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 metastasis 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/biophenotypic/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
of detecting CTCs as a prognostic and/or surrogate marker of treatment response has been established in several cancer types such as breast cancer,\textsuperscript{4} CRC,\textsuperscript{5} and prostate cancer.\textsuperscript{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.\textsuperscript{7} A meta-analysis performed on 12 studies of stage IV CRC provides the strongest level of evidence for the prognostic utility of CTCs.\textsuperscript{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.\textsuperscript{9–13} However, recent studies showed that only enumerated CTCs were not enough to reflect the heterogeneous condition of tumors.\textsuperscript{3,14,15} CTCs disseminate from primary tumors by undergoing phenotypic changes that allow the cells to penetrate blood vessels.\textsuperscript{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.\textsuperscript{17}

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

The family with sequence similarity 172, member A (FAM172A), was first identified in human aortic endothelial cells in 2009. Then, several studies had investigated its functional relationship with cancer. Feng et al found that FAM172A was down-regulated among hepatocellular carcinoma patients. It plays an important role in cell cycle control and tumor cell proliferation.\textsuperscript{21} The protein expression of FAM172A in colorectal cancerous tissues is significantly lower than that in adjacent tissues. It suppressed the proliferative potential and promoted apoptotic and invasive potentials of colon cancer cells.\textsuperscript{11,22} However, in papillary thyroid carcinoma (PTC), it has been found that FAM172A expression in cancerous tissues was significantly higher than that in carcinoma adjacent tissues and normal thyroid tissues. FAM172A accelerated PTC cell proliferation via activation of the p38 MAPK signaling pathway.\textsuperscript{23}

As FAM172A is closely related to CRC proliferation and invasion,\textsuperscript{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 First Affiliated Hospital of Wenzhou Medical University from March 2015 to December 2015. This study was approved by the ethical committee of The First Affiliated Hospital of Wenzhou Medical University. All patients provided written informed consent to participate in this study.

Blood samples were collected before surgery or adjuvant chemotherapy from patients with early stages and before palliative chemotherapy from those with advanced disease. Blood samples (5 mL) were drawn into heparinized tubes and stored at 4°C within 4 hours.

### CTC identification

Erythrocytes were removed using a red blood cell lysis buffer containing ammonium chloride (NH\textsubscript{4}Cl) 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.\textsuperscript{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.\textsuperscript{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).

| 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  | 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|
| CTC counts     |     |    |
| ≥1 CTC/5 mL     | 34  | 75.6|
| ≥3 CTC/5 mL     | 28  | 62.2|

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

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 clinical/pathological variables and the presence of CTCs in blood was analyzed by chi-square test, which is shown in Table 2. Correlation was not found among positive CTCs and most of the clinical/pathological features. Only stage (52.0% in stages I–II, 75.0% in stages III–IV, \( P = 0.114 \)) and carcinoembryonic antigen (CEA) level (50.0% in CEA ≤5 ng/mL, 76.2% in CEA >5 ng/mL, \( P = 0.071 \)) correlated 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. Nevertheless, how FAM172A expressed in CTCs and their clinical value were still unknown.
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.

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 FAM172A in CTCs was 60.7%, with 56.3% in epithelial CTCs, 58.6% in biophenotypic CTCs, and 68.8% in mCTCs. FAM172A expression in mCTCs was significantly higher than that in epithelial CTCs, which implied that FAM172A may correlate with tumor malignancy, promoting cancer cell metastasis and invasion.

The hypothesis was proved when the relationship between FAM172A gene expression and characteristics of CRC patients was analyzed, which is shown in Table 3. We observed a significant association between FAM172A 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 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

### 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             |   |             |         |               |         |
| 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            |         |

**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 37.5%, \( P = 0.199 \)) 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 \( \geq 3 \) 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 3** Demographics of patients with CTCs \( \geq 3 \) 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                |                                |         |                                 |         |
| Colon                         | 64.0                           | 0.151   | 56.0                            | 0.977   |
| Rectal                        | 52.6                           |         | 55.6                            |         |
| Tumor size                    |                                |         |                                 |         |
| \( \leq 5 \) 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                           |                                |         |                                 |         |
| \( \leq 5 \) ng/mL            | 60.4                           | 0.082   | 55.6                            | 0.780   |
| > 5 ng/mL                     | 58.8                           |         | 60.0                            |         |
| Ki-67                         |                                |         |                                 |         |
| \( \leq 60 \)                 | 48.8                           | 0.003   | 52.0                            | 0.553   |
| > 60                          | 71.3                           |         | 61.1                            |         |

**Abbreviations:** CTCs, circulating tumor cells; mCTCs, mesenchymal circulating tumor cells; TnM, tumor–node–metastasis; CEA, carcinoembryonic antigen.
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.\textsuperscript{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.

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.\textsuperscript{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%).\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{19}

Classifying CTCs by EMT markers helps to identify the more aggressive CTC subpopulation and provides useful evidence for determining an appropriate clinical approach.\textsuperscript{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.\textsuperscript{1} The last few years have seen a significant expansion in the number of available systemic therapies to treat mCRC.\textsuperscript{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.

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