

Incorporating MicroRNA into Molecular Phenotypes of Circulating Tumor Cells Enhances the Prognostic Accuracy for Patients with Metastatic Breast Cancer

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Disclosures of potential conflicts of interest may be found at the end of this article.

Key Words. Breast cancer • Circulating tumor cells • MicroRNA • Prognosis • Metastasis

ABSTRACT

Background. The molecular phenotype of circulating tumor cells (CTCs) was associated with clinical outcome of patients with breast cancer. CTCs isolated from patients with metastatic breast cancer (MBC) display a unique microRNA (miRNA) expression profile. The aim of this study was to enhance the prognostic accuracy of the CTC phenotype in patients with MBC, by incorporating miRNA into a combined prediction model.

Subjects, Materials, and Methods. CTCs were detected by CellSearch and enriched by magnetic cell sorting. miRNA deep sequencing and quantitative polymerase chain reaction were used to screen and verify potentially CTC-specific miRNA candidates. Patients with MBC were enrolled from two independent cohorts, and overall survival (OS) and chemotherapy response were analyzed.

Results. We screened and identified that miR-106b was an upregulated molecule in patients with MBC with CTC $\geq 5/7.5$

mL ($n = 16$) compared with patients with CTC = 0/7.5 mL ($n = 16$) and healthy donors ($n = 8$). The expression of CTC-specific miR-106b correlated with vimentin and E-cadherin in CTC and acted as an independent factor for predicting OS (hazard ratio 2.157, 95% confidence interval [CI] 1.098–4.239, $p = .026$). Although CTC-specific miR-106b, E-cadherin, and vimentin showed a prognostic potential independently, the prognostic performance for OS based on the combination of three markers was significantly enhanced in Cohort 1 (area under the curve [AUC] 0.752, 95% CI 0.658–0.847, $n = 128$) and further validated in Cohort 2 (AUC 0.726, 95% CI 0.595–0.856, $n = 91$). Besides, a combined model incorporating miR-106b was associated with therapy response.

Conclusion. The phenotypic assemblies of CTC incorporating miR-106b show enhanced prognostic accuracy of overall survival in patients with MBC. *The Oncologist* 2019;24:1–11

Implications for Practice: In order to enhance the prognostic accuracy of the circulating tumor cell (CTC) phenotype in patients with metastatic breast cancer (MBC), this study screened and identified a CTC-specific microRNA (miRNA), miR-106b, as an upregulated molecule based on the comparison of miRNA profile between CTCs, primary tumors, and healthy blood donors. By incorporating miR-106b into a combined prediction model, the prognostic accuracy of the CTC phenotype for patients with MBC was greatly improved in both the training and validation cohorts. This work provides clinical evidence supporting the prognostic potential of CTC-specific miRNA for patients with MBC. These results indicate that developing CTC-specific miRNAs as new biomarkers will help to further optimize personalized therapy.

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INTRODUCTION

Metastasis is associated with the presence of peripheral blood circulating tumor cells (CTCs), which are suggested to be potential seeds for hematogenous cancer metastasis [1, 2]. Several studies have demonstrated that the presence of CTCs before the initiation and after the completion of adjuvant chemotherapy is associated with poor clinical outcome [3, 4]. In metastatic breast cancer (MBC), the assessment of CTCs before and shortly after the initiation of chemotherapy may predict progression-free survival (PFS) and overall survival (OS) [5, 6]. Although the presence of chromosomal alterations confirmed the malignant nature of CTCs [7, 8], only some are capable of promoting metastasis [9]. Regarding their individual gene expression, characterizing the molecular phenotypes of the CTCs, rather than their blood concentration and/or number, is essential to further understand their metastatic potential as well as identify novel markers related to patients' prognosis.

Epithelial-mesenchymal transition (EMT) is considered to be the crucial event in the metastatic process of cancer, involving aberrant expression of the EMT-related molecules and the acquisition of a migratory mesenchymal phenotype [10]. The morphological and phenotypical changes of EMT undergone by cancer cells also exist in CTCs of breast cancer [11]. CTCs that undergo EMT lose cell-cell contacts and polarity, down-regulate epithelial-associated genes, and acquire mesenchymal gene expression [12–14]. E-cadherin and vimentin act as the core protein of the epithelial adherence junction [15, 16]. Several studies have demonstrated that vimentin is expressed in CTCs of patients with breast cancer [17, 18]. CTCs displaying upregulated vimentin were associated with clinical response to therapy and disease progression [19, 20], suggesting that the molecular phenotype of CTCs including EMT was associated with the clinical outcome of patients with breast cancer.

It has been reported that CTCs isolated from a cohort of patients with MBC by the CellSearch Profile Kit display a unique miRNA expression profile [21], indicating that an extensive miRNA characterization of CTCs will hold great promise and improve the currently available prognostic models on the basis of primary tissue. However, little is known regarding the correlation between the noncoding molecules in CTCs and EMT-related characteristics as well as their potential clinical relevance.

In the current study, in order to identify new miRNAs for CTC phenotype classifications and enhance the prognostic value of the CTC phenotype for patients with MBC, we screened the CTC-specific miRNAs based on the comparison of miRNA deep sequencing between CTCs and primary tumors and verified miR-106b as a key molecule with the highest upregulation in CTCs. Furthermore, we quantified the expression level of miR-106b in CTCs of patients with MBC and investigated the prognostic role of the CTC phenotype incorporating CTC-specific miR-106b for patients with MBC in two independent cohorts.

SUBJECTS, MATERIALS, AND METHODS

Patients

This study enrolled patients initially diagnosed with MBC between March 2012 and December 2015 in the Sun Yat-sen

Memorial Hospital (SYSMH; Guangzhou, China) and the Sun Yat-sen University Cancer Center (SYSUCC; Guangzhou, China). All patients were required to have clinical and radiologic evidence of MBC with either measurable or evaluable disease. Metastatic diseases were measured or evaluated using clinical and radiological methods in accordance with the RECIST version 1.1 criteria. All patients had Eastern Cooperative Oncology Group performance status 0–1.

Prior adjuvant chemotherapy and/or hormone treatment were allowed. The estrogen receptor (ER) and the progesterone receptor (PR) status of the primary tumors were determined by immunohistochemistry. Human epidermal growth receptor 2 (HER2) expression was evaluated using the HercepTest (Dako, Denmark) and assessed according to the DAKO-score; samples with score 2+ were further analyzed by fluorescent in situ hybridization.

Before treatment, all patients had a complete clinical and laboratory evaluation, chest and abdominal computed tomography scans, and whole-body bone scan. Reassessment of the disease status was also performed every 8 weeks. Response to treatment was assessed using the RECIST 1.1 criteria.

Blood Sample Collection

Prior to the administration of systemic therapy, 7.5 mL of blood was drawn from patients in CellSave tubes (Veridex LLC) for CTC enumeration by CellSearch. CTC enumeration and characterization were confirmed by independent reviewers.

Fifty milliliters of fresh blood was collected for sorting and enriching CTCs using magnetic activated cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) if CTC count $\geq 5/7.5$ mL after primary enumeration. To avoid contamination with epithelial cells from the skin, all blood samples were obtained at the middle of vein puncture after the first 1 mL of blood was discarded.

Enumeration of CTCs Using the CellSearch System

Enumeration of CTCs was performed at the central laboratory of our institution using the U.S. Food and Drug Administration-approved CellSearch system by using CELLSEARCH Circulating Tumor Cell Kit. The process has been described in the manufacturer's instructions. Unfavorable CTC enumeration was defined as ≥ 5 CTCs in 7.5 mL of peripheral blood.

CTCs Sorting and Enrichment

CTCs were isolated from 50 mL EDTA blood by MACS. Briefly, samples were layered over Ficoll-Paque (1.077, density) and centrifuged at 400g for 30 minutes at room temperature; peripheral blood mononuclear cells (PBMCs) were present at the interphase. PBMCs were resuspended at 5×10^7 cells in 300 mL solution containing 100 mL FcR Blocking Reagent (130-059-901, Miltenyi Biotec), 100 mL CD45 Microbeads (130-045-801, Miltenyi Biotec), and 100 mL CD15 Microbeads (130-091-058, Miltenyi Biotec). After depletion of CD45+ and CD15+ cells by magnetic separation with autoMACS Pro Separator, 100 mL CD326 (also known as Epithelial Cell Adhesion Molecule, EpCAM) Microbeads (130-095-500, Miltenyi Biotec) per 5×10^7 cells was added for 30-minute incubation at 4°C. The magnetically charged CD326+ and CD326- cell

fractions were eluted as the EpCAM+ and EpCAM− CTCs. The purity of epithelial cells was determined by immunofluorescent staining with an anticytokeratin antibody (10 µg/mL, ab41825; Abcam, UK), which was more than 95%. For a cell to be identified as a CTC, it had to meet two criteria: (a) positive staining for a tumor-specific marker by immunocytochemistry (cytokeratin) and (b) positive scoring upon review by the cytopathologist.

Screening and Quantification of CTC-Specific miRNAs

Total RNA was extracted from primary tumors and CTCs enriched by EpCAM-based CellSearch from 50 mL of blood of six patients with MBC. MiRNA Deep Sequencing on Illumina HiSeq 2500 sequencing platform with 10 M reads (Illumina, San Diego, CA; Guangzhou RiboBio Company, Guangzhou, China) was used to screen CTC-specific miRNA candidates from these samples. Real-time polymerase chain reaction (PCR) was used to quantify CTC-specific miRNAs expression levels. Complementary DNA (cDNA) was generated using the miScript Reverse Transcription (RT) Kit (Qiagen GmbH, Hilden, Germany). Briefly, real-time PCR was performed using the miScript SYBR Green PCR Kit (Qiagen) on an Mx3005P QPCR System (Stratagene, La Jolla, CA). The specificity of this RT-PCR technique was confirmed by dissociation curve analysis using the Mx3005P QPCR System (Stratagene) according to the manufacturer's instruction. Bulge-Loop miRNA primers were offered by RiboBio Company, Guangzhou, China. Transcripts of U6 small RNA were quantified for normalization of the levels of miRNAs [22, 23].

Δ Ct values were used to normalize and calculate the concentration of miRNAs. The experiment was repeated three times and the data were analyzed blind. The $2^{-\Delta\Delta Ct}$ value was the difference in Δ Ct between patients and controls, and the normalized miRNAs expression levels were calculated with the formula $2^{-\Delta\Delta Ct}$.

Quantification of EMT-Related Molecules in CTCs

RNA was extracted from CTCs using the Recover All Total Nucleic Acid Isolation Kit (Ambion, Life Technologies, Carlsbad, CA) as described. Total RNA from each sample was quantified by NanoDrop ND-1000 (ThermoFisher). cDNA was synthesized from total RNA using the ImProm-II Reverse Transcriptase system (Promega) according to the manufacturer's protocol. The mRNA levels were measured by quantitative real-time PCR, which was performed in an ABI 7500 Real-time PCR system using SYBR Green PCR Master Mix (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as standards for the absolute quantification of each cDNA. The amount of target gene expression could be calculated from the standard curve, which plotted the cycle threshold (Ct) value and the corresponding value of the amount of input DNA. The quantitative PCR primers used are listed in supplemental online Table 1.

Statistical Analysis

Statistical analyses were performed using SPSS version 17.0 (SPSS, Chicago, IL). Student's *t* test and one-way analysis of variance analyses were performed to analyze the differences between the groups. A chi-square test was applied to analyze the relationship between miR-106b expression and

EMT-related molecules of CTC. OS was defined as the time elapsed between inclusion and death from any cause. Survival curves were plotted according to the Kaplan-Meier method. Statistical significance between survival curves was assessed using the log-rank test. Multivariate analysis was done by the Cox proportional hazards model with prognostic factors. The Cox regression model was performed for the combined three prognostic factors. The receiver operating characteristic (ROC) curves were employed to test the sensitivity and specificity of variables in predicting OS. For the other analyses, a *p* value of <.05 was considered to be statistically significant.

RESULTS

Patient Characteristics

From March 2012 to December 2015, a total of 128 patients with MBC in SYSMH (Cohort 1) and 91 patients with MBC in SYSUCC (Cohort 2) were enrolled in this study. Cohort 1 was used as a training set in our study, whereas Cohort 2 was used as a validation set. A flow chart outlining the selection of patients from SYSMH (Cohort 1) is shown in supplemental online Figure 1. The patients from Cohort 2 were selected and investigated in a similar manner as those in Cohort 1. All enrolled patients had five or more CTCs in 7.5 mL of blood enumerated at baseline section by CellSearch. Patient characteristics of Cohort 1 and Cohort 2 at baseline are summarized in Table 1 and supplemental online Table 2. The median age (range) at initial diagnosis of these patients with breast cancer was 51 (23–69) years, and the median age at study entry was 56 (29–72) years. Notably, the majority of patients had ER-positive (ER+; 96/128, 75%), PR-positive (87/128, 67.9%), and HER2-negative (101/128, 78.9%) primary tumors. In most patients, the tumor had disseminated into more than one organ (87/128, 68%), and approximately half of the patients had both bone and visceral metastases (70/128, 54.7%). All patients received front-line chemotherapy. Among them, 16 (12.5%) patients received fluorouracil, epirubicin, and cyclophosphamide/epirubicin and cyclophosphamide regimen, 42 (32.8%) patients received epirubicin and taxane chemotherapy regimen, and 53 (41.4 %) patients received taxane chemotherapy regimen. Moreover, there were 17 (13.3 %) patients who received chemotherapy regimens without epirubicin and taxane. In addition, 24 of 27 (88.9 %) patients with HER2-positive tumors received anti-HER2 therapy (trastuzumab or lapatinib or both). All ER+ patients received endocrine therapy. Follow-up data were available for 128 patients with a median follow-up of 13.1 months (range, 2.5–29.7 months) for OS.

Selection of Potentially CTC-Specific miRNA Candidates

To find out CTC-specific miRNAs, we first used an MiRNA Deep Sequencing to screen the miRNA candidates in primary tumors and CTCs enriched by EpCAM-based CellSearch from 50 mL of blood of six patients with MBC (supplemental online Table 3) among 2,588 miRNAs in the MiRNA Sequencing (Fig. 1) based on the following standards: fold changes were more than 5 folds compared with the primary tumor in all six

Table 1. Characteristics of patients in Cohort 1 and Cohort 2

| Characteristics | Cohort 1, n (%) | Cohort 2, n (%) | Total | p value |
|---|--------------------|--------------------|-------|---------|
| ER status | | | | .01 |
| Negative | 32 (25.0) | 10 (11.0) | 42 | |
| Positive | 96 (75.0) | 81 (89.0) | 177 | |
| PR status | | | | .003 |
| Negative | 41 (32.0) | 13 (14.3) | 54 | |
| Positive | 87 (68.0) | 78 (85.7) | 165 | |
| HER2 status | | | | .286 |
| Negative | 101 (78.9) | 77 (84.6) | 178 | |
| Positive | 27 (21.1) | 14 (15.4) | 41 | |
| KI67 status | | | | .244 |
| Negative | 49 (38.3) | 42 (46.2) | 91 | |
| Positive | 79 (61.7) | 49 (53.8) | 128 | |
| Metastasis site | | | | .906 |
| Bone | 18 (14.1) | 12 (13.2) | 30 | |
| Visceral/local | 40 (31.3) | 31 (34.1) | 71 | |
| Both | 70 (54.7) | 48 (52.7) | 118 | |
| Number of metastasis sites | | | | .844 |
| 1 | 41 (32.0) | 28 (30.8) | 69 | |
| ≥2 | 87 (68.0) | 63 (69.2) | 150 | |
| Molecular subtype | | | | .331 |
| Luminal A | 41 (32.0) | 29 (31.9) | 70 | |
| Luminal B | 56 (43.8) | 31 (34.1) | 87 | |
| HER2+ | 9 (7.0) | 7 (7.7) | 16 | |
| TNBC | 22 (17.2) | 24 (26.4) | 46 | |
| Treatment before study | | | | .01 |
| Hormonal therapy | | | | |
| Yes | 96 (75.0) | 81 (89.0) | 177 | |
| No | 32 (25.0) | 10 (11.0) | 42 | |
| Front-line chemotherapy | | | | .063 |
| FEC/EC | 16 (12.5) | 11 (12.1) | 27 | |
| TE | 42 (32.8) | 29 (31.9) | 71 | |
| T | 53 (41.4) | 38 (41.8) | 91 | |
| Other | 17 (13.3) | 13 (14.3) | 30 | |
| Anti-HER2 therapy (trastuzumab, lapatinib) | | | | .185 |
| Yes | 24 (18.8) | 11 (12.1) | 35 | |
| No | 104 (81.3) | 80 (87.9) | 184 | |
| Radiological response after first cycle of chemotherapy | | | | .924 |
| Non-PD | 81 (63.3) | 57 (62.6) | 138 | |
| PD | 47 (36.7) | 34 (37.4) | 81 | |

(continued)

Table 1. (continued)

| Characteristics | Cohort 1, n (%) | Cohort 2, n (%) | Total | p value |
|-----------------|--------------------|--------------------|-------|---------|
| Survival status | | | | <.0001 |
| Alive | 47 (36.7) | 77 (84.6) | 124 | |
| Dead | 81 (63.3) | 14 (15.4) | 95 | |

p value is calculated by chi-square test or Fisher's exact test.

Abbreviations: E, epirubicin; EC, epirubicin combined with cyclophosphamide; ER, estrogen receptor; FEC, epirubicin combined with cyclophosphamide with fluorouracil; HER2, human epidermal growth receptor 2; PD, progressive disease; PR, progesterone receptor; T, docetaxel; TE, epirubicin combined with docetaxel; TNBC, triple-negative breast cancer.

patients with MBC, basal expression level of normalized intensity were more than 100, miRNA candidates were reported to play functional roles in promoting breast cancer metastasis. Finally, 10 miRNAs were picked out as upregulated candidates (supplemental online Table 4), and these 10 upregulated miRNAs could be sensitivity nonhomogeneously amplified with a minimum number of miRNA in sensitive multiplex stem-loop cDNA approach with the TaqMan-based linear pre-amplification method. We further compared these 10 miRNA transcript levels in cells enriched by MACS (CMs) of blood from healthy blood donors (HBDs; $n = 8$) and CTC samples from patients with MBC ($n = 32$) by RT-PCR (supplemental online Table 3). We observed that only miR-106b was significantly upregulated in CTCs ≥ 5 samples ($n = 16$) compared with CTCs = 0 samples ($n = 16$) and HBD-CSs (supplemental online Table 5). This suggests that miR-106b might be a potentially CTC-specific miRNA, and we selected it for our further investigation.

Association of miR-106b and EMT-Related Molecules in CTC

It has been reported that miR-106b regulates the EMT of cancer cells and promotes tumor progression [24]. It is also noted that, upon EMT inducement, cancer cells exhibit morphological changes and a couple of EMT-related molecules including E-cadherin, vimentin, ck19, Snail, and N-cadherin. Therefore, we further quantified the expression level of miR-106b and the above EMT-related molecules in CTCs using quantitative RT-PCR, and analyzed their correlations. As a result, we found that the expression of CTC-specific miR-106b was positively correlated with vimentin ($r = .687$, $p < .001$; Fig. 2A) and negatively correlated with the expression level of E-cadherin ($r = -.672$, $p < .001$; Fig. 2B) but had no significant correlations with other molecules (supplemental online Fig. 2). In subtype analysis, we found that the expression level of CTC-specific miR-106b was significantly higher in patients with triple-negative breast cancer (TNBC) compared with non-TNBC subtypes ($p = .011$; Fig. 2C). These data suggest that miR-106b is a specific biomarker for EMT-related phenotype of CTC.

Association of CTC-Specific miR-106b, E-cadherin, and Vimentin with Clinical Outcome

It is well known that the number and phenotypes of CTC correlates with prognosis of patients with MBC [17, 25].

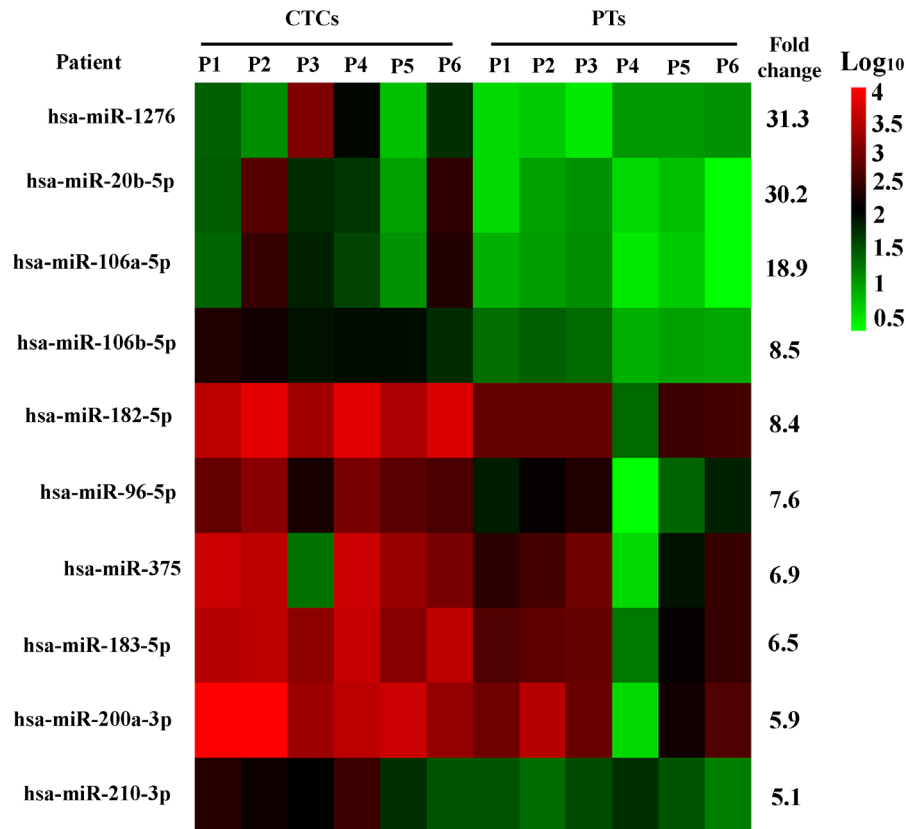


Figure 1. MiRNA Deep Sequencing reveals differentially expressed microRNAs between primary tumors and CTCs from six patients with metastatic breast cancer.

Abbreviations: CTCs, circulating tumor cells; P, patient; PTs, primary tumors.

Table 2. Univariate and multivariate analysis for overall survival

| Variables | Univariate analysis | | | | Multivariate analysis | | | |
|---|---------------------|--------|-------|----------------|-----------------------|--------|-------|----------------|
| | HR | 95% CI | | <i>p</i> value | HR | 95% CI | | <i>p</i> value |
| | | Lower | Upper | | | Lower | Upper | |
| ER status (positive vs. negative) | 0.73 | 0.42 | 1.26 | .254 | 0.935 | 0.522 | 1.677 | .822 |
| PR status (positive vs. negative) | 1.03 | 0.52 | 2.07 | .925 | 0.803 | 0.385 | 1.676 | .559 |
| HER2 status (positive vs. negative) | 1.55 | 0.92 | 2.62 | .098 | 1.37 | 0.771 | 2.435 | .283 |
| Ki67 status ($\geq 14\%$ vs. $< 14\%$) | 0.91 | 0.58 | 1.43 | .687 | 0.756 | 0.47 | 1.216 | .248 |
| Metastasis site (visceral vs. bone) | 1.52 | 1.16 | 1.96 | .002 | 0.781 | 0.56 | 1.089 | .144 |
| Number of metastasis site (≥ 2 vs. 1) | 1.29 | 0.81 | 2.04 | .282 | 1.162 | 0.676 | 1.998 | .588 |
| E-cadherin | 1.624 | 1.017 | 2.594 | .042 | 1.45 | 0.891 | 2.36 | .135 |
| Vimentin | 1.993 | 1.218 | 3.259 | .006 | 1.345 | 0.765 | 2.364 | .304 |
| miR-106b (high- vs. low-expression) | 2.707 | 1.463 | 5.008 | .002 | 2.157 | 1.098 | 4.239 | .026 |

p value is calculated by chi-square test or Fisher's exact test.

Abbreviations: CI, confidence interval; ER, estrogen receptor; HER2, human epidermal growth receptor 2; HR, hazard ratio; PR, progesterone receptor.

Therefore, we further investigated the clinical relevance of the above CTC-specific molecules with the prognosis of patients with MBC. First, we individually assessed the prognostic performance of CTC-specific miR-106b, E-cadherin, and vimentin by plotting the ROC curve in 128 patients. Consistent with other studies [26, 27], in our study, CTC-specific E-cadherin and vimentin showed a prognostic value for patients with MBC, respectively (E-cadherin, AUC = 0.637, Fig. 3A; vimentin, AUC = 0.642, Fig. 3B). The lower-expression group

of E-cadherin (hazard ratio [HR] 1.624, 95% confidence interval [CI] 1.017–2.594, *p* = .042; Fig. 3C) and higher-expression group of vimentin (HR 1.993, 95% CI 1.218–3.259, *p* = .006; Fig. 3D) showed poorer OS in Kaplan-Meier curve analysis. Interestingly, higher prognostic performance of CTC-specific miR-106b for patients with MBC was observed in ROC analysis (AUC = 0.670, 95% CI 0.572–0.769; Fig. 3E). In order to further analyze the association between CTC-specific miR-106b and prognosis, an optimal cutoff value (0.0105) was

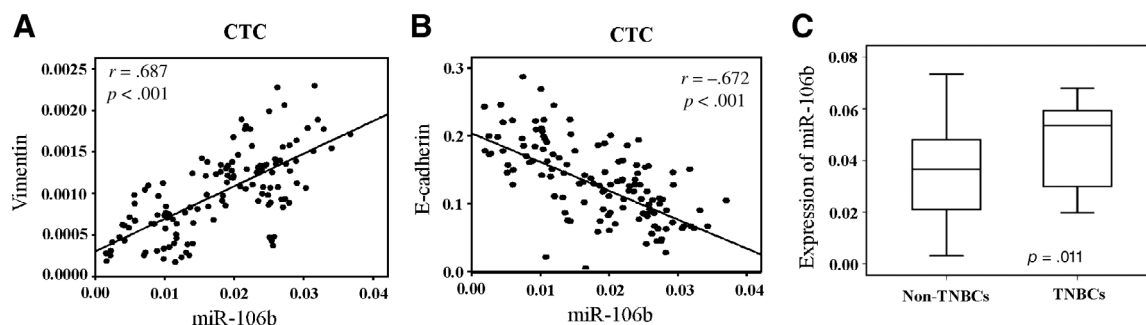


Figure 2. MiR-106b is a specific biomarker for CTC phenotypes. Correlation of the expression of miR-106b with vimentin (A, $n = 128$) and E-cadherin (B, $n = 128$). (C): Quantification of miR-106b by real-time quantitative polymerase chain reaction in TNBCs ($n = 16$) and non-TNBCs ($n = 112$).

Abbreviations: CTC, circulating tumor cell; TNBCs, triple-negative breast cancers.

determined by ROC analysis and Youden index calculated by sensitivity and specificity, which was widely used for determination of optimal cutoff value in diagnostic test and prognosis prediction by other studies [28]. Based on this cutoff value, 128 patients with MBC of Cohort 1 and 91 of Cohort 2 were divided into miR-106 high-expression and low-expression subgroups. As shown by Kaplan-Meier curve analysis, the miR-106 high-expression group was correlated with a poorer OS (HR 2.707, 95% CI 1.463–5.008, $p = .002$; Fig. 3F), compared with the low-expression group.

In addition, univariate analysis found that patients with visceral metastasis (HR 1.52, 95% CI 1.16–1.96, $p = .002$), high expression of miR-106 and vimentin, and low expression of E-cadherin were more likely to have poor OS. However, in multivariate analysis, only CTC-specific miR-106b was an independent factor for predicting OS (HR 2.157, 95% CI 1.098–4.239, $p = .026$; Table 2).

Assessment and Validation of the Prognostic Accuracy of the Three-Molecule Combination Including CTC-Specific miR-106b, E-cadherin, and Vimentin

Because of the limited prognostic accuracy of CTC-specific E-cadherin and vimentin and relative higher prognostic performance of CTC-specific miR-106b for patients with MBC, we further incorporated miR-106b into a Cox regression model to compare the prognostic accuracy between the multimolecule assemblies and single biomarker. As a result, we found that the new model combining the expression of three molecules was significantly more accurate than any other single biomarker evaluated in our study for predicting overall survival (AUC 0.752, 95% CI 0.658–0.847; Fig. 4A).

This result was further validated in Cohort 2. In Cohort 2, the clinicopathological characterization of 91 selected patients with MBC was similar to that of patients from SYSMH (Cohort 1), including 81 (89%) ER-positive patients and 14 (15.4%) HER2-positive patients (supplemental online Table 2). Notably, most patients were Luminal subtype (60 of 91, 65.9%) and triple negative (24 of 91, 26.4%), and only 7 patients were HER2+ subtype. In addition, we found that the distribution of miR-106b expression in Cohort 2 was similar to Cohort 1 (supplemental online Fig. 3). Therefore, we used the same cutoff value to divide Cohort 2 into miR-106b high- and low-expression subgroups. We verified that the new model combining the expression of three molecules

also showed a significantly prognostic value for predicting overall survival in the validation cohort (AUC 0.726, 95% CI 0.595–0.856; Fig. 4B).

In subtype analysis, we made ROC analysis to test the prognostic accuracy in all 219 patients (Cohort 1 and Cohort 2) and found that the prognostic accuracy of the three-molecule combination including CTC-specific miR-106b, E-cadherin, and vimentin was still significant in luminal subtypes (AUC = 0.693, 95% CI 0.609–0.776, $n = 157$; supplemental online Fig. 4A) and the TNBC subtype (AUC = 0.710, 95% CI 0.522–0.898, $n = 48$; supplemental online Fig. 4B).

Association of the Three-Molecule Combination and Clinical Response

The correlation between expression of CTC-specific miR-106b and clinical response was further analyzed. Patients with progression at the radiological examination following two cycles of first-line chemotherapy showed higher expression of CTC-specific miR-106b (Fig. 5A, $p < .01$). Further analysis showed that the combination of CTC-specific miR-106b containing E-cadherin and vimentin could significantly predict the clinical response to therapy in patients with MBC (AUC 0.691, 95% CI 0.598–0.784; Fig. 5B). This result was further validated in Cohort 2 with 91 patients with MBC (AUC 0.661, 95% CI 0.519–0.804; Fig. 5C).

DISCUSSION

In this study, we found that miR-106b was significantly upregulated in CTCs compared with primary tumor and CMs of blood from HBDs. The expression level of CTC-specific miR-106b was correlated with EMT-related phenotype of CTC. In addition, high expression of CTC-specific miR-106b was correlated with a poor OS and acted as an independent factor for predicting OS. More importantly, by incorporating miRNA expressions into a combined prediction model, the prognostic accuracy of CTC phenotype in patients with MBC was significantly enhanced.

Quantification of CTCs in breast [29], colorectal [30], and pancreatic cancer [31] has been shown to correlate with survival. However, less than 0.01% of CTCs seem to survive in the target organ for colonization and subsequent growth. An intriguing area of active research is whether molecular characterization of CTC before clinical manifestation could better predict metastatic risk and facilitate individualized

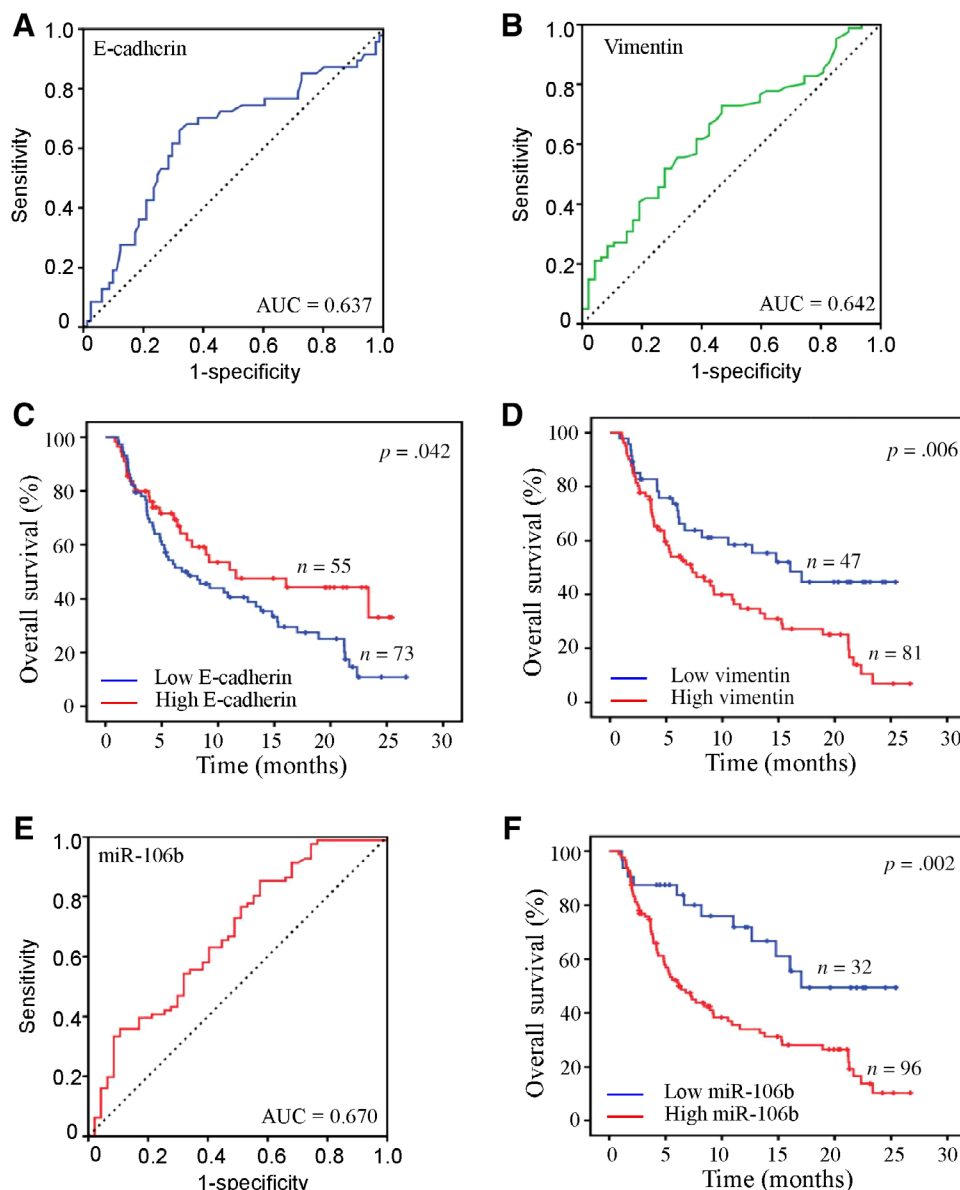


Figure 3. Association of circulating tumor cell (CTC)-specific molecules with clinical outcome. Receiver operating characteristic curves evaluated the prognostic accuracy for E-cadherin (A), vimentin (B), and miR-106b (E) in CTCs. Kaplan-Meier survival curves for E-cadherin (C), vimentin (D), and miR-106b (F) in CTCs. Abbreviation: AUC, area under the curve.

therapeutic strategies to inhibit target organ colonization and metastatic growth. Of note, the activation of an EMT is favorable for the CTC population [32]. It has been reported that the presence of mesenchymal markers such as vimentin on CTCs more accurately predicted worse prognosis than the expression of cytokeratin [20, 33].

In our study, we found that CTC-specific miR106b was positively related to the expression level of vimentin in CTCs. It has been reported that overexpression of miR-106b was observed in a variety of human tumors, which plays an oncogenic role in tumor progression and prognosis, including colorectal cancer [34, 35], gastric cancer [36, 37], hepatocellular carcinoma [38, 39], glioma tumors [40], renal cell carcinoma [41, 42], and head and neck squamous cell carcinomas [43]. MiR-106b plays a functional role in promoting proliferation, migration, and invasion of cancer cells both

in vitro and in vivo [44–46]. Clinical data show that high expression level of miR-106b is associated with metastasis and poor prognosis [34, 47]. Consistent with these findings, we found that patients with MBC with high CTC-specific miR-106b expression had shorter progression-free survival, suggesting that this phenotypic attribute becomes salient when considering each of the steps in the cascade of events required for metastatic success.

Our clinical data showed that in both the training and validation set, the combination of CTC-specific miR-106b, E-cadherin, and vimentin was significantly more accurate than any single molecule assessed in our study for predicting overall survival and clinical response to therapy. Several lines of explanation may be responsible for this enhanced prognostic value of this molecule combination. First, molecular phenotypes of CTCs were associated with various behavior

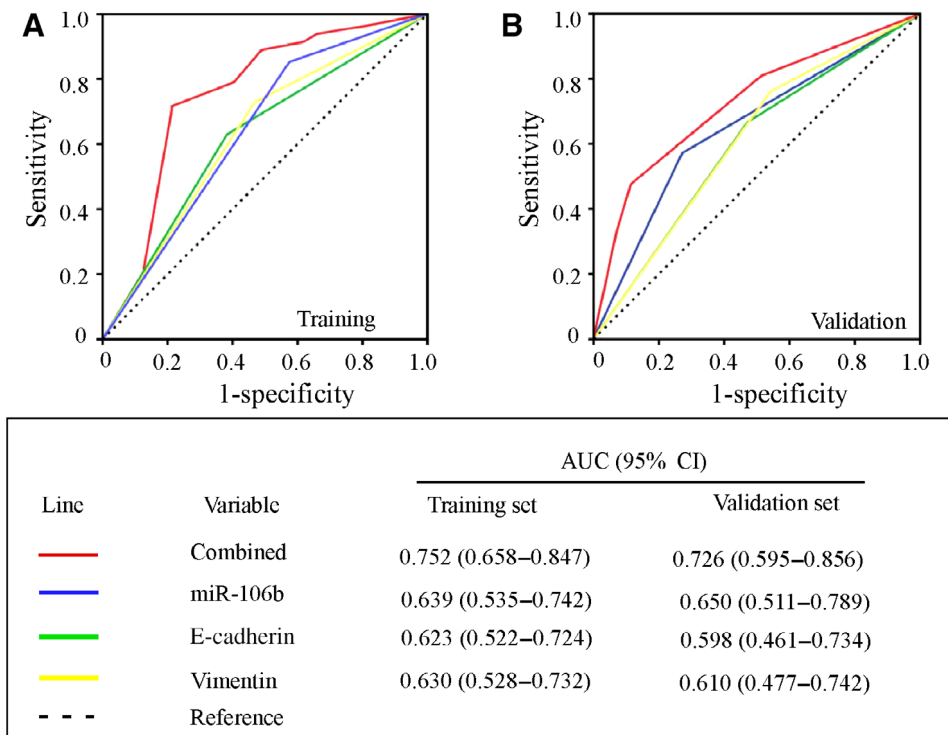


Figure 4. Comparison of the prognostic accuracy of single and combined factors. **(A):** Training set. **(B):** Validation set. Abbreviations: AUC, area under the curve; CI, confidence interval.

of cancer cells including anoikis resistance metastasis and therapeutic resistance. It has been reported that miR-106b promotes anoikis resistance [24] and EMT processes in breast cancer cells by targeting *RB* [48] and *Snail* gene [49]. Acquisition of a more malignant phenotype for CTC might easily explain the resistance to elimination strategies such as chemotherapy, local antigrowth signaling, and immune attack by the host. Second, according to our results, CTC-specific miR-106b was expressed at a higher level in mesenchymal-like CTCs that highly expressed vimentin, suggesting that there is a coordination between miR-106b and EMT-related phenotypes of CTCs. Third, mechanically, our previous study demonstrated that miR-106b determines the effect of transforming growth factor β , a key EMT inducer, on the tumor behavior of breast cancer cells by targeting *RB* [48]. Several studies have also demonstrated that a variety of genes were identified as the target of miR-106b including caspase-7 [47], PTEN [50], and Smad7 [17, 20, 51], which contribute to miR-106b-mediated EMT of cancer cells. In this scenario, CTC-specific miR-106b plays a critical role in regulating the EMT of CTCs, indicating that CTCs with higher expression of vimentin and miR-106b show a higher motility in the bloodstream and more easily interact with the intravascular microenvironment, extravagate through the microvasculature, and interact with the metastatic microenvironment of target organs. Consistent with this notion, the clinical relevance was further demonstrated by our clinical data that CTC-specific miR-106b was an independent factor for predicting overall survival and therapy response of patients with MBC. These are responsible for incorporating miR-106b into the molecular phenotype of CTC and could further enhance the prognostic accuracy of CTC.

Increasing evidence shows that the phenotype of CTCs was related to disease prognosis and holds great promise in guiding treatment decision making in patients with breast cancer. Therefore, identifying new molecules to refine the molecular phenotype of CTC will help the clinicians to better formulate appropriate therapy strategies for individual patients. The EMT-related biomarkers including vimentin and E-cadherin have been reported to detect the phenotype of CTC [17, 20, 52], and CTCs displaying upregulated vimentin were associated with clinical response to therapy and disease progression [17, 20]. With the development of sequencing technology and in-depth understanding of the mechanism for cancer progression, it is necessary to take into account more gene information and then characterize the detailed molecular phenotype of CTC to improve the prognostic performance of established CTC-related clinical models. In our study, we screened and validated that miR-106b was an upregulated biomarker in patients with MBC. The expression of CTC-specific miR-106b was correlated with vimentin and E-cadherin in CTC and acted as an independent factor for predicting OS in patients with MBC. The prognostic performance for OS based on the combination of three markers (miR-106b, E-cadherin, and vimentin) was significantly enhanced and can be further validated in an external validation cohort. Our results suggest that incorporating miRNA into molecular phenotypes of circulating tumor cells enhances the prognostic accuracy. In addition, this new model is feasible, efficient, and cost-effective for clinical application.

In our study, there was still some weakness that deserves to be acknowledged. First, the molecular mechanisms by which miR-106b and EMT-related molecules in CTCs regulate tumor progression were not presented in this study. However, several

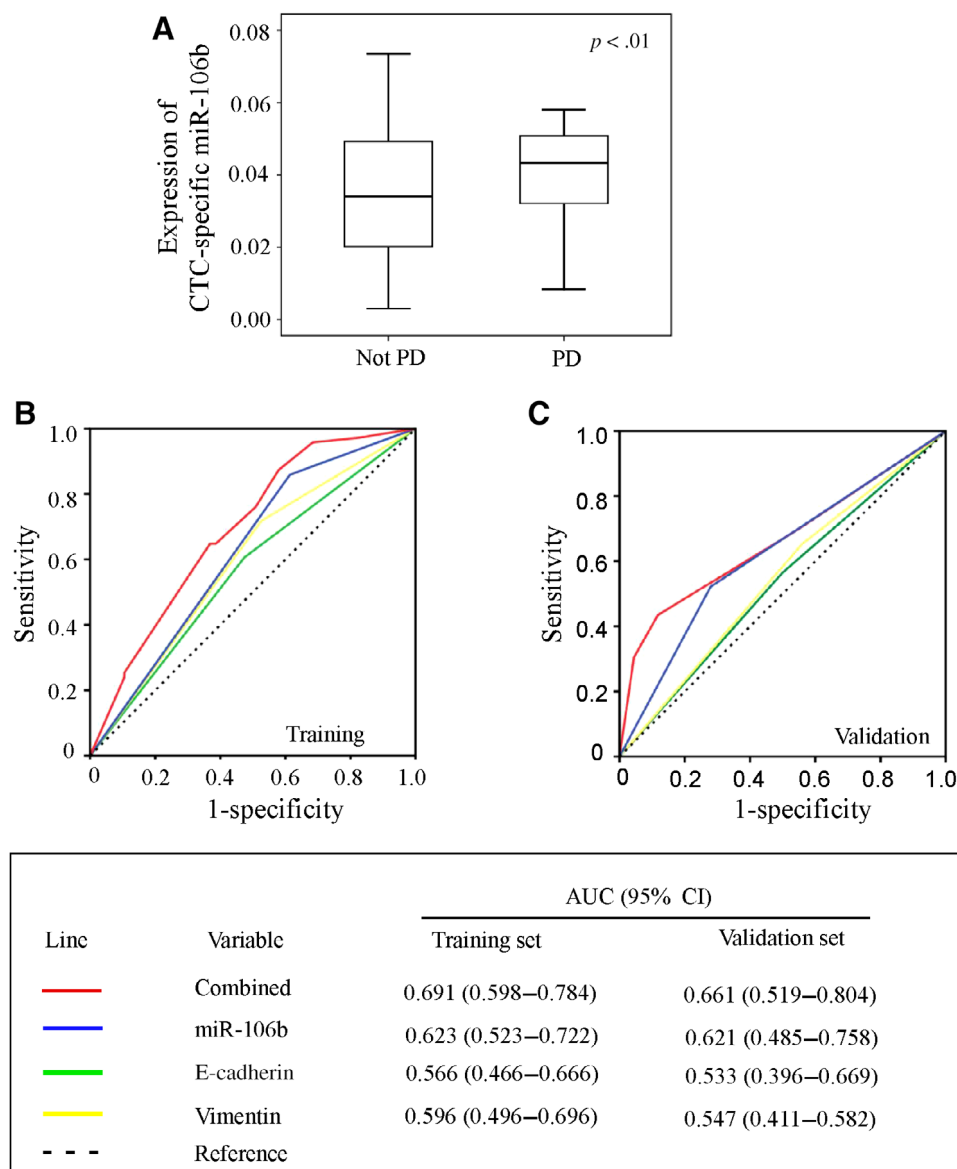


Figure 5. (A): Correlations between expression of CTC-specific miR-106b and clinical response. Comparison of the prognostic accuracy of single and combined factors in the training set **(B)** and validation set **(C)**.

Abbreviations: AUC, area under the curve; CI, confidence interval; CTC, circulating tumor cell; PD, progressive disease.

reasons are responsible for this limitation. As far as we know, the isolation and enrichment as well as ex vivo culture of viable CTCs are still technically challenging, and the frequency of CTC in the peripheral blood of patients with solid tumors varies among individual patients with different prior systemic therapies. In recent years, although a few papers reported that the genome alternation of CTC lines and suspended cancer cells may be related to the drug sensitivity in both in vitro and xenografts models in breast cancer [53], the evidence is indirect and not strong enough because of the above limitations. Second, the downstream targeted molecules of miR-106b related to EMT should be further tested in our future studies.

CONCLUSION

Our work provides clinical evidence supporting the prognostic potential of CTC-specific miRNA for patients with MBC.

These results indicate that developing CTC-specific miRNAs as new biomarkers will help to further optimize personalized therapy.

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DISCLOSURES

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