MiR-335 inhibits migration of breast cancer cells through targeting oncprotein c-Met

Yue Gao · Fan Zeng · Jia-Yan Wu · Hai-Yu Li · Jian-Jun Fan · Li Mai · Ji Zhang · Dong-Mei Ma · Yun Li · Fang-zhou Song

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Abstract Metastasis is the leading cause of death in patients with breast cancer and aberrantly expressed microRNAs (miRNAs) are highly associated with this process. A previous study has shown that miR-335 is downregulated in breast cancer and can suppress tumor invasion and metastasis. Emerging evidences indicate that c-Met is implicated in cell scattering, migration, and invasion. However, little is known about the relationship between miR-335 expression and c-Met alteration in breast cancer. In the present study, we found that miR-335 expression was downregulated and c-Met protein expression was upregulated in two human breast cell lines. MiR-335 was found to negatively regulate c-Met protein level by directly targeting its 3′ untranslated region (UTR). Forced expression of miR-335 decreased c-Met expression at protein levels and consequently diminished hepatocyte growth factor (HGF)-induced phosphorylation of c-Met and subsequently inhibited HGF promotion of breast cancer cell migration in a c-Met-dependent manner. MiR-335 expression was increased after 5-aza-2′-deoxycytidine (5-AZA-CdR) treatment, and 5-AZA-CdR treatment resulted in the same phenotype as the effect of miR-335 overexpression. Taken together, these results demonstrate that miR-335 suppresses breast cancer cell migration by negatively regulating the HGF/c-Met pathway.

Keywords Breast cancer · miR-335 · c-Met · HGF · Metastasis · 5-AZA-CdR

Introduction

Breast cancer is a significant health problem for women and a leading cause of cancer mortality worldwide [1]. However, improvement in early detection and treatment, tumor invasion, and metastasis are still responsible for breast cancer mortality, and the molecular mechanisms associated with metastatic ability in breast cancer are poorly understood [2].

MicroRNAs (miRNAs) are small noncoding RNAs which can suppress gene expression at the post-transcriptional level by cleaving messenger RNA (mRNA) molecules or by inhibiting their translation [3]. MiRNAs play fundamental roles in a broad range of biological processes, including cell growth [4], differentiation [5], embryo development [6], and apoptosis [7]. Recent studies have demonstrated that mutations or aberrant expression of miRNAs correlates with human carcinogenesis and cancer progression, indicating that some miRNAs can function as tumor suppressor genes or oncogenes [8]. For instance, miR-335 [9] and miR-31 [10] are downregulated in breast cancer and can suppress tumor invasion and metastasis, whereas upregulation of miR-9 [11] and miR-10b [12] can increase breast cancer cell invasion and metastasis.

c-Met tyrosine kinase is a disulfide-linked heterodimer composed of a completely extracellular alpha chain (50 kDa) and a single-pass transmembrane beta chain (145 kDa). c-Met plays a key role in the control of invasive growth not only during tumorigenesis but also in embryonic development, organ development, and inflammatory responses [13, 14]. Hepatocyte growth factor (HGF) is the natural ligand for the receptor c-Met [15]. Upon stimulation of HGF, c-Met becomes phosphorylated and initiates a range of downstream signaling pathways, eventually inducing the
tumor invasion and migration [16, 17]. Previous studies demonstrated that certain miRNAs, such as miR-340 [18], miR-34a [19], and miR-198 [20], were able to inhibit migration and invasion through regulation of c-Met gene expressions. Indeed, c-Met is implicated in cell scattering, migration, and invasion. MiR-335 has been found to suppress breast cancer cell metastasis and migration through targeting of the transcription factor SOX4 and extracellular matrix component tenascin C [9]. As a novel and attractive therapeutic strategy, one miRNA can simultaneously regulate the expression of hundreds of different genes. However, the relationship between miR-335 expression and c-Met alteration in breast cancer remains unknown.

Therefore, in this study, we first determined the expression of miR-335 using quantitative reverse transcriptase (qRT)–PCR and the protein expression of c-Met using Western blotting in different breast cancer cells with diverse invasive ability. We demonstrated that miR-335 was frequently down-regulated in MDA-MB-231 and BT-20 cells, while c-Met was obviously upregulated. In addition, we identified c-Met was a direct and functional target of miR-335. Furthermore, we showed that miR-335 negatively regulated c-Met expression and subsequently diminishes HGF-stimulated migration and invasion of breast cancer cells.

Materials and methods

Cell lines and cultures

The human breast cancer cell lines MCF-10A, MCF-7, BT-20, BT-474, SK-BR-3, MDA-MB-231, and MDA-MB-435S, ZR-75-30, T47D, and BCaP-37 were obtained from the American Type Culture Collection (Rockville, Md). MCF-7 cells were grown in RPMI 1640 medium and MDA-MB-231 and MDA-MB-468 in Leibovitz’s L-15 cell culture medium with 10 % fetal bovine serum at 37 °C in a humidified atmosphere of 95 % air and 5 % CO2.

Total RNA extraction and qRT–PCR

Total RNA was extracted from cultured cells using TRIzol (Invitrogen) or miRVana miRNA Isolation Kit (Ambion) according to the manufacturer’s protocols. For quantitative real-time PCR, RNA was first reversely transcribed into complementary DNA (cDNA) using SuperScript III reverse transcriptase (Invitrogen). After that, qPCR was performed with an SYBR Green I real-time PCR kit (GenePharma, Shanghai, China) according to the manufacturer’s instructions with a Stratagene M×3000P Real Time PCR machine (Agilent Technologies). U6 small nuclear RNA was used as an internal control. The PCR amplification protocol was as follows: an initial 95 °C for 5 min and 50 cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s.

Transient miRNA and siRNA transfection

The miRNAs and small interfering RNAs (siRNAs) were synthesized by GenePharma (Shanghai, China). The miRNA mimics are small double-stranded RNAs that can enter the miRNA-bioprocessing pathway. The sequences of negative control (GenePharma) were nonhomologous to any human genome sequences and used to eliminate the potential nonsequence-specific effects. Cells (1.0×106 per well) were seeded and grown overnight in six-well plates. The next day, they were transfected with siRNAs and miRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Total protein was prepared 48 h after transfection and used for Western blot analysis.

Western blot analysis

The cells were washed twice with cold PBS, and total cellular protein was extracted using a modified RIPA buffer with 0.5 % sodium dodecyl sulfate (SDS) in the presence of proteinase inhibitor cocktail (Complete mini, Roche). The protein concentration was then determined by a protein assay kit (Bio-Rad), and equal amounts of protein lysates were separated on SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5 % nonfat milk and incubated with primary antibodies at 4 °C overnight. The next day, the membranes were washed and then incubated with rabbit anti-c-Met monoclonal antibody. The intensity of protein bands was quantified using image J software (National Institutes of Health, Bethesda, MD).

Luciferase assay

A dual-luciferase reporter vector psiCHECK2 (Promega) was used to generate luciferase reporter constructs. The full-length 3’ untranslated region (UTR) of c-Met was amplified by PCR from human genomic DNA with primers of 5’-CATCTCGA GGAGCGGCAGCCCCAGAACAGG-3’ (sense) and 5’-CATGCCGCGCATGATTTCAGAAACACTATTA-3’ (antisense) and cloned downstream Renilla luciferase in a psiCHECK2 vector. Cells were seeded in 24-well plates cultured overnight and then cotransfected with 100 ng of psICHECK2-C-MET-WT, psICHECK2-C-MET-mut, and 200 nmol/L of miR-335 or negative control by using Lipofectamine 2000. Luciferase assays were performed 48 h after transfection using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was then normalized to Renilla luciferase activity. All experiments were performed in triplicate.
Cell migration assay

Transwell insert with a pore size of 8 μm from Corning, Inc. (Corning, NY) was used to determine tumor cell migration capacity. MDA-MB-231 and BT-20 cells were first transfected with miR-335 mimic or control RNA. After 24 h, the cells were starved in medium without fetal calf serum (FCS) for 24 h, and then the cells were resuspended in the FCS-free medium and placed in the top chambers in triplicate. HGF (2 or 10 ng/mL) was added to the lower chamber as a chemoattractant for 12–24 h. The cells remaining on the upper membrane were removed with cotton wool, whereas the cells that had migrated to the bottom of the membrane were then fixed with 95% ethanol and stained with 0.1% crystal violet. Five visual fields of each insert were randomly chosen and photographed under a light microscope at ×200 magnification. All experiments were performed in triplicate.

Drug treatment

The cells were plated into 24-well plates at a density of 5 × 10⁴ cells/well, and 5-aza-2′-deoxycytidine (5-AZA-CdR) was added to the cells after they had adhered to the plate. The cells were incubated with 5-AZA-CdR for approximately 96 h and then harvested to assess miR-335 expression and c-Met protein expression and performed cell migration assay.

Statistics analysis

A two-tailed Student’s t test was used to evaluate the significance of the differences between two groups of data in all pertinent experiments. All the experiments were similarly done at least three times. All statistical analyses were performed using the SPSS statistical package (11.5, Chicago, IL, USA). P<0.05 was considered significant.

Results

miR-335 and c-Met are inversely expressed in breast cancer cells

To address the biological significance of the c-Met-miR-335 interaction in breast cancer, we initially examined c-Met and miR-335 expression levels in breast cancer cell lines. c-Met protein levels were obviously higher in breast cancer BT-20 and MDA-MB-231 cells but relatively lower in nonmalignant human breast epithelial MCF-10A cells and other breast cancer cells (Fig. 1a). In contrast, miR-335 levels were shown to be significantly decreased in BT-20 and MDA-MB-231 cells but increased in MCF-10A cells and other breast cancer cells (Fig. 1b). A highly significant negative correlation between c-Met protein and miR-335 was observed, a low expression of miR-335 being associated with high amounts of c-Met protein (Fig. 1c). These results indicate that miR-335 expression is negatively related to c-Met expression in breast cancer cell lines.

c-Met is a direct target of miR-335

To validate whether c-Met gene is a target of miR-335, two algorithm programs (PicTar [21] and TargetScan Release [22]) were used to predict miRNAs targeting the 3′UTR of c-Met. A putative miR-335 binding site was found in the 3′UTR of c-Met which was highly conserved in some species (Fig. 2a). To...
determine whether c-Met was regulated by miR-335 through direct binding to its 3′UTR, we cloned the 3′UTR of c-Met containing the putative miR-335 binding site into a luciferase reporter construct (Fig. 2b). Meanwhile, we also constructed a mutated 3′UTR bearing four mismatched nucleotides in the seed sequence of miR-335 (Fig. 2b). The BT-20 and MDA-MB-231 cells were transfected with the luciferase reporter gene fused to the c-Met 3′UTR or mutant c-Met 3′UTR with miR-335 mimics or control. Luciferase reporter assays showed that miR-335 transfection caused a remarkable decrease in relative luciferase activity in the presence of wild-type c-Met 3′UTR (Fig. 2c). However, the luciferase activity with the mutated 3′UTR was not affected by miR-335 (Fig. 2c). These results suggest that miR-335 can specifically bind to the 3′UTR of c-Met mRNA and specifically suppress target gene expression. The effect of miR-335 on endogenous c-Met protein expression was subsequently evaluated in BT-20, MDA-MB-231, and MDA-MB-435S cells by Western blot. MiR-335 mimic significantly reduced the expression of c-Met protein, whereas the expression of c-Met

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**Fig. 2** C-Met is a direct target of miR-335. 

**a** The complementary sequences and evolutionary conservation of the miR-335 binding site in c-Met 3′UTR from different mammalian species are shown. **b** The wild-type or mutant (underlined) binding site of c-Met-3′UTR for miR-335 is shown. **c** The effect of miR-335 on luciferase intensity controlled by the wild-type or mutant 3′UTR of c-Met was determined by luciferase assay. **d** Western blot assays of c-Met protein after transfection with miR-335 mimic, mimic control, or miR-335 ASO and ASO control in BT-20, MDA-MB-231, and MDA-MB-435S cells. GAPDH served as an internal control.
protein was upregulated after forced expression of miR-335 ASO (Fig. 2d). These results indicate that miR-335 regulates c-Met at the post-transcriptional level.

Forced expression of miR-335 diminishes HGF-promoted cell motility

HGF interacts with its cognate receptor c-Met and induces autophosphorylation of tyrosine residues in c-Met to promote cell migration and invasion [23]. The level of c-Met phosphorylation in BT-20 and MDA-MB-231 cells after HGF stimulation was investigated. The total expression of c-Met was detected by Western blot analysis with anti-c-Met antibody. Western blot results indicated that miR-335 mimic significantly reduced the expression of c-Met protein (Fig. 3a). But no significant differences were observed in the total expression of c-Met in response to stimulation with HGF and without HGF treatment (Fig. 3a). Phosphorylation of c-Met was detected by Western blot analysis with anti-phosphotyrosine monoclonal antibody. The phosphorylation levels of c-Met in HGF-treated cells were higher than the control cells without HGF treatment. Moreover, HGF induced c-Met phosphorylation in a dose-dependent manner. In addition, the phosphorylated c-Met in miR-335 mimic-treated cells were extensively lower than those in mimic control-treated cells after HGF treatment. These results suggest that downregulation of c-Met with miR-335 mimic inhibits HGF-enhanced phosphorylation (Fig. 3a).

Subsequently, we tested whether miR-335 had an effect on the migration of breast cancer cells. BT-20 and MDA-MB-231 cells were transfected with miR-335 mimics or miR-335 control and then treated with or without HGF for evaluation of BT-20 and MDA-MB-231 cells transfected with miR-335 mimic or mimic control with or without 2 or 10 ng/mL of HGF treatment. c Transwell migration assays of BT-20 and MDA-MB-231 cells transfected with miR-335 mimic or mimic control with 10 ng/mL of HGF treatment.

**Fig. 3** Forced expression of miR-335 abrogates HGF-stimulated cell motility. a BT-20 and MDA-MB-231 cells were transfected with miR-335 mimic or mimic control for 48 h and then with or without 2 or 10 ng/mL of HGF treatment for 15 min. Western blot analysis was performed to detect phosphorylated c-Met or total c-Met. b Transwell migration assays

![Image](image_url)
of cell migration activity. The cell migration assay showed that HGF stimulation resulted in higher migration capacity of cancer cells (Fig. 3b, c). Moreover, HGF with dose-dependent manner increased the motility of BT-20 and MDA-MB-231 cells by inducing tyrosine phosphorylation of c-Met (Fig. 3b, c). In contrast, the miR-335 significantly decreased the HGF-induced migration of BT-20 and MDA-MB-231 cells (Fig. 3b, c). Taken together, the results above suggest that miR-335 is a potent suppressor of BT-20 and MDA-MB-231 cell migration through downregulation of the c-Met.

c-Met reintroduction restores miR-335-repressed cell motility

To further investigate the relationship between c-Met and miR-335-mediated cell motility inhibition, we overexpressed miR-335 with c-Met together in BT-20 and MDA-MB-231 cells. The endogenous c-Met protein expression was evaluated by Western blot, and cell migration was analyzed by use of transwell assay. In the absence of HGF, overexpression of miR-335 with c-Met could restore the total protein expression of c-Met, but no significant differences were observed in the phosphorylation levels of c-Met (Fig. 4a). Moreover, we observed that overexpression of miR-335 with c-Met could not reverse the miR-335-repressed cell migration without stimulation by HGF (Fig. 4b). On the contrary, the phosphorylation levels of c-Met were restored by forced expression of c-Met with HGF treatment (Fig. 4c). In addition, in the presence of HGF treatment, overexpression of miR-335 with c-Met rescued miR-335-repressed cell migration (Fig. 4d). Therefore, we conclude that overexpression of c-Met counteracts the repressive effects of miR-335 on cell migration.

5-AZA-CdR diminishes HGF-promoted cell motility

Aberrant DNA methylation within the promoter regions of tumor suppressor genes is an epigenetic mechanism that can inhibit transcriptional initiation and thereby silence these genes [24]. 5-AZA-CdR, a potent and specific inhibitor of DNA methylation, has been shown to reactivate the expression of genes [24]. Evidence indicates that miR-335 expression is regulated through epigenetic means via promoter methylation of a specific CpG island in the miR-335/Mest promoter during cancer progression [25]. To examine the functional significance of DNA methylation in transcriptional repression of the miR-335 gene, BT-20 and MDA-MB-231 cells were treated with the DNA methyltransferase inhibitor, 5-AZA-CdR. The level of miR-335 transcripts was measured by quantitative real-time PCR, and the protein level of c-Met was measured by Western blot. We observed that 5-AZA-CdR with a dose-dependent manner significantly increased endogenous miR-335 expression (Fig. 5a) and decreased the protein expression level of c-Met in BT-20 and MDA-MB-231 cells (Fig. 5b). This finding is consistent with the notion that miR-335 downregulates the expression of c-Met at the translational level. To determine the effect of 5-AZA-CdR on cancer cell motility, BT-20 and MDA-MB-231 cells were treated with 5-

![Fig. 4](image-url) Forced expression of c-Met restores miR-335 expression that diminished HGF-stimulated cell migration. a and b BT-20 and MDA-MB-231 cells were transfected with miR-335 mimic, mimic control, c-Met siRNA, or miR-335 mimic plus vector expressing c-Met cDNA for 48 h. Western blot analysis was performed to detect phosphorylated c-Met or total c-Met. Transwell migration assay was performed in the absence of HGF treatment. c and d BT-20 and MDA-MB-231 cells were transfected with miR-335 mimic, mimic control, c-Met siRNA, or miR-335 mimic plus vector expressing c-Met cDNA for 48 h and then with 10 ng/mL of HGF treatment for 15 min. Western blot analysis was performed to detect phosphorylated c-Met or total c-Met. Transwell migration assay was performed in the presence of HGF treatment.
AZA-CdR. Cell migration without stimulation by HGF was not significantly altered by 5-AZA-CdR (Fig. 5c). In contrast, the cells incubated with 5-AZA-CdR had relatively lower levels of cell migration with HGF treatment (Fig. 5d). These results are similar to those observed for miR-335 overexpression. Therefore, we conclude that 5-AZA-CdR diminishes HGF-promoted cell motility through reactivating the expression of miR-335.

**Discussion**

A large amount of evidence has demonstrated that alterations in the expression of miRNAs contribute to the pathogenesis of most human cancers and that they can act as oncogenes or tumor suppressors [8]. Tumor metastasis is the main cause of mortality in patients with solid cancer. Thus, a better understanding of the gene networks orchestrated by these miRNAs is important for the development of effective therapeutic approaches leading to the improvement of survival outcome of patients with breast cancer. Previous studies suggest that certain miRNAs, such as miR-340 [18], miR-34a [19], and miR-198 [20], play an important role in the regulation of cancer metastasis by directly targeting c-Met gene. Emerging evidence has reported that miR-335 is downregulated in breast cancer and suppresses cell metastasis and migration through targeting of the transcription factor SOX4 and extracellular matrix component tenascin C [9]. Each miRNA can potentially interact with several mRNA targets via perfect or imperfect base pairing, primarily in the 3′UTR portion. However, the role of miR-335 on c-Met is still largely unknown.

In our recent study, we further confirmed that miR-335 expression was significantly downregulated in breast cancer cell lines, BT-20 and MDA-MB-231 cells, and that loss of miR-335 expression was associated with upregulation of c-Met protein expression level. We identified c-Met as the potential target gene of miR-335 genes using bioinformatic analyses. Using the luciferase report assay, we observed that miR-335 reduced the luciferase intensity controlled by the wild-type 3′UTR of c-Met, while there was no effect on the mutant 3′UTR with several binding sites mutated, indicating that c-Met was a direct target gene for miR-335. We found that breast cancer cells transfected with miR-335 mimics had dramatically lowered levels of c-Met protein compared with the control cells. Moreover, miR-335 was a potential metastasis suppressor for breast cancer as miR-335 expression significantly diminished HGF-stimulated migration of breast cancer cells.

Cancer cells, including breast cancer cells, overexpress c-Met oncogene [26–29]. c-Met is a member of the tyrosine kinase-type receptor family, and binding of HGF induces autophosphorylation of tyrosine residues in c-Met [23]. HGF and its receptor c-Met are powerful factors associated with the progression and prognosis of patients with breast cancer [26, 27]. The HGF/c-Met pathway has been demonstrated to

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contribute to tumor invasion and metastasis of breast cancer [27, 30]. A previous report suggests that the migration and invasiveness of breast cancer cells, in response to HGF, can be manipulated by using a U1/ribozyme targeting the expression of human c-Met [31]. Our data indicated that HGF, in a dose-dependent manner, induced c-Met phosphorylation. Forced expression of miR-335 abrogated HGF-stimulated c-Met phosphorylation and subsequent cell migration by diminishing c-Met expression.

HGF is a powerful motility and invasion promoter in breast cancer cells. Targeting the HGF receptor, c-Met, is a powerful approach in combating the action of HGF in breast cancer. Thus, multiple c-Met pathway inhibitors have been evaluated in the clinic [32]. Aberrant DNA methylation of CpG islands within the promoter regions is known to be an epigenetic aberration, leading to the inactivation of tumor-suppressive gene in cancer. The DNA methyltransferase (DNMT) inhibitor 5-AZA-CdR can irreversibly inhibit the activity of DNMT and cause passive demethylation, which reactivates the expression of genes [24]. Our study showed that 5-AZA-CdR treatment significantly increased miR-335 expression. The cells incubated with 5-AZA-CdR had relatively lower levels of c-Met protein expression. In addition, the cell migration capacity was decreased after 5-AZA-CdR treatment and HGF stimulation. 5-AZA-CdR leads to the same phenotype as the effect of miR-335 on breast cancer cells.

In conclusion, the present study indicates that 5-AZA-CdR increases miR-335 expression, and miR-335 diminishes HGF-stimulated cell migration by targeting c-Met expression, functioning as a tumor suppressor. These findings will facilitate a better understanding of the molecular pathogenesis of breast cancer and imply that the overexpression of miR-335 may be utilized as a novel strategy for breast cancer therapy.

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Conflicts of interest None

References

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