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Original Article

Expression of vascular endothelial growth factor in circulating tumor cells for prediction of colorectal cancer

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Abstract: Objectives: Previous studies used enumerated circulating tumor cells (CTCs) to predict prognosis and therapeutic effect in several types of cancers. However, increasing evidence showed that only enumerated CTCs were not enough to reflect the heterogeneity of tumor. Therefore, we classified different metastasis-potential of CTCs from colorectal cancer patients, in order to improve accuracy of the prognosis by CTCs. Methods: Blood samples were collected from 45 primary colorectal cancer patients. CTCs were enriched by blood filtration, and the RNA in situ hybridization method was used to identify and discriminate subgroups of CTCs. Afterwards, vascular endothelial growth factor (VEGF) expression in individual CTCs was measured. Results: Three CTCs subgroups (epithelial/biophenotypic/mesenchymal CTCs) were identified using epithelial-mesenchymal transition (EMT) markers. In our research, mesenchymal CTCs significantly increased along with tumor progression, including developing distant metastasis and vascular invasion. Furthermore, VEGF expression rate in mesenchymal CTCs was significantly higher than that of epithelial CTCs, which suggested that VEGF may be correlated with tumor malignancy. This hypothesis was further verified by VEGF expression in mesenchymal CTCs strictly related to tumor aggressiveness factors. Finally, we revealed that mesenchymal CTCs and VEGF expression may predict high risk subgroups in stage II colorectal cancer. Conclusions: Our research proved that CTCs could serve as feasible surrogate samples to detect gene expression as a predictive biomarker for tumor evaluation.

Keywords: Colorectal cancer, circulating tumor cells (CTCs), epithelial-mesenchymal transition (EMT), VEGF

Introduction

Despite improvements in surveillance and clinical treatment strategies, the prognosis of colorectal cancer (CRC) remains very poor due to high incidence of recurrence and metastasis, approximately 20% to 45% of those who undergo curative resection subsequently develop local tumor recurrence or metastasis at distant sites [1]. The lack of effective methods for timely diagnosis and monitoring anticancer treatment response is the main obstacle preventing improvement of overall survival (OS) of patients with CRC.

Traditional clinico-pathological parameters and serologic tumor markers offer limited information covering CRC diagnosis, prognosis prediction, and monitor the therapeutic response in a real-time manner. Therefore, there is an urgent requirement to develop a reliable and versatile method for discriminating high-risk factors of recurrent patients and continuous surveillance of antitumor treatment response [2].

The spread of circulating tumor cells (CTCs) in the blood plays a major role in the initiation of metastases and tumor recurrence after surgery [3]. The clinical relevance of detecting CTCs as a prognostic and/or surrogate marker of treatment response has been established in several cancer types, such as breast cancer [4], colorectal cancer [5], and prostate cancer [6]. In a multicenter prospective study including 456 patients with metastatic colorectal cancer, which demonstrated that CTCs levels before treatment were an independent prognostic factor for PFS and OS [7]. A meta-analysis performed on 12 studies of stage IV CRC provides the strongest level of evidence for the

Table 1. Demographics of patients included in the study (n=45)

Characteristic	n	%
Age		
≤60	26	57.8
>60	19	42.2
Gender		
Male	18	40
Female	27	60
Tumor location		
Colon	30	66.7
Rectal	15	33.3
Tumor size		
≤5 cm	32	71.1
>5 cm	13	28.9
Tumor grade		
Low	8	17.8
Moderate	37	82.2
Vascular invasion		
No	32	71.1
Yes	13	28.9
Depth of invasion		
T1-T3	21	46.7
T4	24	53.3
Lymphatic metastasis		
No	20	44.4
Yes	25	55.6
Distant metastasis		
No	39	86.7
Yes	6	13.3
TNM stage		
I	5	11.1
II	20	44.4
III	14	31.1
IV	6	13.3
CEA		
≤5 ng/mL	24	53.3
>5 ng/mL	21	46.7
Ki-67		
≤60	24	53.3
>60	21	46.7
CTCs counts		
≥1 CTCs/5 mL	34	75.6
≥3 CTCs/5 mL	28	62.2

prognostic utility of CTCs [8]. These studies confirm the association between CTCs in patients with metastatic disease, and worse PFS and OS.

Most of these researches focus on the correlation of CTCs enumeration with prognosis [9-13]. However, recent studies showed that only enumerated CTCs was not enough to reflect the heterogeneous condition of the tumor [3, 14, 15]. CTCs disseminate from primary tumors by undergoing phenotypic changes that allow the cells to penetrate blood vessels [16]. These changes are accompanied by a process described as epithelial-mesenchymal transition (EMT), which is a complicated process that plays an essential role in metastasis [17].

Some recent reports have provided evidence that CTCs exhibit dynamic changes in epithelial and mesenchymal composition [18-20]. Mesenchymal CTCs are associated with metastasis and resistance to chemotherapy. These encourage future studies regarding the expression of EMT-related markers in CTCs and cancer progression.

Vascular endothelial growth factor (VEGF), originally known as vascular permeability factor (VPF), is a sub-family of growth factors, to be specific, the platelet-derived growth factor family of cystine-knot growth factors [21]. It is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis. It is part of the system that restores the oxygen supply to tissues when blood circulation is inadequate such as in hypoxic conditions [22]. When VEGF is overexpressed, it may lead to disease. Solid cancers could not grow beyond a limited size without an adequate blood supply, while cancers that can express VEGF are able to grow and metastasize. As VEGF may be related to CRC, and how VEGF expressed in CTCs and their clinical value were still unknown, it would be highly interesting to detect VEGF expression in CTCs, to get a deeper understanding of the role VEGF play in EMT process.

The aim of this study was to discriminate different metastasis potential of CTCs and explore VEGF expression in individual CTCs, in order to find the correlations of CTCs subgroups and VEGF expression in CTCs with the commonest clinical and morphological variables of CRC patients.

Methods

Patient samples and blood collection

This prospective single-institution study enrolled 45 patients with the following criteria: (1)

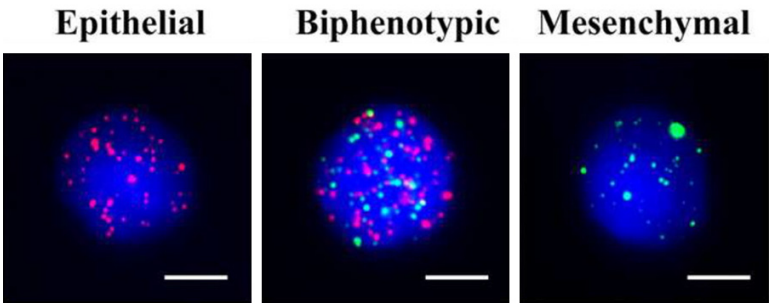

Figure 1. Representative images of three subgroups of CTCs isolated from patients with colorectal cancer, based on RNA-ISH staining of E (red dots) and M (green dots) markers. The scale bar is 10 μm. Note: E: epithelial; M: mesenchymal.

signed inform consent, (2) newly diagnosed non-metastatic colon having histological diagnosis, (3) newly diagnosed metastatic colorectal cancer, and (4) absence of other concomitant or previous malignant disease.

Patients were recruited by Cancer Hospital of China Medical University from Mar. 2015 to Dec. 2015. This study was approved by the Ethical Committee of Cancer Hospital of China Medical University. All patients had given informed consent to be included in this study.

Blood samples were collected before surgery or adjuvant chemotherapy for patients with early stages and before palliative chemotherapy in those with advanced disease. Blood samples (5 ml) were drawn into heparinized tubes and stored at 4 degrees within 4 hours.

CTCs identification

Erythrocytes were removed using a red blood cell lysis buffer containing ammonium chloride (NH_4Cl), and then transferred to the filtration tube and filtered with the help of a pump valve. CTCs were isolated using a calibrated membrane with 8-μm diameter pores [23].

The cells on the membrane were hybridized for 2 hours, and un-bound probes were washed three times with PBS. Subsequently, samples were incubated with preamplifier solution for 20 minutes, and then incubated with amplifier solution (Three types of fluorescently labeled probes, which had been conjugated with the fluorescent dyes: Alexa Fluor 594 (EpCAM and CK8/18/19), Alexa Fluor 488 (vimentin and twist), and Alexa Fluor 647 (CD45)). Finally, the cells were stained with DAPI for 5 minutes then analyzed with a fluorescence microscope [18, 19].

The leukocytes were characterized as CD45+DAPI+ cells. CTCs were defined with three subgroups: (1) epithelial marker-positive CD45-DAPI+ cells (Epithelial CTCs); (2) biophenotypic epithelial/mesenchymal marker-positive CD45-DAPI+ cells (Biophenotypic CTCs); (3) mesenchymal marker-positive CD45-DAPI+ cells (Mesenchymal CTCs).

Statistical methods

Correlation of CTCs with clinical variables was done by contingency table analysis using the chi-square test. Continuous data were compared using nonparametric tests (Mann-Whitney test for comparison between two groups and Kruskal-Wallis test for comparison among three or more groups). All analyses were conducted by using SPSS 20.0. For all analyses, $P<0.05$ was considered statistically significant.

Results

Patient demographics

Blood samples for CTCs assessment were taken in 45 consecutive patients with primary colorectal cancer. Clinical and morphological characteristics of the assessable 45 patients are summarized in **Table 1**. The median number of CTCs isolated was 4 (range 0-31).

Mesenchymal CTCs closely related to hematogenous metastasis

The CTCs could be classified into three subpopulations according to the EMT markers that expressed, including epithelial CTCs, biophenotypic CTCs, and mesenchymal CTCs. The typical photos were shown in **Figure 1**.

Overall, presence of ≥3 CTCs/5 ml was detected in 28 of 45 patients (62.2%), which was defined as CTCs positive. Mesenchymal CTCs (mCTCs) were found in 26 enrolled patients; ≥1 mCTCs/5 ml was defined as mCTCs positive.

Correlation between typical clinical/pathological variables and the presence of CTCs in blood

Table 2. Correlation among CTCs and clinical/morphological variables (n=45)

Characteristic	n	≥3 CTCs/ 5 mL	P	≥1 mCTCs/ 5 mL	P
Tumor location					
Colon	30	20	0.384	18	0.670
Rectal	15	8		8	
Tumor size					
≤5 cm	32	21	0.460	16	0.097
>5 cm	13	7		10	
Tumor grade					
Low	8	6	0.411	7	0.113
Moderate	37	22		19	
Vascular invasion					
No	32	18	0.195	15	0.020
Yes	13	10		11	
Depth of invasion					
T1-T3	21	10	0.203	8	0.058
T4	24	18		18	
Lymphatic metastasis					
No	20	13	0.615	10	0.434
Yes	25	15		16	
Distant metastasis					
No	39	23	0.252	20	0.024
Yes	6	5		6	
TNM stage					
I-II	25	13	0.114	12	0.138
III-IV	20	15		14	
CEA					
≤5 ng/mL	24	12	0.071	11	0.083
>5 ng/mL	21	16		15	
Ki-67					
≤60	22	11	0.299	11	0.302
>60	23	17		15	

Note: CTCs, Circulating tumor cells; mCTCs, Mesenchymal circulating tumor cells; CEA, Carcinoembryonic antigen.

was analyzed by Chi square test [24], which was shown in **Table 2**. Correlation was not found among positive CTCs and most of the clinico-pathologic features. Only stage (52.0% in stage I-II, 75.0% in stage III-IV, *P*=0.114) and CEA level (50.0% in CEA≤5 ng/mL, 76.2% in CEA>5 ng/mL, *P*=0.071) correlated with positive CTCs. However, the difference was not statistically significant (**Table 2**).

Among colorectal cancer patients, mesenchymal CTCs percentage significantly increased along with tumor progression. A significant association of mCTCs positivity and the development of distant metastases in CRC patients could be observed. The mCTCs was detected in all patients with distant metastasis, which significant higher than those without developed distant metastasis (100% *vs.* 51.3%, *P*=0.024). In addition, mCTCs was also closing related to vascular invasion. Our study showed that mesenchymal CTCs were more common to be found in patients with vascular invasion (84.6% *vs.* 46.9%, *P*=0.020). There was also a clear association between the presences of mCTCs and depth of invasion and/or TNM stage. However, the difference was not statistically significant (**Table 2**).

Considering the significantly higher percentage of mCTCs in the more aggressive status, we hypothesize that mCTCs detection can be a surrogate marker of tumor aggressiveness.

VEGF gene expression in CTCs aggressively correlated with tumorous

Furthermore, in our platform, CTCs can be captured and then used for further analysis gene expression. This is attractive because we can obtain genome information from the cancers via CTCs without invasive procedures, and detected genetic change in real time, which has the potential to provide predictive information to guide the selection of therapy.

Twenty-eight patients with CTCs positive (≥3 CTCs/mL) were enrolled for evaluated biomarker expression. VEGF+CTC were detected in 20/28 patients (71.4%). Photographs of CTCs with VEGF expression were shown in **Figure 2**.

Previous studies have shown wide molecular and cellular heterogeneity of CTCs from the same types of cancer and even from the same patient. Our research found that the overall expression rate of VEGF in CTCs was 60.7%, with 56.3% in epithelial CTCs, 58.6% in biophenotypic CTCs, and 68.8% in mesenchymal CTCs. VEGF expressed in mesenchymal CTCs significantly higher than in epithelial CTCs, which indicates that VEGF may correlated with tumor

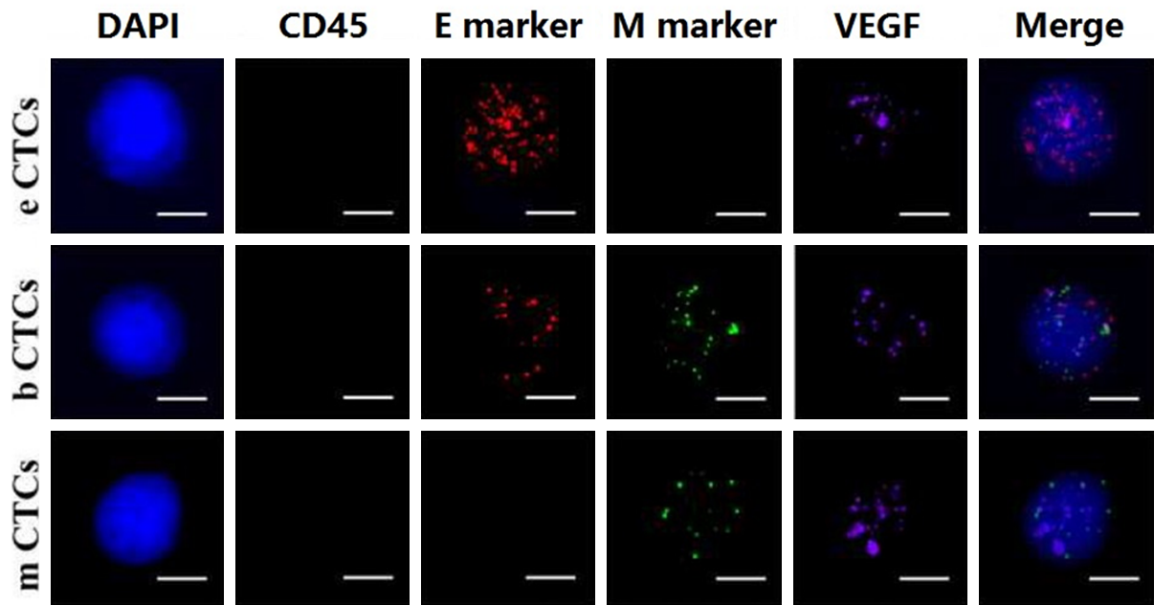

Figure 2. Representative images of VEGF expression in three subgroups of CTCs isolated from patients with colorectal cancer, based on RNA-ISH staining of E (red dots), M (green dots) markers, VEGF (purple dots) markers. The scale bar is 10 μm. Note: E: epithelial; M: mesenchymal; eCTCs: epithelial circulating tumor cells; bCTCs: biophenotypic circulating tumor cells; mCTCs: mesenchymal circulating tumor cells.

malignancy, promoting cancer cells metastasis and invasion.

The hypothesis was supported when analyzed the relationship between VEGF gene expression and characteristics of colorectal cancer patients, which showed in **Table 3**. We observed a significant association between VEGF expression and depth of invasion in CRC patients (68.1% in T1-T3 *vs.* 51.3% in T4, P=0.024). Besides, higher Ki-67 value showed higher VEGF expression rate (71.3% in Ki-67 ≤60 *vs.* 48.8% in Ki-67 >60, P=0.003). In addition, VEGF expression rate in mesenchymal CTCs closely correlated with metastasis-associated clinico-pathologic features, such as vascular invasion (78.9% *vs.* 37.5%, P=0.007) and depth of invasion (77.3% in T1-T3 *vs.* 33.3% in T4, P=0.004) in CRC patients. These results indicate that combining circulating tumor cells subgroups with VEGF gene expression may enhance clinical prediction of CRC cancer metastasis.

CTCs/VEGF detection may predict high risk subgroups in stage II colorectal cancer

For patients diagnosed at stage II, correlations between CTCs and prognostic subgroups were analyzed. CTCs detection would be an easy and reproducible test to select high-risk stage II patient candidates for adjuvant chemotherapy. At the present time, high-risk stage II is defined by clinical/pathological prognostic factors such as T4, perforation, acute bowel obstruction, undifferentiated tumors, high preoperative CEA levels or <12 lymph nodes removed [24].

The correlation of CTCs or VEGF detection and prognostic subgroups in stage II colorectal cancer was shown in **Table 4**. We found that mCTCs positive rate (66.7% *vs.* 37.5%, P=0.199) and VEGF expression positive rate (54.5% *vs.* 22.2%, P=0.142) in high risk groups were both higher than low risk groups, although there were no statistically significant differences between them.

Limited by sample quantity, although our study did not given absolute evidence to discriminate high/low risk of prognostic subgroups, there is potential clinical value that the potential of CTCs to better in aiding selection of patients high risk groups. As currently prescribed based on clinico-pathological criteria, there is controversial evidence regarding the benefits of chemotherapy.

Discussion

Blood sampling has advantages of less invasive, less painful, easy to perform, and better

Table 3. Demographics of patients with CTCs≥3 used for analysis VEGF expression rate with clinico-pathologic features (n=28)

Characteristic	VEGF expression in CTCs (%)	P	VEGF expression in mCTCs (%)	P
Tumor location				
Colon	64.0	0.151	56.0	0.977
Rectal	52.6		55.6	
Tumor size				
≤5 cm	56.6	0.164	51.9	0.497
>5 cm	68.0		62.5	
Tumor grade				
Low	75.0	0.523	60.0	0.841
Moderate	69.3		55.3	
Vascular invasion				
No	58.7	0.619	37.5	0.007
Yes	62.7		78.9	
Depth of invasion				
T1-T3	51.3	0.024	33.3	0.004
T4	68.1		77.3	
Lymphatic metastasis				
No	64.5	0.250	52.6	0.708
Yes	55.8		58.3	
Distant metastasis				
No	58.1	0.297	50.0	0.190
Yes	69.6		72.7	
TNM stage				
I-II	63.8	0.378	52.4	0.658
III-IV	57.1		59.1	
CEA				
≤5 ng/mL	60.4	0.082	55.6	0.780
>5 ng/mL	58.8		60.0	
Ki-67				
≤60	48.8	0.003	52.0	0.553
>60	71.3		61.1	

Note: CTCs, Circulating tumor cells; mCTCs, Mesenchymal circulating tumor cells; CEA, Carcinoembryonic antigen.

Table 4. CTCs/ VEGF detection and prognostic subgroups in stage II colorectal cancer

Prognostic subgroups	CTCs positive (%)	P	mCTCs positive (%)	P	VEGF expression positive (%)	P
Low risk	5 (50.0)	0.653	3 (37.5)	0.199	2 (22.2)	0.142
High risk	6 (60.0)		8 (66.7)		6 (54.5)	

accepted. For monitoring, the efficacy of therapy with detection and characterization of CTCs in blood sampling might be a new option for therapeutic interventions. CTCs enumeration via the CellSearch™ System is FDA-cleared for use as an aid in monitoring patients with metastatic colorectal cancers. The presence prior to treatment of ≥3 CTCs for colorectal cancer is associated with decreased progression-free and overall survival, and is prognostic, regardless of therapy used. This enrichment approach involves the attachment of magnetic particles to EpCAM expression on the cell surface for separation of CTCs from the sample using magnetic fields. Although frequently used, CellSearch™ System needs to be interpreted with caution [25].

The presence of non-tumor epithelial cells within the bloodstream may contribute to false-positive results. It has been noted that patients with benign disease of the colon exhibited "tumor cells" as detected with the CellSearch™ system (11.3%) [26]. Besides, this approach would miss CTCs that have low levels of EpCAM expression, and fail to detect the most aggressive CTCs subpopulation, which may have undergone EMT [27]. For example, the rarity of CTCs in early CRC was illustrated in a study of 20 consecutive patients undergoing curative resection for stages I to III CRC [28]. The detection rate using CellSearch™ System was 5% in the pre-operative samples, using a cut-off of 2 CTCs/7.5 ml. Although the cascades of cancer metastasis formation are not fully understood, the epithelial-mesenchymal transition (EMT) process is believed to have a great role in these cascades [29].

In this paper, the above results were taken into consideration. In order to minimize CTCs losses as much as possible, we isolate CTCs via a fil-

ter-based method, which entrap non-blood-derived cells because of their bigger size and inflexibility. Afterward, an RNA in situ hybridization (RNA-ISH) method based on the branched DNA signal amplification technology was used to classify the CTCs according to EMT markers, an antibody cocktail consisting of EpCAM, CK8/18/19, vimentin, twist was used to identify the CTCs. And exclude hematopoietic cells using CD45 markers [19].

Classifying CTCs by EMT markers helps to identify the more aggressive CTCs subpopulation and provides useful evidence for determining an appropriate clinical approach [30]. Therefore, our research could provide better prognostic information on the probability of metastasis in early stage cancer patients.

Colorectal cancer is the third leading cause of cancer death in China [1]. In the last few years, a significant expansion in the number of available systemic therapies to treat metastatic colorectal cancer (mCRC) was investigated [31]. However, with increasing options, there is also greater complexity for decision making. A biomarker obtained in a noninvasive manner would be of great potential for clinical application on guiding therapy.

Circulating tumor cells (CTCs) has great potential application as liquid biopsies to prognosticating disease and guiding treatment in colorectal cancers. It is worthwhile to study their role in determining the genome information of tumor metastasis, providing biomarker detection for targeted therapies and determination of drug resistance [32, 33]. VEGF had shown important roles in regulating colorectal cancer proliferation and metastasis. In this work, significant association of mCTCs positivity and the development of distant metastases in CRC patients were observed. Patients with distant metastasis were significant higher than those without distant metastasis.

In addition, mCTCs also closed related to vascular invasion. The mCTCs positive rate and VEGF expression positive rate in high risk groups were both higher than low risk groups. Combining circulating tumor cells subgroups with VEGF gene expression may enhance clinical prediction of CRC cancer metastasis, which could help us better understand the mechanism of tumor metastasis.

Conclusions

In summary, CTCs from 45 primary colorectal cancer patients were enriched by blood filtration. The RNA in situ hybridization method was used to identify and discriminate subgroups of CTCs, and VEGF expression in individual CTCs was measured. The mCTCs positive rate and VEGF expression positive rate were obviously higher in high risk groups, suggesting VEGF may correlate with tumor malignancy. Our research proved that CTCs could be served as feasible surrogate samples to detect gene expression as a predictive biomarker for tumor evaluation, which provides a more accurate route for prediction of high CRC risk in future.

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Disclosure of conflict of interest

None.

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