disease in prostate cancer, no significant association has been found between lymph node metastasis and CTC detection before [24], which is consistent with our results. Therefore, the presence of CTCs might not be an effective evaluation method for diagnosing lymph node metastasis in prostate cancer.

In the present study, 6 CTCs per 7.5 ml blood were observed in a patient with neither local invasion nor distant metastasis, but with a cancer thrombus in vessel. Actually, when the CTCs disseminate through the systemic vasculature, the tumor cells can gather to form circulating tumor microemboli, then constituting the hematogenous route of metastasis and highly metastatic, which may happen before the clinical diagnosis of distant metastasis [25, 26]. Therefore, the high metastasis ability of cancer thrombus causes the presence of a considerable number of CTCs in the blood without hematogenous metastasis observed clinically. This case indicates that CTC is a highly sensitive marker for monitoring cancer metastasis.

Invasion to the neighboring tissue before distant metastasis is an important part of the metastatic cascade [6]. However, previous studies [27-30] did not analyze the connection between CTC and local invasion, which play an important role in the metastatic cascade. In the present study, we found that the number of CTCs identified in patients with local invasion is relatively higher than it is in patients without local invasion, implying a significant association between CTCs and local invasion in prostate cancer. Also, the relationship between the CTCs and local invasion suggests that the blood circulatory system might participate in the process of invasion. Therefore, CTC in the blood is a more sensitive biomarker for metastasis, not only for distant metastasis but also for local invasion in prostate cancer.

As local invasion is the early part of the process of tumor metastasis, it would be useful in the clinical management of cancer. Once the local invasion is verified, effective treatment can be given in time to prevent the tumor metastasis and further malignant development at an early date, improving the survival rate [31-33]. CTCs in the blood are a relatively sensitive biomarker for monitoring tumor invasion and metastasis for the prognosis evaluation. We found that the CTC count also correlates with the survival period. Similar results have been reported, namely that patients with less than 5 CTCs tend to survive longer than those with 5 or more CTCs (more than 4.00 years vs 0.7 years, log-rank $P = 0.002$) [34]. Moreover, another large cohort study concluded that the number of CTCs is the most accurate and independent predictor of overall survival (OS) in castration-resistant prostate cancer [35]. However, for the patients with cancer thrombi in their vessels and local invasions, it was difficult to detect the CTCs in time by conventional means. The presence of CTCs is necessary for generating distant metastasis (mainly bone lesions) during the course of prostate cancer.

In the present study, CTCs were identified in a high proportion of patients with prostate cancer. The presence of CTCs is associated with local invasion and distant metastasis, but it is not associated with lymph node metastasis. The relationship between CTCs and the spreading of the cancer to nearby sites was shown. The number of CTCs in patients with

local invasion were higher than they were in those without local invasion. In addition, our study suggests that CTCs are not only an early detection biomarker of spread, but they are also a meaningful prognostic marker.

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Disclosure of conflict of interest

None.

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