Original Article
The diagnostic and clinical value of mir-433 in circulating tumor cell for breast cancer

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Abstract: Objective: This study is to investigate the expression of miRNA-433 in tumor tissues, circulating tumor cells and mammary gland of patients with breast cancer, and to investigate the mechanism of infiltration and invasion of breast cancer. Methods: From January 2013 to March 2015, blood and breast duct fluid specimen were collected from 56 patients with breast cancer. Blood and breast duct fluid specimen from 31 patients without breast cancer were collected as the control group. Breast cancer tumor specimens were collected and peritumoral tissues were also collected as a negative control group. Circulating cell sorting kit was used to collect and count the circulating tumor cell in the blood. The qRT-PCR was used to detect miRNA-433 and metastasis-associated in colon cancer 1 (MACC1) mRNA. Western Blot and ELISA were performed to detect level of MACC1 protein. Results: Compared with the control groups, the circulating tumor cells in the blood of patients with breast cancer were significantly increased (P < 0.05). The expression of MACC1 mRNA and MACC1 protein in tumor tissues, mononuclear cells in the blood and breast duct fluid were significantly up-regulated (P < 0.05). However, the expression of miRNA-433 was significantly down regulated in these samples (P < 0.05). Conclusions: MACC1 expression increase may be related to the expression of miRNA-433. MiRNA-433 may regulate the infiltration and invasion of breast cancer by MACC1. Especially, miRNA-433 in circulating tumor cells may become a marker of breast cancer.

Keywords: Circulating tumor cells (CTCs), miRNA-433, breast cancer, metastasis-associated in colon cancer 1 (MACC1)

Introduction
The incidence of breast cancer is increasing rapidly, and breast cancer ranks in first place in female malignant tumor [1, 2]. Breast cancer is one of the most common malignant tumors, and every year there are about 1.5 million women are diagnosed with breast cancer [3]. It has become a female malignant tumor with the highest incidence and mortality rate and a serious threat to women’s health and life [3-5]. It is reported that the genes of P53 [6], adenomatous polyposis coli (APC) [7], K-ras [8], and C-myc [9] play important roles in the development of breast cancer.

Tumor transfer refers to the tumor cells shed from the primary site, transfer to other sites through lymphatic vessel and blood vessel, and continue to grow into the same type of tumor with the primary tumor. This tumor is called metastatic tumor, which is the leading cause of death in most cancers [10, 11]. Tumor metastasis through the blood and lymph circulation system in the body is the most important factor affecting the treatment of cancer patients [12-14]. Modern cancer detection methods mainly include radiological imaging and the detection of serum tumor markers. However, there methods are still unable to meet the actual clinical needs [15].

Metastasis-associated in colon cancer 1 (MACC1) is firstly found in colon cancer tissue and MACC1 is closely related to the invasion and metastasis of colon cancer, liver cancer, renal cell carcinoma, gastric cancer, and ovarian cancer [16-18]. The relationship between
MACC1 and breast cancer are very close. It was found that MACC1 expression was changed in breast cancer tissues [19]. Some evidence suggests that MACC1 plays an important role in breast cancer, but there are few reports on the upstream regulators of MACC1. The MACC1 gene is located on chromosome 7 (7p21.1), containing seven exons and six introns. The cDNA contains 2559 nucleotides and encodes 852 amino acid residues. Stein et al. [20] showed that MACC1 activated hepatocyte growth factor (HGF)/c-Met signal transduction pathway by combining with c-Met promoter, which increased the number of c-Met protein. The increased c-Met protein combined with more HGF, and HGF induced the transfer of MACC1 from cytoplasm to the nucleus, promoting the positive feedback loop of MACC1 and finally resulting in cell growth, cell migration, angiogenesis, cell motility, invasion and metastasis [21]. Another research reported that the combination of HGF and c-Met could activate downstream signaling factors such as Ras/MAPK/ERK, PI3K/Akt and STAT to promote cell proliferation, survival and migration [22].

In this study, qRT-PCR, Western Blot, gene bioinformatics prediction and ELISA techniques were used to detect MACC1 mRNA and protein expression in tumor tissue of breast cancer patients, circulating tumor cells and breast duct lavage. The relationship between miRNA-433 and MACC1 was analyzed.

Materials and methods

Research object and selection criteria

From January 2013 to March 2015, blood and breast duct lavage fluid specimen were collected from 56 patients in our hospital with breast cancer. These patients were diagnosed by Fiberoptic Ductoscopy and finally chose to do surgical resection. As control, blood and breast duct lavage fluid specimen were also collected from 31 control patients without breast cancer. Tumor tissues were collected from the 56 breast cancer patients and peritumoral tissues were also collected from these 56 breast cancer patients. The 56 breast cancer patients aged 27 to 58 years old, with a median age of 39 years. The control patients aged 25 to 62 years old, with a median age of 42 years. All of these patients were the first onset and they were not treated with hormone, radiotherapy or chemotherapy before surgery. These 56 breast cancer patients included 49 cases of invasive ductal carcinoma, 5 cases of invasive carcinoma of special type, and 2 cases of intraductal carcinoma. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of Nanjing Medical University.

Reagents and instruments

The miRcute miRNA Isolation Kit, miRcute miRNA first strand cDNA synthesis Kit, miRcute miRNA fluorescent quantitative detection kit (FP401), SuperReal PreMix (SYBR Green) (FP204), and TIANScript II first strand cDNA synthesis Kit (KR107) were all purchased from TIANGEN (Beijing, China). The machine for qRT-PCR was purchased from Bio-Rad (PCR-iQ5; Bio-Rad Laboratories, Hercules, CA, USA). The polyclonal rabbit anti human anti-MACC1 antibody (ab106005) and the polyclonal rabbit anti human anti-β-actin antibody (ab129348) were from Abcam (Abcam, Burlingame, CA, USA). The secondary antibody (goat anti-rabbit) was from Abcam (ab6721; Abcam, Burlingame, CA, USA). Trizol reagent was from YEASEN biological company (10606ES60; Shanghai, China), BCA protein assay kit was from Real-Times (Beijing) Biotechnology Co., Ltd. (RTP7102; Beijing, China). The MACC1 ELISA Kit was from Shanghai Wuhao Trade Co., Ltd. (sE981667Hu, Shanghai, China). The Image lab 3.0 software and the circulating cell sorting Kit was from Beijing Biolaunching Technologies CO., Ltd. (Beijing, China). The solution for separation of human peripheral blood mononuclear cells was from Shanghai Lengton Bioscience Co. Ltd. (LTS1071-1; Shanghai, China).

Sample collection

Tumor tissues and peritumoral tissues were cryopreserved in liquid nitrogen for further test. Peripheral blood was collected in the morning when fasting and stored at -20°C after anticoagulant. The peripheral blood mononuclear cells and circulating tumor cells were isolated from peripheral blood. The breast duct lavage fluid was collected.

Isolation of mononuclear cells

Peripheral blood was mixed with PBS (1:1) and centrifugated at 2000 rpm for 15 min. After
Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequence (5'-3')</th>
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<tbody>
<tr>
<td>MACC1_Forward</td>
<td>5'-GCAGACAAAGAATCAGAGAAAG-3'</td>
</tr>
<tr>
<td>MACC1_Reverse</td>
<td>5'-GATGAGACGTCGACTAATCT-3'</td>
</tr>
<tr>
<td>GAPDH_Forward</td>
<td>5'-ATGCTGGCGCTGAGTACGTC-3'</td>
</tr>
<tr>
<td>GAPDH_Reverse</td>
<td>5'-GGTCATGAGTCTCCAGCAGATA-3'</td>
</tr>
<tr>
<td>U6_Foreward</td>
<td>5'-GCTCTGGAGACGATATATATATAAAT-3'</td>
</tr>
<tr>
<td>U6_Reverse</td>
<td>5'-CGCTTCAGAATTTGCAGCTCAT-3'</td>
</tr>
<tr>
<td>miR-433_Foreward</td>
<td>5'-GGATCATGATGGGCTCCT-3'</td>
</tr>
<tr>
<td>miR-433_Reverse</td>
<td>5'-CAGTGCCTGTCAGTGGAGT-3'</td>
</tr>
</tbody>
</table>

centrifugation, blood was divided into four layers. The second layer was mononuclear cells and was collected. The mononuclear cells were further washed for 2 times and centrifuged at 2000 rpm for 15 min.

Collection of circulating tumor cells

Circulating tumor cells were collected with the circulating cell sorting Kit according to the kit instructions. Briefly, before filtering, 1 ml Screen Cell® LC dilution buffer was added to 6 ml blood samples. After incubation for 2 min, 1.6 ml of tissue culture medium was added in order to separate living cells. After filtering, the ScreenCell® MB fixator was removed for collecting circulating tumor cells.

Collection of breast duct lavage fluid

The front section of the ductoscopy was embedded into the duct with suspicious lesions or sub-section of the duct opening. Then, normal saline was injected with l ml syringe to lavage the breast duct. The lavage was performed 5-7 times, with 0.2–0.5 ml normal saline each time. And, each time after injection, the duct lavage fluid was sucked out under negative pressure.

Quantitative RT-PCR (qRT-PCR)

To detect MACC1, Trizol method was used to extract total RNAs from samples. UV spectrophotometer was used to measure A260/A280 to test the purity of RNAs. And then these RNAs were transcribed into cDNA. The results were calculated using \(2^{-\Delta\Delta Ct}\) method. The ratio miRNA-433/U6 was calculated. Primers sequences for qRT-PCR were given in Table 1.

Western blot

Total proteins were extracted and separated by 10% SDS-PAGE. Then proteins were transferred onto nitrocellulose membrane. After blocking with non-fat milk, the membrane was incubated with primary antibodies of anti-MACC1 (1:1000) and anti-β-actin (1:5000) at 4°C overnight. After washing, the membrane was then incubated with goat anti-rabbit secondary antibody (1:2000) at room temperature for 1 h. Finally, the membrane was developed by enhanced chemiluminescence plus reagent. The Image lad 3.0 was used to analyze the grey values. β-actin was used as an internal control. The relative expression level of MACC1 was calculated based on the grey value of β-actin.

ELISA

ELISA was performed according to the instructions of MACC1 ELISA Kit. Briefly, blank wells and wells for standard sample and duct lavage fluid were set up. In the wells for duct lavage fluid, 50 ul duct lavage fluid were added. In the wells for standard sample, 10 ul standard sample and 40 ul dilution solution were added. Nothing was added to the blank wells. Besides the blank wells, each well was added with 100 ul horseradish peroxidase (HRP) labeled detection antibody. The plate was sealed and incubated for 1 h at room temperature. After washing for 5 times, the substrate (50 ul substrate A and 50 ul substrate B) was added to each well and incubated at 37°C for 15 min. The reaction was terminated by adding 50 ul stopping solution to each well. OD value was measured at a wavelength of 450 nm.

Bioinformatics prediction

Bioinformatics prediction is currently the basis and main tool for the study of miRNA function. In order to further explore the regulation mechanism of MACC1, the softwares of miRanda, TargetSeam, PicTar, MiRanda, and BibiServ were used to predict the possible regulatory gene of MACC1. And miR-433 was predicted as one of the genes that could regulate MACC1 (Figure 1).
Statistical analysis

All data were processed using the statistical package SPSS18.0. Data were presented as mean ± standard deviation (SD). The normality of the data was tested. Comparison among multiple sets of measurement data was performed using one-way ANOVA. When there was homogeneity of variance, LSD and SNK methods were used. Otherwise, Tamhane’s T2 or Dunnett’s T3 method was used. P < 0.05 was considered statistically significant.

Results

The number of blood circulating tumor cells

In order to determine the number of circulating tumor cells in blood, circulating tumor cells were sorted using circulating cell sorting kit. As shown in Figure 2, the number of circulating tumor cells in breast cancer patients was significantly more than that in control patients (P < 0.05). This result suggests that in the blood of breast cancer patients, the number of circulating tumor cells has significantly increased.

Levels of MACC1 mRNA in samples

The qRT-PCR was conducted to detect levels of MACC1 mRNA in tumor tissues, peritumoral tissues, the blood mononuclear cells and the breast duct lavage fluid. Compared with that in peritumoral tissues, MACC1 mRNA level in tumor tissues of breast cancer patients was significantly higher (Figure 3A) (P < 0.05). Similarly, compared with the control patients, levels of MACC1 mRNA in the blood mononuclear cells of breast cancer patients (Figure 3B) and in the breast duct lavage fluid of breast cancer patients (Figure 3C) were upregulated. And, the differences were statistically significant (P < 0.05). These results indicate that MACC1 may play roles in breast cancer.

Protein expression level of MACC1 in tumor tissues, peritumoral tissues, and blood mononuclear cells

To determine MACC1 protein level in tumor tissues, peritumoral tissues, and blood mononuclear cells, Western Blot was performed. As shown in Figure 4A, MACC1 protein level in tumor tissues of breast cancer patients was significantly higher than that in peritumoral tissues of breast cancer patients (P < 0.05). Compared with the control patients, level of MACC1 protein in was increased in blood mononuclear cells of breast cancer patients (Figure 4B), and the difference was statistically significant (P < 0.05). The trend of change in MACC1 protein was consistent with that in MACC1 mRNA. The results suggest that MACC1 is upregulated both at the transcriptional level and protein expression level and that this upregulation of MACC1 may play a certain role in breast cancer.

On the other hand, the protein expression of MACC1 in blood mononuclear cells also significantly increased (P < 0.05). Since that the blood is a common way for cancer metastasis, the upregulation of MACC1 protein expression in blood may have an impact on the metastasis of breast cancer and may be involved in the tumor metastasis through the blood circulation. And, since that the number of circulating
tumor cells in blood of breast cancer patients was increased, the upregulation of MACC1 protein expression in blood may be caused by the increase of circulating tumor cells.

**Protein expression of MACC1 in breast duct lavage fluid**

Protein expression of MACC1 in the breast duct lavage fluid was detected using ELISA. Compared with the control patients, the expression of MACC1 protein in breast duct lavage fluid of breast cancer patients increased, and the difference was statistically significant (P < 0.05) (Figure 5). The trend of change in MACC1 protein in the breast duct lavage fluid was consistent with that in MACC1 mRNA. Because of the invasion and infiltration of breast cancer to surrounding area, the MACC1 protein in the duct lavage fluid may be caused by the high expression of MACC1 protein in the detached tumor cells.

The expression level of miRNA-433 in tumor tissues, peritumoral tissues, blood mononuclear cells, and duct lavage fluid

The qRT-PCR technology was used to detect the expression of miRNA-433 in tumor tissues, peritumoral tissues, the blood mononuclear cells and duct lavage fluid. As shown in Figure 6A, miRNA-433 level in tumor tissues of breast cancer patients was significantly lower than that in peritumoral tissues of breast cancer patients (P < 0.05). Similarly, miRNA level in the blood mononuclear cells (Figure 6B) and duct lavage fluid (Figure 6C) was reduced in breast cancer patients. And, the difference between control patients and breast cancer patients was statistically significant (P < 0.05). We pre-
dicted that the MACC1 was one of the target genes of miRNA-433. Thus, the results suggest that miRNA-433 may play a regulatory role in breast cancer and that miRNA-433 may regulate the transcriptional level of MACC1 to finally affect the protein expression of MACC1.

**Discussion**

In this study, we detected the mRNA and protein expression of MACC1 in the tumor tissues, peritumoral tissues, circulating tumor cells and duct lavage fluid. The expression of MACC1 upstream gene miR-433 was also analyzed in various specimens. The effect of miRNA-433 on the regulation of its downstream gene MACC1 in breast cancer was discussed.

Circulating tumor cells may exist in the early stage of tumor and may be the cause for the recurrence and metastasis of tumor [23]. Circulating tumor cells and cancer stem cells have been confirmed to be related. Cancer

![Figure 4. Analysis of MACC1 protein expression level. MACC1 protein expression level was detected with Western Blot. Representative and quantitative Western Blot results were shown on the left panel and the right panel, respectively. A. Expression level of MACC1 protein in tumor tissues and peritumoral tissues. Compared with peritumoral tissues, **P < 0.01. B. Expression level of MACC1 protein in blood mononuclear cells of control patients and breast cancer patients. Compared with control patients, *P < 0.05.](image)

![Figure 5. Analysis of MACC1 protein expression level in breast duct lavage fluid. MACC1 protein expression level in breast duct lavage fluid was detected with ELISA. Compared with control patients, **P < 0.01.](image)
stem cells have a highly invasive and metastatic potential as that of circulating tumor cells, and some cancer stem cells can also produce drug resistance [24, 25]. This correlation is important for tumor recurrence and metastasis caused by circulating tumor cells. The tumor stem cells theory thinks that only a few cells in the tumor tissues can proliferate, and most of the tumor cells have limited proliferative ability or no proliferation ability. Only cancer stem cells is the main cell source of tumor occurrence and distant metastasis [26], while the circulating tumor cells with the ability of invasion and metastasis can also lead to tumor distant metastasis. So finding a way to detect circulating tumor cells has great significance to the early diagnosis, early treatment and prognosis of breast cancer.

In this study, we found that MACC1 expression at mRNA level and protein level was significantly increased in tumor tissues, blood mononuclear cells and duct lavage fluid. The increase of MACC1 in tumor tissues suggests that MACC1 may serve as a carcinogenetic gene in breast cancer and may regulate the invasion and metastasis of breast cancer. And, the increase of MACC1 in circulating tumor cells might indicate that MACC1 increase is likely involved in the tumor metastasis by way of circulating tumor cells. Additionally, MACC1 increase in duct lavage may mean that MACC1 is involved in local invasion of breast cancer. Collectively, our results indicate that MACC1 may be of great significance for early tumor diagnosis and prognosis prediction of breast cancer [27].

MiRNAs play an important role in tumorigenesis through regulating expression of proteins [21, 22]. MiRNAs may regulate the MACC1 mRNA and inhibit their translation [20]. Research results show that miRNA-433 may become new targets for cancer prevention, diagnosis and treatment [20]. To further investigate the regulation mechanism of MACC1, we analyzed the
expression of miRNA. We used bioinformatics method to predict the possible regulatory miRNA of MACC1. We found that the relationship of miRNA-433 with MACC1 was very close and that miRNA-433 was likely to be an upstream regulatory miRNA of MACC1. And, we found that miRNA-433 was reduced in tumor tissues, blood mononuclear cells and duct lavage fluid. Thus, it is possible that miRNA-433 regulates the expression of MACC1. However, this possibility still needs further verification.

In conclusion, the number of circulating tumor cells in the blood of breast cancer patients was significantly increased. The expression of MACC1 in tumor tissues, blood mononuclear cells and duct lavage fluid were significantly increased. And this increase may be related to the expression of miRNA-433. MiRNA-433 may regulate the infiltration and invasion of breast cancer by MACC1. Especially, the miRNA-433 of circulating tumor cells may become a marker of breast cancer.

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Disclosure of conflict of interest

None.

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