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ORIGINAL RESEARCH

FAM172A expression in circulating tumor cells for prediction of high-risk subgroups of colorectal cancer

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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 tumors. Therefore, we classified different metastatic potentials of CTCs from colorectal cancer (CRC) patients to improve the accuracy of prognosis by CTCs.

Methods: Blood samples were collected from 45 primary CRC patients. CTCs were enriched by blood filtration, and the RNA in situ hybridization method was used to identify and discriminate subgroups of CTCs. Later, FAM172A expression in individual CTCs was measured.

Results: Three CTC subgroups (epithelial/hybrid phenotypic/mesenchymal CTCs) were identified using epithelial–mesenchymal transition markers. In our research, mesenchymal CTCs significantly increased along with tumor progression, including developing distant metastasis and vascular invasion. Furthermore, FAM172A expression rate in mesenchymal CTCs was significantly higher than that in epithelial CTCs, which suggested that FAM172A may correlate with tumor malignancy. This hypothesis was further verified by FAM172A expression in mesenchymal CTCs strictly related to tumor aggressiveness factors. Finally, we revealed that mesenchymal CTCs and FAM172A expression may predict high-risk subgroups in stage II CRC.

Conclusion: 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, epithelial–mesenchymal transition, FAM172A

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; ~20%–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 clinicopathological parameters and serologic tumor markers offer limited information covering CRC diagnosis, prognosis prediction, and monitoring of the therapeutic response in a real-time manner. Therefore, there is an urgent need 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

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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] CRC,[5] and prostate cancer.[6]

A multicenter prospective study including 456 patients with metastatic colorectal cancer (mCRC) demonstrated that CTC levels before treatment were an independent prognostic factor for progression-free survival (PFS) and OS.[7] A meta-analysis performed on 12 studies of stage IV CRC provides the strongest level of evidence for the 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 studies focus on the correlation of CTC enumeration with prognosis.[9–13] However, recent studies showed that only enumerated CTCs were not enough to reflect the heterogeneous condition of tumors.[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 (mCTCs) are associated with metastasis and resistance to chemotherapy. The[y] encourage future studies regarding the expression of EMT related markers in CTCs and cancer progression.

The family with sequence similarity 17[2], mem[b]er A (FAM172A), was first identified in human aortic [e]ndothelial cells in 2009. Then, several stu[d]ies had investigated its functional relationship with c[a]ncer. Feng et al found that FAM172A was down-regulated a[m]ong hepatocellular carcinoma patients. It play[s] an important r[o]le in cell cycle control and tumor cell p[r]oliferation.[21] The protein expression of FAM172A in c[o]rrect[e]d cancerous tissues is significantly lower than that in a[d]jacent tis[s]ues. It suppressed the proliferative p[o]tential and pro[m]oted apoptotic and invasive potentials of colon[i]c cancer cells.[11,22] However, in papillary thyroid carcin[o]ma (PTC), it has been found that FAM172A expression in can[c]erous tissues was significantly higher than that in carcinoma adjacent tissues and normal thyroid tissues. FAM172A accelerated PTC cell proliferation via activation of the *p*38 MAPK signaling pathway.[23]

As FAM172A is closely related to CRC proliferation and invasion,[23,24] it would be highly interesting to detect FAM172A expression in CTCs to get a deeper understanding of the role FAM172A plays in EMT process.

The aim of this study was to discriminate different metastasis potentials of CTCs and explore FAM172A expression in individual CTCs to determine the correlations of CTC subgroups and FAM172A 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) signed informed consent, 2) newly diagnosed nonmetastatic colon having histological diagnosis, 3) newly diagnosed mCRC, and 4) absence of other concomitant or previous malignant diseases.

Patients were recruited by The Fir[st] A[ffi]liated Hospital of Wenzhou Medical Universit[y] from Mar[ch] 2015 to December 2015. This study was a[p]proved by [t]he ethical committee of The First A[ffi]liated H[o]spital o[f] Wenzhou Medical University. All [p]atients p[r]ovided [w]ritten informed consent to participate in [t]his st[u]dy.

Blood sample[s] were coll[e]cted bef[o]re surgery or adjuvant chemotherap[y] fro[m] patients w[i]th early stages and before palliative chemothera[py] from those with advanced disease. Blood [s]amp[l]es (5 mL) [w]ere drawn into heparinized tubes and [s]tored at 4°C within 4 hours.

CTC identif[i]cation

Erythrocytes were removed using a red blood cell lysis buffer co[n]ta[i]ning ammonium chloride (NH_4Cl) and then transferred [t]o the filtration tube and filtered with the help of a pump valve. CTCs were isolated using a calibrated membrane with 8 µm diameter pores.[25]

The cells on the membrane were hybridized for 2 hours, and unbound probes were washed three times with phosphate-buffered saline (PBS). Subsequently, samples were incubated with a preamplifier solution for 20 minutes and then incubated with an 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 4′,6-diamidino-2-phenylindole (DAPI) for 5 minutes and then analyzed with a fluorescence microscope.[18,19]

The leukocytes were characterized as CD45+DAPI+ cells. CTCs were defined with the following three subgroups: 1) epithelial marker-positive CD45–DAPI+ cells (epithelial CTCs); 2) biophenotypic epithelial/mesenchymal marker-positive CD45–DAPI+ cells (biophenotypic CTCs); and 3) mesenchymal marker-positive CD45–DAPI+ cells (mCTCs).

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 using SPSS 20.0. For all analyses, $P<0.05$ was considered statistically significant.

Results

Patient demographics

Blood samples for CTC assessment were taken from 45 consecutive patients with primary CRC. 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).

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

Characteristics	n	%
Age		
≤60 years	26	57.8
>60 years	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	
Tumor grade		
Low	8	17
Moderate	37	
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
CTC counts		
≥1 CTCs/5 mL	34	75.6
≥3 CTCs/5 mL	28	62.2

Abbreviations: TNM, tumor–node–metastasis; CEA, carcinoembryonic antigen; CTCs, circulating tumor cells.

mCTCs 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 mCTCs; typical photographs are shown in Figure 1.

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

Correlation between typical clinicopathological variables and the presence of CTCs in blood was analyzed by chi-square test,[26] which is shown in Table 1. Correlation was not found among positive CTC and most of the clinicopathological features. Only stage (50.0% in stages I–II, 75.0% in stage III–IV, $P=0.114$) and carcinoembryonic antigen (CEA) level (50.0% in CEA ≤5 ng/mL, 76.2% in CEA >5 ng/mL, $P=0.071$) were related with positive CTCs, although it was not statistically significant (Table 2).

Among CRC patients, mCTC percentage significantly increased along with tumor progression; we observed a significant association between mCTC positivity and the development of distant metastases in CRC patients. Significantly higher mCTC levels were detected in patients with distant metastasis than in patients without distant metastasis (100% vs 51.3%, $P=0.024$). In addition, mCTCs were also closely related to vascular invasion. Our study showed that mCTCs were more commonly found in patients with vascular invasion (84.6% vs 46.9%, $P=0.020$). There was also a clear association between the presence of mCTCs and depth of invasion and/or tumor–node–metastasis stage, although it was not statistically significant (Table 2).

The significantly higher percentage of mCTCs in the more aggressive status prompts us to hypothesize whether mCTC detection can be a surrogate marker of tumor aggressiveness.

FAM172A gene expression in CTCs aggressively correlated with tumors

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

Recent report showed that FAM172A suppressed the proliferative and invasive potentials of CRC cell lines.[22] Nevertheless, how FAM172A expressed in CTCs and their clinical value were still unknown.

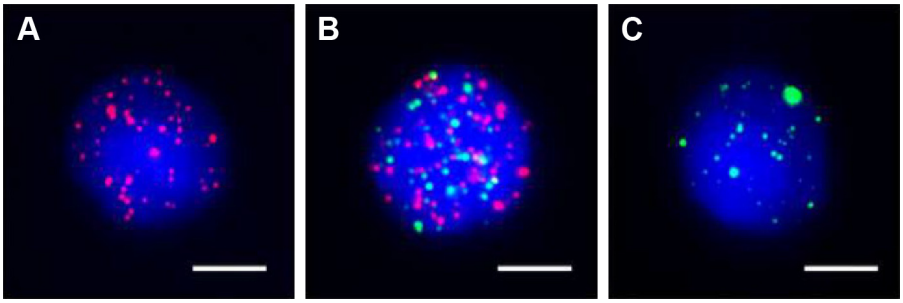

Figure 1 Representative images of three subgroups of CTCs isolated from patients with CRC, based on RNA-ISH staining of E (red dots) and M (green dots) markers.
Notes: (**A**) Epithelial, (**B**) Biphenotypic, (**C**) Mesenchymal. The scale bar is 10 μm.
Abbreviations: CTCs, circulating tumor cells; CRC, colorectal cancer; RNA-ISH, RNA in situ hybridization; E, epithelial; M, mesenchymal.

Twenty-eight patients with CTC positivity (≥3 CTCs/mL) were enrolled for evaluating biomarker expression. FAM172A + CTC were detected in 20 of 28 patients (71.4%). Photographs of CTCs with FAM172A expression are shown in Figure 2.

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

Characteristics	n	≥3 CTCs/ 5 mL	*P*-value	≥1 mCTCs/ 5 mL	*P*-value
Tumor location			0.384		0.670
Colon	30	20		18	
Rectal	15	8		8	
Tumor size			0.460		097
≤5 cm	32	21		16	
>5 cm	13	7		10	
Tumor grade			0.411		0.113
Low	8	6		7	
Moderate	37	22		19	
Vascular invasion			.195		0.020
No	32	18		15	
Yes	13	10		11	
Depth of invasion			03		0.058
T1–T3	21	10		8	
T4	24	18		18	
Lymphatic metast			0.6		0.434
No	20	8		10	
Yes	2			16	
Distant metastas			0.252		0.024
No	39	23		20	
Yes	5	5		6	
TNM stage			0.114		0.138
I–II	25	13		12	
III–IV	20	15		14	
CEA			0.071		0.083
≤5 ng/mL	24	12		11	
>5 ng/mL	21	16		15	
Ki-67			0.299		0.302
≤60	22	11		11	
>60	23	17		15	

Abbreviations: CTCs, circulating tumor cells; mCTCs, mesenchymal circulating tumor cells; TNM, tumor–node–metastasis; CEA, carcinoembryonic antigen.

Previous studies have sho wide olecular a d cellular heterogeneity of CTCs fro he same type f ca cer and even from the same patient. ur res ch found hat the overall expression rate o AM in CT was 60.7%, with 56.3% in epith l CTCs, 58 % i biophenotypic CTCs, and 68.8% mC s. FAM172A expression in mCTCs was sig ntly highe han that in epithelial CTCs, which impl d that FAM172A may correlate with tumor malignancy, pro oting cance ell metastasis and invasion.

hypothesi was proved when the relationship between FAM1 e expression and characteristics of CRC pa ts was analyzed, which is shown in Table 3. We observed significant association between FAM172A expression and depth of invasion in CRC patients (68.1% in T1–T3 vs 51.3% T4, P=0.024). Besides, higher Ki-67 value showed higher FAM172A expression rate (71.3% in Ki-67 ≤60 vs 48.8% in Ki-67 >60, P=0.003). In addition, FAM172A expression rate in mCTCs is closely correlated with metastasis-associated clinicopathological 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, which meant that combining CTC subgroups with FAM172A gene expression may enhance clinical prediction of CRC metastasis.

CTC/FAM172A detection may predict high-risk subgroups in stage II CRC

For patients diagnosed at stage II, correlations between CTCs and prognostic subgroups were analyzed. CTC 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.[26]

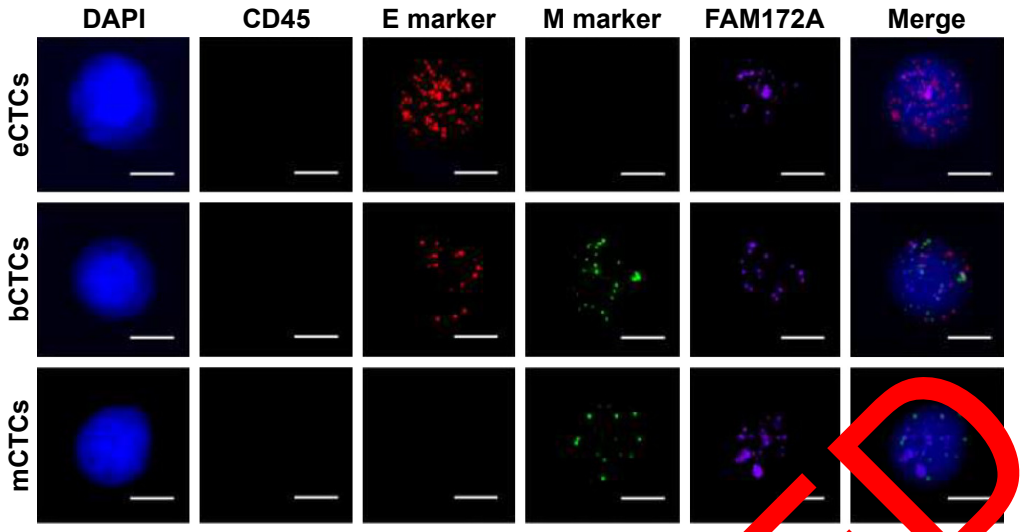

Figure 2 Representative images of FAM172A expression in three subgroups of CTCs isolated from patients with C based o NA-ISH staining of E (red dots), M (green dots), and FAM172A (purple dots) markers.
Note: The scale bar is 10 μm.
Abbreviations: CTCs, circulating tumor cells; CRC, colorectal cancer; CD45, leukocyte common antigen; DA 4′,6-diamidino-2 nylin e; RNA-ISH, RNA in situ hybridization; E, epithelial; M, mesenchymal; eCTCs, epithelial circulating tumor cells; bCTCs, biophenotypic circulatin mo ls; mCTCs, mes mal circulating tumor cells.

Table 3 Demographics of patients with CTCs ≥3 used for analysis of FAM172A expression rate with clinicopathological features (n=28)

Characteristics	FAM172A expression in CTCs (%)	*P*-value	FAM172A expression in mCTCs (%)	*P* value
Tumor location		0.151		0.9
Colon	64.0		56.0	
Rectal	52.6		5	
Tumor size		0.164		0.497
≤5 cm	56.6		5	
>5 cm	68.0		62.5	
Tumor grade				0.841
Low	75.0		60.0	
Moderate	69.3		55.3	
Vascular invasion		0.619		0.007
No	5		37.5	
Yes	7		78.9	
Depth of invasion		0.02		0.004
T1–T3	51.3		33.3	
T4	68.1		77.3	
Lymphati metastasis		0.250		0.708
No			52.6	
Yes	55.8		58.3	
Distant metas s		0.297		0.190
No	58.1		50.0	
Yes	69.6		72.7	
TNM stage		0.378		0.658
I–II	63.8		52.4	
III–IV	57.1		59.1	
CEA		0.082		0.780
≤5 ng/mL	60.4		55.6	
>5 ng/mL	58.8		60.0	
Ki-67		0.003		0.553
≤60	48.8		52.0	
>60	71.3		61.1	

Abbreviations: CTCs, circulating tumor cells; mCTCs, mesenchymal circulating tumor cells; TNM, tumor–node–metastasis; CEA, carcinoembryonic antigen.

The correlation between CTCs or FAM172A detection and prognostic subgroups in stage II CRC is shown in Table 4. We found that both mCTC positivity rate (66.7% vs 5% =0.199) and FAM172A expression positivity rate (54.5% vs 22.2%, *P*=0.142) in high-risk groups were higher than those in low-risk groups, although there were no statistically significant differences between them.

Limited by sample quantity, although our study had not proven confident evidence to discriminate high/low risk of prognostic subgroups, the potential of CTCs to better aid in selection of high-risk group patients has potential clinical value, as there is controversial evidence regarding the benefits of chemotherapy, which is currently prescribed based on clinicopathological criteria.

Discussion

Blood sampling is less invasive, less painful, easy to perform, and better accepted sampling. For monitoring the efficacy of therapy, detection and characterization of CTCs by blood sampling might be a new option for therapeutic interventions. CTC enumeration via the CellSearch™ system is approved by the US Food and Drug Administration for use as an aid in monitoring patients with mCRCs. The presence of ≥3 CTCs for CRC prior to treatment is associated with decreased PFS and OS and is prognostic, regardless of the 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.

Table 4 CTCs/FAM172A detection and prognostic subgroups in stage II colorectal cancer

Prognostic subgroups	CTCs positivity rate (%)	*P*-value	mCTCs positivity rate (%)	*P*-value	FAM172A expression positive (%)	*P*-value
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)	

Abbreviations: CTCs, circulating tumor cells; mCTCs, mesenchymal circulating tumor cells.

Although frequently used, the CellSearch system needs to be interpreted with caution.[27]

The presence of nontumor 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%).[28] Besides, this approach would miss CTCs that have low levels of EpCAM expression and fail to detect the most aggressive CTC subpopulation, which may have undergone EMT.[29] For example, the rarity of CTCs in early CRC was illustrated in a study of 20 consecutive patients undergoing curative resection for stages I–III CRC.[30] The detection rate using CellSearch system was 5% in the preoperative samples, using a cutoff of 2 CTCs/7.5 mL. Although the cascades of cancer metastasis formation are not fully understood, the EMT process is believed to have a great role in these cascades.[31]

In this study, we have taken the above into consideration. In order to minimize CTC losses as much as possible, we isolated CTCs via a filter-based method, which entraps nonblood-derived cells because of their bigger size and inflexibility. Later, 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 and an antibody cocktail consisting of EpCAM, CK8/18/19, vimentin, and twist was used to identify the CTCs. CD45 markers were used to exclude hematopoietic cells.[19]

Classifying CTCs by EMT markers helps to identify the more aggressive CTC subpopulation and provides useful evidence for determining an appropriate clinical approach.[32] Therefore, our research presented here has the potential to provide better prognostic information on the probability of metastasis in early-stage cancer patients.

CRC is the third leading cause of cancer death in China.[1] The last few years have seen a significant expansion in the number of available systemic therapies to treat mCRC.[33] However, with increasing options comes greater complexity in decision making. A biomarker that could be obtained in a noninvasive manner to guide therapy would thus be of great potential clinical utility.

CTCs hold great potential as liquid biopsies to prognosticate disease and guide treatment in CRCs. It is worthwhile to study their important role in determining the genome information of tumor metastasis, providing biomarker detection for targeted therapies and determination of drug resistance.[34,35] FAM172A had shown important roles in regulating CRC proliferation and metastasis. In this work, a significant association between mCTC positivity and the development of distant metastases in CRC patients was observed. Greater number of patients with distant metastasis in CRC were identified than those without distant metastasis.

In addition, mCTCs were also closely related to vascular invasion. Both mCTC positivity rate and FAM172A expression positivity rate in high-risk groups were higher than those in low-risk groups. Combining CTC subgroups with FAM172A gene expression may enhance clinical prediction of CRC metastasis, which could help us better understand the mechanism of tumor metastasis involved in it.

Conclusion

CTCs from 45 primary CRC patients were enriched by blood filtration. The RNA-ISH method was used to identify and discriminate subgroups of CTCs, and FAM172A expression in individual CTCs was measured. The mCTC positivity rate and FAM172A expression positivity rate were obviously higher in high-risk groups, suggesting that FAM172A may correlate with tumor malignancy. Our research proved that CTCs could serve 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.

Disclosure

The authors report no conflicts of interest in this work.

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