Upregulated microRNA-301a in breast cancer promotes tumor metastasis by targeting PTEN and activating Wnt/β-catenin signaling

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Abstract

MicroRNAs (miRNAs) are strongly implicated in many cancers, including breast cancer. Recently, microRNA-301a (miR-301a) has been proved to play a substantial role in gastric cancer, but its functions in the context of breast cancer remain unknown. Here we report that miR-301a was markedly upregulated in primary tumor samples from patients with distant metastases and pro-metastatic breast cancer cell lines. Gain-of-function and loss-of-function studies showed that ectopic overexpression of miR-301a promoted breast cancer cell migration, invasion and metastasis both in vitro and in vivo. Notably, Wnt/β-catenin signaling was hyperactivated in metastatic breast cancer cells that express miR-301a, and mediated miR-301a-induced invasion and metastasis. Furthermore, miR-301a directly targeted and suppressed PTEN, one negative regulator of the Wnt/β-catenin signaling cascade. These results demonstrate that miR-301a maintains constitutively activated Wnt/β-catenin signaling by directly targeting PTEN, which promotes breast cancer invasion and metastasis. Taken together, our findings reveal a new regulatory mechanism of miR-301a and suggest that miR-301a might be a potential target in breast cancer therapy.

1. Introduction

In contrast to well-confined primary tumors, metastatic disease is always intractable because of its surgically inoperable nature and the resistance of disseminated cancer cells to existing therapeutic drugs. Indeed, it is not the primary tumors, but the distant metastases are responsible for about 90% of cancer-associated mortality (Spano et al., 2012). These clinical realities apply to a variety of cancer types including breast cancer, which alone is expected to account for 25% of all new cancer cases among women worldwide (Anand et al., 2008). Despite much advancement in knowledge from more than a century of research, metastasis remains one of the most enigmatic aspects of the disease and the molecular pathways underlying each step are still obscure. Elucidation of the precise molecular mechanism that governs the metastasis process is critical for decreasing mortality by breast cancer.

Wnt/β-catenin signaling has been shown to play an important role in the development and promotion of cancer metastasis (Cai et al., 2013). Upon activation, the WNT signals will stabilize and finally lead to accumulation of β-catenin. Activated β-catenin dissociates with E-cadherin, dissembling the adherens and enters the nucleus to turn on the expression of target genes, most of which show invasion-promotion functions (Tian et al., 2011). It is well recognized that Wnt/β-catenin signaling is subject to multiple levels of control (Fang et al., 2013). Thus, identifying the regulators of the Wnt/β-catenin signaling pathway in breast cancer is biologically and clinically important for future development of anti-metastatic strategies.

Recently, accumulating data have pointed to a central regulatory role for miRNAs in the initiation and development of breast cancer (Fonseca-Sánchez et al., 2013). These small (19–25 nucleotides), non-coding RNAs can modulate the expression of their cognate target genes by binding to the 3′-untranslated region (UTR) of target mRNAs, causing either translational inhibition or mRNA cleavage (Shi et al., 2013). Bioinformatic predictions indicate that miRNAs regulate more than 30% of the protein-coding genes (Nohata et al., 2011). The dysregulation of miRNA expression appears to be a general trait of metastatic breast cancer (Dombkowski et al., 2011). Thus, it is of particular interest to identify miRNAs that might interfere with β-catenin signaling and thereby lead to cancer invasion and metastasis.

MiRNA-301a has attracted much attention due to its important role in various biological and pathological processes, including development, differentiation, inflammation, apoptosis and cancer (Wa Lee et al., 2011; Wang et al., 2013; Xu et al., 2013). However, whether miR-301a is involved in the progression of breast cancer and the underlying mechanism remains poorly understood. In the present study, we showed that miR-301a was associated with breast cancer development and metastasis by directly targeting PTEN to activate the Wnt/β-catenin pathway, suggesting an oncogenic role of miR-301a in breast cancer.
Our findings suggested that the inhibition of miR-301a represents a promising therapeutic strategy for breast cancer treatment.

2. Materials and methods

2.1. Cell lines and patient samples

Breast cancer cell lines (MCF7, T47D, BT474, MDA-MB-231, MDA-MB-435, MDA-MB-468, and 4T1) were purchased from ATCC and maintained in DMEM supplemented with 10% FBS. NBECS were obtained from Clonetics. A total of 10 pairs of human breast cancer specimens were obtained from patients who underwent surgery at the second Affiliated Hospital, Harbin Medical University. The study was approved by the Ethics Review Committee of the Institutional Review Board of the hospital, and written informed consent was obtained from every patient.

2.2. Plasmid construction and luciferase assay

The ORFs of PTEN generated by PCR amplification were cloned into mammalian expression vector pcDNA 3.1 (Invitrogen; Life Technologies). A 293 bp fragment from the 3’ UTR of PTEN containing the miR-301a binding sites was amplified and cloned downstream to the luciferase gene in a modified pCI3 control vector. The miR-301a expression plasmid was cloned into pMSCV-puro (Clontech Laboratories Inc.) to generate plasmid pMSCV-miR-301a. pMSCV-miR-301a was cotransfected using the standard calcium phosphate transfection method. The reporter plasmids containing wild-type (CTTTTGGATC; TOP) or mutated (CTTTTCGCC; TOPflash) TCF/LEF DNA binding sites were purchased from Upstate Biotechnology. The sense strand sequences of siRNAs targeting both human and mouse cells, were as follows: siPTEN, 5′-GACGGCGCAGAUAAUAGCA-3′; siTCF4, 5′-AAGUCCGAGAAAGGAAUCUGA-3′; siLEF1, 5′-UCAGAGUICGACUCCAAAAC-3′; Indicated cells were cultured in 24-well plates, and transfected with 100 ng luciferase reporter plasmid and 5 ng pRL-TK vector expressing the Renilla luciferase (Promega, USA) using Lipofectamine 2000 reagent (Invitrogen). After 48 h, cells were harvested, lysed and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. Renilla luciferase was used for normalization. All assays were performed in triplicate and repeated at least three times.

2.3. Quantitative real-time PCR analysis

miRNAs were isolated from cells or tissues using a miRNeasy Mini Kit (Qiagen, USA). Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. The RT and PCR primers for miR-301a and U6 were purchased from Ribobio (Guangzhou, China). The levels of PTEN were examined using the forward primer, 5′-TGATTGACCTAGACGAGCT-3′; and the reverse primer, 5′-GGTCTCAATAACGCTTGAGAC-3′. Reverse transcription PCR was done using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). qRT-PCR was performed using SYBR Premix Ex Taq II (TaKaRa) and the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). U6 level was used as internal controls, the forward primer, 5′-CTGCGTTCGACGAGCACAT-3′; and the reverse primer, 5′-AGCCTTCAGAATTTCCTGCTGTC-3′. Fold changes were calculated using the 2 − ΔΔCt method. Each experiment was performed in triplicate.

2.4. Western blot analysis

Cell lysates were prepared in RIPA buffer and total protein content was quantified by the BCA assay (Pierce). The following primary antibodies were used: anti-β-catenin, anti-TCF4, and anti-PTEN (Santa Cruz, USA); Nuclear extracts were prepared using the Nuclear Extraction Kit (Active Motif), according to the manufacturer’s instructions.

2.5. Migration, invasion wound-healing assays

For transwell migration assays, 6 × 104 infected cells were plated into the top chamber of the insert (8 µm pore size; BD Bioscience, USA). For invasion assays, 2 × 105 cells were plated in the top chamber of the insert precoated with Matrigel (BD Bioscience). In both assays, cells were plated in medium without serum, and medium supplemented with 10% FBS was used as a chemoattractant in the lower chamber. The cells were incubated for 48 h and cells that migrated to the underside of the membrane were fixed with methanol and stained with crystal violet, imaged, and counted. Wound-healing assay was also done to assess cell migration. Cells were grown in complete medium to a confluent monolayer and were scraped by 200-µl pipette tips to create an artificial wound. Each experiment was performed in triplicate.

2.6. Animal experiments

For tumor growth assays, a total of 2 × 106 indicated cells were injected into the tail vein of BALB/c nude mice (6 mice per group). After 40 days, the animals were sacrificed and lung metastatic colonization was monitored and quantified. H&E staining was performed on sections from paraffin-embedded samples for histological evidence of the tumor phenotype. All experimental procedures involving animals were approved by Harbin Medical University Experimental Animal Care Commission.

2.7. Immunofluorescence staining

Cells were fixed with 4% formaldehyde, permeabilized with 0.5% Triton, and followed by incubating with an anti-β-catenin antibody overnight. After being washed, cells were incubated with Alexa Fluor 594-labeled secondary antibody (Invitrogen) for 45 min. Cells were counterstained with DAPI to label nuclei. The presence of β-catenin was visualized under an immunofluorescence microscope.

2.8. Statistical analysis

Data were expressed as the mean ± SD from at least three independent experiments. The Student’s t-test was used to compare the differences between groups. All statistical analyses were performed using SPSS 15.0 software. P < 0.05 was considered statistically significant.

2.9. Study approval

For the use of materials for research purposes, study approval was granted by the Harbin Medical University Institutional Animal Care and Use Committee.

3. Results

3.1. MiR-301a is significantly up-regulated in metastatic breast cancer cell lines and tissues

We first analyzed the expression level of miR-301a in a panel of breast cancer cell lines with different degrees of differentiation and metastatic ability. We observed that miR-301a was relatively higher in a series of tested human breast cancer cell lines as compared with that found in primary human normal breast epithelial cells (NBECS) and spontaneously immortal MCF10A cell line. Moreover, its expression was most pronounced in cell lines known to be highly metastatic (MDA-MB-231, MDA-MB-468, and MDA-MB-435) compared with that in non- or low-metastatic breast cancer cell lines (BT474, T47D, and MCF7) (Fig. 1A). By contrast, such a change was not observed in the expression of miR-1 or miR-21 (data not shown), suggesting the likelihood that miR-301a is associated with the development of breast cancer metastasis. To further understand the clinical relevance of the above
findings in human breast cancer, the expression of miR-301a was examined in 20 human breast cancer tissue specimens. As shown in Fig. 1B, the expression level of miR-301a was significantly elevated in the primary tumors of 10 patients with distant metastasis, as compared with that in primary tumors from 10 patients without detectable distant metastasis. The results of this analysis suggest a strong correlation between miR-301a and distant metastasis. Based on this expression pattern, we therefore chose MCF7, and MDA-MB-231 cells for the following gain-of-function and loss-of-function studies, respectively.

3.2. MiR-301a promotes the pro-metastatic phenotype of breast cancer

To understand the biological effect of miR-301a deregulation on the invasiveness of breast cancer cells, in vitro gain-of-function analyses were performed through the retrovirally stable expression of miR-301a in MCF-7 cell line. Matrigel-coated (for invasion) or -uncoated (for migration) Transwell assay showed that miR-301a overexpression markedly promoted the invasion and migration of the MCF7 cells (Fig. 2A). In addition, wound healing assay showed that miR-301a
overexpression increased the migration of MCF7 cells compared with that of the vector control cells (Fig. 2B).

To further probe the pro-invasive role of miR-301a in breast cancer, we examined the effect of inhibiting miR-301a on the phenotype of a highly metastatic MDA-MB-231 breast cancer cell line that expresses miR-301a at an elevated level. As expected, inhibition of miR-301a drastically decreases the invasive capabilities of MDA-MB-231 cells (Figs. 2A and B). Collectively, our data suggest that miR-301a greatly contributes to the development of breast cancer metastasis and invasion.

Next we asked whether miR-301a could promote metastasis in vivo. The MCF7/miR-301a cells and their control cells were injected into the tail vein of nude mice. Strikingly, mice bearing MCF7/miR-301a tumors displayed prominent lung metastasis, whereas no visible metastasis was observed in mice transplanted with control MCF7 cells (Figs. 2C and D). To further validate whether miR-301a is required for the observed enhanced metastasis in vivo, antagonir-301a was applied to inhibit endogenous expression of miR-301a. As shown in Fig. 2C, the ability of MDA-MB-231 cells to cause lung metastasis was markedly

Fig. 3. Wnt/β-catenin signaling mediates the effects of miR-301a. (A) Altered nuclear translocation of β-catenin induced by miR-301a expression. Nuclear fractions of indicated cells were analyzed by Western blot. Lamin B1 was used as a loading control. (B) Subcellular β-catenin localization in indicated cells was examined by immunofluorescence staining. (C) Indicated cells were transfected with TOPflash or FOPflash and Renilla pRL-TK plasmids, and subjected to dual-luciferase assays 48 h after transfection. Reporter activity was normalized by Renilla luciferase activity. (D) Depletion of TCF4 or LEF1 with specific siRNA in indicated cells confirmed by Western blot analysis (right panel); quantification of invading cells in a Matrigel-coated Transwell assay (left panel), and luciferase-reported TCF/LEF transcriptional activity in indicated cells (middle panel). (E) Overexpression of TCF4 or LEF1 in miR-301a-silenced MDA-MB-231 cells confirmed by Western blot analysis (right panel); quantification of invading cells impacted by overexpression of TCF4 or LEF1 in Transwell assay (left panel), and luciferase-reported TCF/LEF transcriptional activity in indicated cells (middle panel). *P < 0.05, **P < 0.01.
impaired by intraperitoneal administration of antagonir-301a. Histological analyses further revealed that miR-301a could promote metastasis of breast cancer cell lines (Fig. 2C). Together, these data indicate that miR-301a plays a critical role in breast cancer metastasis in vivo.

3.3. miR-301a activates Wnt/β-catenin pathway which mediated miR-301a-induced metastasis

Given the critical role of the Wnt/β-catenin pathway in tumorigenesis and metastasis, we probed whether miR-301a activates Wnt/β-catenin signaling. Subcellular fractionation and immunofluorescence staining assays indicated that overexpression of miR-301a resulted in nuclear accumulation of β-catenin in MCF7 cells, suggesting that miR-301a might activate Wnt/β-catenin signaling (Figs. 3A and B). Furthermore, miR-301a overexpression markedly increased the activity of β-catenin in MCF7 cells, as determined by β-catenin reporter assay (Fig. 3C). However, miR-301a inhibitor decreased the nuclear translocation of β-catenin and subsequent TCF/LEF activities in MDA-MB-231 cells (Figs. 3B and C). These data suggest that miR-301a overexpression can enhance β-catenin nuclear translocation and promote the transcriptional activity of TCF/LEF.

To further validate the role of β-catenin activation in miR-301a-induced cell invasion, we knocked down TCF4 or LEF1 in miR-301a-transduced MCF7 cells (Fig. 3D). As shown in Fig. 3D, inhibition of β-catenin signaling not only reduced TCF/LEF transcriptional activity, but also abrogated miR-301a-induced invasiveness. On the other hand, activation of β-catenin signaling by ectopically expressing TCF4 or LEF1 in miR-301a-inhibited MDA-MB-231 cells (Fig. 3E) mimicked the impact of miR-301a-induced cell invasion and TCF/LEF transcriptional activity (Fig. 3E). Taken together, these data indicate that Wnt/β-catenin signaling is a functional mediator for miR-301a-induced proliferation and metastasis in the breast cancer cell lines.

3.4. MiR-301a activates β-catenin signaling via directly targeting PTEN

To elucidate the underlying mechanisms by which miR-301a activates β-catenin signaling, we explored miR-340 targets using the TargetScan (http://www.targetscan.org) and miRanda (http://www.microrna.org) bioinformatics algorithms. Our analysis revealed that Wnt/β-catenin signaling was mainly enriched by predicted target of miR-301a. Among the predicted targets, PTEN was specifically noted as a negative regulator of β-catenin signaling. To validate this, we performed luciferase reporter assays using pGL3-PTEN-3′-UTR reporters cotransfected with increasing amounts (10, 20, and 50 nM) of miR-301a mimic and mutant oligonucleotides in MCF7 cell lines, or with increasing amounts (10, 20, and 50 nM) of miR-301a inhibitor oligonucleotides. Western blot analysis confirmed the transfection of PTEN-targeting siRNAs in MCF7 cells (left panel); quantification of invading MCF7 cells transfected with indicated siRNAs (middle panel); and luciferase assay of TCF/LEF transcriptional activity in indicated cells transfected with specific siRNA (left panel). *P < 0.05, **P < 0.01.
of Wnt/\(\beta\)-catenin signaling. Bioinformatics tools were used to predict that PTEN was a putatively potential target of miR-301a based on target sequences at the PTEN 3′UTR (Fig. 4A). Western blotting analysis indicated that the expression level of PTEN was reduced in miR-301a-overexpressing cells, whereas miR-301a inhibition elevated the level of PTEN (Fig. 4B). Similar changes were found at the mRNA level (Fig. 4C). Furthermore, reporter assays showed that the activity of luciferase linked with the 3′ UTR of PTEN was repressed in a dose-dependent manner in miR-301a mimic-transfected MCF7 cells, compared with those in control cells (Fig. 4D). Conversely, inhibition of miR-301a caused a marked increase in luciferase reporter activities under the control of the 3′ UTR of PTEN (Fig. 4D). Notably, mutations brought into the seed sequence of miR-301a abolished its suppressive effects (Fig. 4D). Together, our results indicate that miR-301a directly decreases PTEN expression in breast cancer cell lines.

To explore the functional significance of PTEN in the invasive capability of breast cancer cell lines and \(\beta\)-catenin activation induced by miR-301a, we investigated the impact of its depletion via siRNAs. As shown in Fig. 4E, silencing PTEN in MCF7 cells led to increased cell invasiveness and the TCF/LEF transcriptional activity. However, reintroduction of PTEN in miR-301a-transduced MCF7 cells at least partly abrogated miR-301a-induced cell invasion and TCF/LEF transcriptional activity (Fig. 4F). In addition, depletion of PTEN dramatically increased the invasiveness and TCF/LEF transcriptional activity of miR-301a-inhibited MDA-MB-231 cells (Fig. 4G). These results demonstrate that PTEN is functionally important for miR-301a-induced cell motility and invasiveness in breast cancer cell lines.

4. Discussion

The unique characteristic that differentiates miRNAs from siRNAs is the ability to bind to imperfectly matched target mRNAs and inhibit translation (Boutz et al., 2007). Thus, a single miRNA is able to silence many target genes that are involved in the same functional event (Wang, 2011). Several miRNAs are known to drive tumorigenesis at the initiation stage and to induce invasive activity at the metastatic stage by regulation of different targets, as with miR-21, miR-374a and miR-146a in the development of some breast cancers (Cai et al., 2013; Han et al., 2012; Pastrello et al., 2010). In this report, we found that miR-301a was overexpressed and acted as an oncogenic miRNA that promoted tumour cellular invasiveness and metastasis in breast cancer, enforcing its oncogenic function via inactivating PTEN, consequently activating Wnt/\(\beta\)-catenin pathway.

Previous evidence has demonstrated that miR-301a is overexpressed in gastric, liver cancers and mesothelioma, which strongly suggests a general role in cancers (Guled et al., 2009; Wang et al., 2013; Zhou et al., 2012). The ability of miR-301a to target multiple cancer suppressors indicates an indiscriminate ability to promote tumour progression and metastasis (Chen et al., 2012). Other researchers have found that miR-301a may also contribute to inflammation development in pancreatic cancer, implying that it has a central role in regulating several signaling pathways, thereby confirming its participation in multifarious processes during cancer development (Ma et al., 2011).

A reduced expression of PTEN has been reported to correlate with poor clinical outcomes in breast, ovarian and liver cancers (Mester & Eng, 2013), and the tumour suppressor function of PTEN has been reported to mainly focus on reducing cells growth (Mester & Eng, 2013). However, in the current study, we indicated a function of PTEN in suppressing the migratory and invasive activity of tumour cells in breast cancer. Consistent with the finding that miR-301a could modulate breast cancer cells invasion, our results further support that miR-301a may control cancer metastasis through downregulating PTEN in breast cancer cells.

Several signaling pathways, including the Wnt/\(\beta\)-catenin, Notch, and Hedgehog pathways, have been found to be aberrantly activated and play important roles in the progression of breast cancer (O’Toole et al., 2013). However, unlike in other tumour types, the mutations in \(\beta\)-catenin are rare, but the expression and nuclear localization of \(\beta\)-catenin are often abnormal in breast cancer (Benhaij et al., 2006). Thus, identifying regulators of \(\beta\)-catenin signaling may provide new insights into the molecular mechanisms underlying breast cancer invasion and metastasis, and facilitate the discovery of novel anti-metastatic targets. Our current data show that downregulation of PTEN mediates the effects of miR-301a on \(\beta\)-catenin activation. Moreover, it has been well characterized that the PTEN/Akt axis is involved in tumor metastasis via \(\beta\)-catenin signaling (Damsky et al., 2011). Our finding strongly supports the notion that the miRNA represents a potent activator of the pathway and subsequent tumor metastasis. While it remains to be clarified whether other mediating signals may also contribute to the pro-metastatic effect of miR-301a, our data provide a biological basis for the potential use of miR-301a as an anti-metastatic target in breast cancer.

In summary, our studies have found that expression of miR-301a was markedly elevated in breast cancer. Functional studies indicated that the modulation of miR-301a promoted breast cancer invasion and metastasis via downregulation of the tumour suppressor PTEN and activation of the Wnt/\(\beta\)-catenin pathway, which suggests that a miR-301a antagonist may have a therapeutic potential for breast cancer treatment.

Conflict of interest

The authors declare no conflict interest.

References