miR-340 suppresses cell migration and invasion by targeting MYO10 in breast cancer

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Abstract. Breast cancer is one of the most common malignant tumors among females, and can seriously affect the physical and mental health and even threaten the lives of women. Recently, research has demonstrated that microRNAs (miRNAs), as a new method of regulation, have been shown to have oncogenic and tumor-suppressive functions in human breast cancer. Detection of their expression may lead to the identification of novel markers for breast cancer. In the present study, we firstly detected miR-340 expression and found lower expression of miR-340 in 6 human breast cancer cell lines by using RT-qPCR. Then by using wound healing assay and Transwell migration and invasion experiments, we focused on the role of miR-340 in the regulation of tumor cell migration and invasion, exploring the relationship between them. The results revealed that induction of miR-340 expression was able to suppress tumor cell migration and invasion, whereas knockdown of miR-340 expression promoted breast cancer cell migration and invasion. At the gene level, MYO10 (myosin X), as a direct miR-340 target gene, mediated the cell migration and invasion. Finally, we verified our research further at the tissue specimen level and in animal experiments. In brief, miR-340 plays an important role in breast cancer progression. Thus, miR-340 may be further explored as a novel biomarker for breast cancer metastasis and prognosis, and potentially a therapeutic target.

Introduction

Breast cancer is a serious disease threatening the health of women. In 2010, the WHO statistics showed that breast cancer alone is expected to account for 28% (207,090) of all new cancer cases among women and is the third leading cause of cancer-related deaths (1). The occurrence of breast cancer involves multiple processes, including enhanced tumor cell proliferation and reduced apoptosis (2), increase in tumor cell migration and invasion capacity (3), formation of vascular mimicry by tumor cells (4) and evasion of immunity of an organism (5). It is known that tumor invasion and metastasis of breast cancer is the main reason for mortality. Therefore, studying the molecular mechanisms of breast cancer cells in the process of invasion and metastasis has meaningful significance for the pathogenesis of breast cancer and its early diagnosis and treatment. Tumor cell migration and invasion is a complex process involving many molecules. In recent years, many scholars have been committed to exploring the mechanisms of breast cancer recurrence, invasion and metastasis. They demonstrated that various molecules involved in cell adhesion, such as integrin, E-cadherin (6) and ICAM-1 (7) are associated with breast cancer invasion and migration. Multiple subtype expression of CD44 in tumors is markedly increased and is positively correlated with tumor capsule invasion and epithelial-to-mesenchymal transition (EMT) (8). Yet, the specific molecular mechanisms involved in breast cancer metastasis remain unknown (9). Recently, it has been suggested that miRNAs regulate the development of breast cancer as a novel gene regulation mechanism.

MicroRNAs (miRNAs) are non-coding single-stranded RNAs with a length of ~18-22 nucleotides, and play an important role in regulating gene expression (10). miRNAs cause target mRNA degradation or translation inhibition through the negative regulation of its target gene mRNAs, acting as tumor promoters or suppressors (11). For example, miR-23a was found to play a role in tumor promotion by downregulating PPP2R5E thereby inhibiting gastric cancer cell apoptosis (12). miR-429 was demonstrated to have cancer-suppressive function by targeting the PAK6 signaling pathway and by inhibiting the migration and invasion of colon cancer cells (13). Research shows that miRNAs can also regulate the malignant process in breast cancer. In human breast cancers, miRNAs can promote the occurrence and development of breast cancer. For example, miR-217 can promote...
breast cancer cell invasive ability and drug resistance (14). miRNA-24-3p can promote the growth of breast cancer cells and inhibit apoptosis (15). More and more studies suggest that miR-340 also plays an important role in the malignant behavior of tumor cells. miR-340 was found to be able to inhibit the cancer stem cell-like function of glioma tumor cells through targeting tissue plasminogen activator (16). In non-small cell lung cancer, miR-34 inhibited tumor cell proliferation and induced apoptosis by targeting P27 multiple negative regulatory factors (17). However, research concerning the regulation of miR-340 in breast cancer is rare. Thus, in the present study, we investigated the effect of miR-340 on cell migration and invasion. As the classical pathway of miRNA regulation is through target genes, we predicted and verified the expression of miRNA and microRNA-24-3p can promote the growth of breast cancer cells and inhibit apoptosis (15). More and more studies suggest that microRNA-24-3p can promote the growth of breast cancer cells and inhibit apoptosis (15). More and more studies suggest that microRNA-24-3p can promote the growth of breast cancer cells and inhibit apoptosis (15).

Materials and methods

Cell lines and cell culture. Human breast cancer cell lines MCF-7, MCF-10A, MDA-MB-231, MDA-MB-468 and SKBR3 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and preserved in our laboratory. Cell culture medium for MCF-7 and MCF-10A cells contained 10% FBS and 1% double resistant RPMI-1640 medium. Cell culture medium for the MDA-MB-231 and MDA-MB-468 cell lines contained 10% FBS and 1% double resistant Leibovitz L-15. Cell culture medium for SKBR3 cells contained 10% FBS and 1% double resistant Dulbecco's modified Eagle's medium (DMEM). All the above cells were cultured in a humidified incubator with 5% CO₂ at 37°C.

RNA extraction and real-time PCR. The process of extracting the total RNA in cells and tissues was carried out according to the instructions of the mirVana miRNA isolation kit (Ambion, Austin, TX, USA). The extraction concentration was measured by spectrophotometer NanoDrop (NanoDrop, Wilmington, DE, USA). Finally, it was standby preserved at -80°C. As for RT-qPCR, we first used reverse transcriptase M-MLV and nucleic acid enzyme inhibitor RiboLock (Applied Biosystems, Foster City, CA, USA) to transcribe RNA into cDNA in reverse. Next, SYBR Green (GenePharma, Shanghai, China) was used to conduct the real-time quantitative PCR reaction with IQ-5 (Bio-Rad Laboratories, Hercules, CA, USA). The PCR procedure consisted of 4 min at 95°C firstly, and 94°C for 30 sec, 50°C for another 30 sec, 72°C for 30 sec by cycles. 2-ΔΔC₅₀ method was used as the quantification approach. Primer information is as follows: miR-340 forward, 5'-GGCGTCTAAT AAAGCAATGAGA-3' and reverse primer, 5'-GTTCGTTGTCGTTGAGTCG-3'; U6 forward, 5'-GGTCCCAGCAGCAGATATACTAAAT-3' and reverse primer, 5'-GGTCTCAACG ATTTTGCGTGTCTA-3'; MYO10 forward, 5'-AAGTGAGGAGGAGCAGGTTAAAACCG-3' and reverse primer, 5'-GCTCGTTCACGAACTGCTTAGC-3'; GAPDH forward, 5'-TGCACCATACCTTCTTCTA-3' and reverse primer, 5'-GGCATGGACAAAGCAATGAGA-3'.

Plasmid, ASO-miRNA and siRNA transfection. The SKBR3 and MCF-7 cells were plated in 6-well plates at a density of 1x10⁸ cells/well and kept overnight. Then, we transferred the miR-340 mimic or ASO-miR-340, to pCMV5/MY010 or si-MY010 (all from GenePharma) using the liposome method. Lipofectamine 2000 was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA); miR-340 mimic, 5'-UAUA AAAGCAUAGAGACUGAUU-3' (sense) and 5'-UCAGUC UCAUUGCUUAUUAAUU-3' (antisense); ASO-miR-340: 5'-UUCUCGCAACGUGUCACUGT-3' (sense) and 5'-ACG UGACCGAGCUAAGGAATT-3' (antisense); MYO10-siRNA, 5'-GCCCAGUCUACAGAUAUGUGTT-3' (sense) and 5'-ACC AUUUCUGAAGGCTT-3' (antisense). Six hours after transfection, the cells were cultured in normal culture medium, and 48 h later, further assessment was conducted.

Protein extraction and western blotting. The transfected cells were lysed with RIPA buffer. The proteins (50 μg) were then subjected to 10% SDS-PAGE electrophoresis. Subsequently, the proteins were transferred to the membranes. The membranes were incubated with 5% blocking buffer and then incubated with antibody at 4°C overnight, and added secondary antibodies (1:1,000; Abcam, Cambridge, MA, USA) for 2 h. The protein expression was accessed by exposing film. To quantitate band intensities, labWorks™ gel imaging and analysis system was adopted for photographing and analyzing the luminance value of each group of target bands.

Luciferase reporter assay. Approximately 3x10⁴ cells were plated in a 12-well plate evenly. Approximately 24 h later, breast cancer cells were transfected with 200 ng miR-340 and 50 ng pGL3-MY010-3'-UTR. Thirty-six hours after transfection, the cells were split, and their fluorescence activity was measured with a dual luciferase system (Promega, Madison, WI, USA).

In vitro scratch test. In vitro scratch test was used to analyze the migration ability of the cells. The cells were transfected in a 6-well plate. After the cell fusion, the surface of the cell layer was scratched with a sterile plastic gun. The cells were then cultured with double-nutrient medium. Forty-eight hours later, we observed scratch healing on the cell layer surface under a microscope.
**Transwell invasion experiment.** The apertures of the bottom membrane of the Transwell chambers or wells (Corning Inc., Corning, NY, USA) was 8 μm. The chambers were coated with Matrigel (Sigma-Aldrich, St. Louis, MO, USA), and were used for detecting the cell invasive ability. The underlayer was filled with 600 μl L15/RPMI-1640/DMEM nutrient solution which contained 10% FBS. The volume of the upper layer of the Transwell was 200 μl inoculated with a certain amount of breast cancer cells, 5x10³ MDAMB-231 or 1x10⁶ MCF-7. The cells were cultured in an incubator at 37°C with 5% CO₂ for 36 or 48 h, and then the well was removed and fixed in liquid, which consisted of methanol and glacial acetic acid at a ratio of 3:1, for 30 min. Then the wells were washed with PBS, stained with 0.1% crystal violet and finally mounted.

**Immunohistochemical (IHC) analysis.** We detected the tissue expression levels of candidate target gene MYO10 with IHC testing technology. The TMA slice was dewaxed. Endogenous peroxidase activity was halted with 3% hydrogen peroxide for 10 min. The section was infiltrated in 0.01 M citric acid buffer (pH 6.0) and then heated in a microwave for 10 min to carry out antigen recovery. Then it was incubated with the primary antibody anti-MYO10 (1:100) for 1 h at room temperature, and the secondary antibody was added after washing with PBS, and the incubation was continued. Finally it was incubated with dimethylbenzidine and counterstained with hematoxylin.

Using a microscope, we observed the staining intensity and the percentage of stained cells with respect to the background. A positive signal was indicated when over 10% of the tumor cells were stained.

**Animal experiment.** All of the animal experiments conformed to the requirements of the ethics committee. MCF-7-transfected cells (1x10⁶) were injected into 6- to 8-week-old nude mice by tail intravenous injection. The mice were sacrificed 5-7 weeks later. Under a microscope, we observed and counted the metastatic pulmonary nodules. IHC staining was also carried out and assessed.

**Statistical analysis.** Statistical software SPSS22 was used. Data are expressed as mean ± standard deviation. A t-test was used for analyzing the data. P<0.05 or P<0.01 were considered to indicate a statistically significant difference.

**Results**

**miR-340 is expressed at a low level and directly targets MYO10 in breast cancer cell lines.** In order to explore the role of miR-340 in breast cancer cells, RT-qPCR technology was applied to analyze miR-340 expression levels in different breast cancer cell lines. We took the expression level of miR-340 in MCF7A cells as a standard 1, and found that miR-340 showed low expression in multiple breast cancer cell lines, compared to the MCF7A cells (Fig. 1A). As the function of miRNA is realized by regulating its corresponding target gene, we searched TargetScan database for the target gene of miR-340. Among several candidate target genes of miR-340, we chose MYO10 as the object of further study. The reason was that previous reports indicated that MYO10 could promote the development of breast cancer, and we predicted that there are three potential targets for miR-340 in the 3'UTR region in MYO10 (Fig. 1B). Meanwhile, we assessed the MYO10 expression level in 8 different breast cancer cell lines using western blot analysis, and found that the expression level was higher in the breast cancer cell lines than that in the MCF10A cells (Fig. 1C). In order to verify that MYO10 is the direct target gene of miR-340, we constructed MYO10-3'UTR, which contained miR-340 binding sites, into the fluorescent reporter vector, and observed the target given situation by detecting luciferase activity. The result suggested that the fluorescence activity of miR-340 decreased due to overexpression, while, the fluorescence activity of the mutant UTR was not significantly changed after the mutation (Fig. 1D). This showed that there was a direct and inverse relationship between miR-340 and MYO10. Overexpression of miR-340 in the breast cancer MDA-MB-231, MDA-MB-468 or SKBR3 cells led to a decrease in the MYO10 protein level at varying degrees. Moreover, the expression of MYO10 was significantly increased after blocking miR-340 expression (Fig. 1E). Thus, we confirmed that miR-340 is expressed at a low level in breast cancer cells and MYO10 is a direct target of miR-340, which negatively regulates its expression.

**Overexpression of MYO10 reverses the inhibition of breast cancer cell migration and invasion mediated by miR-340.** To further explore the regulation of malignant behavior of breast cancer cells by MYO10 and miR-340, we studied breast cancer cell migration and invasion. We used miR-340 mimics and pCMV/PMYO10 to overexpress miR-340 and MYO10 in the MCF-7 cells. First we measured the protein expression level of MYO10 by western blotting when miR-340 and MYO10 were stained respectively or conjointly, and the miR-340 mimics and pCMV/PMYO10 plasmids were verified to be effective (Fig. 2A). Then we assessed the effects of miR-340 and MYO10 on cell migration through in vitro scratch test. As shown in Fig. 2B, when MYO10 was overexpressed alone, cell migration ability was enhanced. When miR-340 and MYO10 were overexpressed together, miR-340 induced cell migration; MYO10 reversed the condition partly. The same result appeared in Transwell invasion and migration experiment. When overexpressing MYO10 alone, the ability of cell invasion and migration increased about 4.5 times. When miR-340 and MYO10 were overexpressed conjointly, miR-340 induced cell migration; MYO10 saved the condition partly. The reduction in cell migration and invasion due to miR-340 may be saved by MYO10 (Fig. 2C).

**Knockdown of MYO10 weakens the enhancement of cell migration and invasion induced by silencing miR-340.** In order to further verify whether MYO10 has a necessary influence on the change in breast cancer cell migration and invasion mediated by miR-340, on the basis of previous experiments, we assessed the function of knockdown of MYO10 and silencing of miR-340. First, we tested MYO10 expression with western blotting, and verified that siRNA MYO10 could effectively knock down the expression of MYO10. MYO10 expression obviously increased after ASO-miR-340 transfection illustrating the effectiveness of the two knockdowns (Fig. 3A). Following...
knockdown of MYO10 alone, cell migration decreased. When cells were transfected with MYO10 siRNA and ASO-miR-340 jointly, ASO-miR-340-induced enhancement of cell migration was inhibited by MYO10 siRNA (Fig. 3B). We found the same results in the Transwell invasion and migration experiments. Following knockdown of MYO10 alone, cell migration and invasion ability decreased by ~35%. Following treatment of MYO10 siRNA and ASO-miR-340 together, cell migration enhancement induced by ASO-miR-340 was inhibited by MYO10 siRNA (Fig. 3C).

Expression level of miR-340 and MYO10 in high metastatic and low metastatic breast cancer specimens. Based on the cytological experiments mentioned above on the expression levels of MYO10 and miR-340 and on their functions, we attempted to validate our conclusions from the levels in tissue specimens. Before the detection of miR-340 expression in the breast cancer specimens, pathological classification was carried out according to the number of cancer cell lymph node metastasis. Those with lymph node metastasis <1 were considered as the no metastasis group, those with lymph node

Figure 1. Expression of miR-340 and its target gene in breast cancer cell lines. (A) Real-time PCR analysis of miR-340 expression in different breast cancer cell lines is shown. (B) Alignment between the predicted miR-340 target sites of MYO10 3'UTR and miR-340 is shown. The conserved 7-bp seed sequence of miR-340 is aligned to MYO10 mRNA. (C) Western blotting of MYO10 expression in different breast cancer cell lines is shown. (D) In the luciferase reporter assay, miR-340 directly interacts with its target gene 3'UTR. (E) Western blotting of MYO10 expression after cells were transiently transfected with miR-340 mimic and ASO-miR-340, respectively. *P<0.01; ns, not significant.
metastasis from 1 to 3 were considered as the low metastasis group, and those with lymph node metastasis >3 were regarded as the high metastasis group. The result revealed that, when compared to the no metastasis group, the expression level of miR‑340 in the high metastasis group was decreased by 60%, while the expression level of miR‑340 in the low metastasis group increased by ~1.2 times (Fig. 4A). These results confirm our previous conclusion that miR‑340 inhibits tumor cell migration and invasion. We detected the expression levels of MYO10 in 10 high metastatic and low metastatic carcinomas using western blotting. We found that in high metastatic carcinomas, the expression level of MYO10 was increased (Fig. 4B), while the MYO10 expression level was decreased in the low metastatic carcinomas (Fig. 4C). Thus, miR‑340 inhibits tumor cell migration and invasion by negative regulation of MYO10 expression.

miR‑340 inhibits lung metastasis of breast cancer in mice and is negatively correlated with MYO10. To further clarify the correlation between miR‑340 and the MYO10, we performed animal experiments in mice by tail vein injection. We injected MCF‑7 cells overexpressing miR‑340 into mice as described earlier. Metastatic lung nodules were counted after the mice were sacrificed. It was found that overexpression of miR‑340 significantly decreased the lung metastasis of breast cancer in the mice. Compared to the control group, lung metastasis decreased by 70% (Fig. 5A). We also confirmed that miR‑340 effectively inhibited the lung metastasis of breast cancer using IHC staining for carcinoembryonic antigen (CEA) (Fig. 5B). We obtained a small portion of the tumor tissues to extract RNA and detect expression levels of miR‑340. The results confirmed that overexpression of miR‑340 was effective (Fig. 5C). Western blotting also demonstrated that the expression of miR‑340 negatively regulated MYO10 expression in the tissues (Fig. 5D). Likewise, using immunohistochemical staining techniques we also found that the expression of MYO10 in the miR‑340 overexpression group was reduced, compared to the control group (Fig. 5E). All of the above confirmed that miR‑340 and MYO10 expression were negatively correlated in the tissues.

Discussion

Tumor cell metastasis is a critical stage which threatens the life of cancer patients and involves a complex cascade of events. Progressing from in situ is the first step, which includes change in tumor cell adhesion, cell migration, invasion of the basement membrane, enzymatic hydrolysis substrate, angiogenesis and survival in the circulatory system (20). Next, tumor cells settle over a long distance.
and grow and form a metastatic tumor. In this series of events, tumor cell migration and invasion of surrounding tissue is a crucial early step. A large number of recent studies indicate that miRNAs play an important role in the migration and invasion of tumor cells (21). For instance, miR-20a/b can inhibit the invasion of prostate cancer cells (22) and miR-140-5p can inhibit the migration of hepatocellular carcinoma cells. Many studies have also reported that in the course of breast cancer development, many miRNAs, which include miR-205, miR-206 and miR-146, can promote or inhibit the migration and invasion of breast cancer cells (24, 25). In the present study, we assessed a number of different breast cancer cell lines and detected low expression of miR-340. A number of studies previously confirmed that miR-340 can play a regulatory role in different types of tumors. For example, in osteosarcoma miR-340 can target ROCK1 and inhibit cell growth and metastasis (26). miR-340 expression in bone marrow was associated with liver metastasis of colon cancer (27). Yet, few reports have reported the relationship between miR-340 and breast cancer cell migration and invasion. Our research focused on the effects of miR-340 on the migration and invasion of breast cancer cells. And our final conclusion is that miR-340 can inhibit the migration and invasion of breast cancer cells.

It is known that miRNAs effect post-transcriptional expression levels by targeting target genes. Thus, we used bioinformatic prediction method to identify the potential target gene of miR-340 as MYO10. the MYO family has 12 members in vertebrates. Their structure is characterized by the actin dependent domain in N-terminal and variable
C-terminal, which contains a number of cytoskeleton, cytoplasmic membrane, signaling molecules and target anchor points of other factors (28). MYO10 role is also crucial in biological function. At the University of North Carolina, the researchers in the Cell and Molecular Physiology Laboratory used scanning electron microscopy to observe the large number of filopodia on the back of HeLa cells. The results suggested that MYO10 is the necessary molecular motor for the formation of filopodia (29). Cox et al (30) found that there is a close connection between MYO10 and cell phagocytosis, and MYO10 tail truncated structure could inhibit the phagocytosis of alveolar macrophages. It was confirmed that the effect of MYO10 is essential in the process of cell adhesion and migration (31). It was also found by many other studies that MYO10 plays a major role in cell endocytosis (32). Yet, reports on the regulation of MYO10 on invasion and gene expression in breast cancer are rare. Our research confirmed that MYO10 plays a promoting role in the migration and invasion of breast cancer cells. Further research found that MYO10 promotes the migration and invasion of breast cancer cells and restores miR-340-mediated inhibition of breast cancer cell migration and invasion. The findings are consistent with previous studies. Thereby it was also confirmed by functional verification that MYO10 is the direct target gene of miR-340. Further research will be carried out to ascertain through which specific molecular mechanisms or signaling pathways is this functional regulation achieved.

Our experiment demonstrated for the first time that in breast cancer cells, miR-340 inhibited the migration and invasion of cancer cells through targeting MYO10. This finding has important significance in tumor metastasis as it may become an important method for the inhibition of tumor metastasis. MYO10 also provides another effective target for the treatment of breast cancer. Therefore, MYO10 is expected to become a new means of diagnosis and treatment of breast cancer metastasis to weaken the migration and invasion of tumor cells through mediating the expression level of miR-340 in breast cancer cells.

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References