MicroRNA-106a-5p functions as an oncogene via regulating PTEN in breast cancer cells

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Abstract: Objective: Breast cancer remains the most common cancer in women, worldwide. It has been shown that microRNAs play essential roles in tumorigenesis and progression in various cancers, including breast cancer. The current study assessed the roles of miR-106a-5p in proliferation, chemotherapy sensitivity, migration, and invasion of breast cancer cells. Methods: MTT assays were performed to examine the proliferation of MCF-7 and MDA-MB-231 breast cancer cells with altered expression of miR-106a-5p, with or without 5-fluorouracil treatment. Transwell migration and Matrigel invasion assays were used to assess migration and invasion of MCF-7 and MDA-MB-231 breast cancer cells. Western blotting was performed to examine protein expression levels. Caspase-Glo3/7 assays were used to examine the effects of miR-106a-5p on 5-fluorouracil-induced apoptosis in MCF-7 and MDA-MB-231 breast cancer cells. Results: Forced expression of miR-106a-5p improved proliferation, migration, and invasion of breast cancer cells. Moreover, miR-106a-5p decreased the sensitivity of MCF-7 and MDA-MB-231 breast cancer cells after 5-fluorouracil treatment. Additionally, 5-fluorouracil-induced apoptosis in breast cancer cells was reduced by miR-106a-5p by regulation of BAX and Bcl-2 expression. Furthermore, miR-106a-5p regulated epithelial to mesenchymal transition (EMT) by altering expression of E-cadherin, N-cadherin, Snail, and VIMENTIN IN BOTH MCF-7 and MDA-MB-231 breast cancer cells. Further examination showed that expression levels of PTEN and p-AKT in MCF-7 and MDA-MB-231 breast cancer cells were changed after aberrant expression of miR-106a-5p. Conclusion: miR-106a-5p plays important roles in tumorigenesis and progression in breast cancer cells. It might act as a potential biomarker, predicting chemotherapy response and prognosis in breast cancer.

Keywords: miR-106a-5p, breast cancer, apoptosis, AKT

Introduction

Breast cancer remains the most common cancer in women, worldwide [1]. Although mortality rates of breast cancer have decreased dramatically in the past three decades, due to the increased screening and improved treatment, breast cancer is the second most common cause of cancer-related deaths in women. It has been estimated that there will be 266,120 new cases of invasive breast cancer, 63,960 new cases of breast carcinoma in situ, and 40,920 breast cancer deaths in 2018 [2]. Chemotherapy plays important roles in breast cancer treatment. Thus, 5-fluorouracil is one of the most common chemotherapy reagents used for adjuvant and neoadjuvant treatment. It has been shown that 5-fluorouracil inhibits proliferation of cancer cells by interfering with the normal metabolic processes of cells [3]. Recently, resistance to 5-FU-based treatments and recurrence have been reported in patients with breast cancer, involving multiple singling pathways, including apoptosis, cell proliferation, and DNA damage repair. Therefore, it is important to understand the molecular mechanisms of chemoresistance in the treatment of breast cancer, discovering more effective therapeutic strategies for breast cancer therapy [4-6].

MicroRNAs (miRNAs) are a group of endogenous and small non-coding RNA molecules of about 18-25 nucleotides. MicroRNAs take part in multiple biological processes by binding to the 3'-untranslated region (3'-UTR) of target mRNAs. They play important roles in post-transcriptional regulation of gene expression [7]. More than 2,000 miRNAs have been identified in humans [8]. A single gene can be regulated by many different miRNAs and a single miRNA
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can target different genes [9]. Aberrant miRNA expression has been reported in a variety of human neoplasms, including breast cancer tissues [10]. Recent studies have shown that the expression profile of particular miRNAs is associated with tumor progression, response to therapy, and prognosis in breast cancer [11]. Expression levels of some miRNAs are upregulated in breast cancer. For example, expression levels of miR-21 are increased in many species of cancer, including breast cancer [12]. Further studies have demonstrated that miR-21 is associated with cancer cell proliferation, metastasis, resistance to chemo- or radio-therapy, and poor prognosis [13-15]. Expression of other miRNAs is downregulated in breast cancer. For instance, it has been found that miR-205 is significantly downregulated in breast tumor tissues. Aberrant miR-205 expression can influence proliferation, metastasis, and invasion of breast cancer cells by targeting HER3 and VEGF [16-18].

The current study examined the function of miR-106a-5p in proliferation, chemotherapy sensitivity, invasion, and migration of breast cancer cells. Results indicate that miR-106a-5p could act as a biomarker and novel therapy target in breast cancer.

Materials and methods

Cell lines

Human breast cancer cell lines MDA-MB-231 and MCF7 were obtained from the American Type Culture Collection (ATCC, USA). They were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 units of penicillin/mL, and 100 mg of streptomycin/ml (Invitrogen, USA). Human breast cancer cells were grown at 37°C in a 5% CO₂ atmosphere.

Cell transfection

Breast cancer cells were seeded in 24-well plates. Transfection was performed using Lipofectamine 2000 (Invitrogen, USA), according to manufacturer protocol. miR-106a-5p and its negative control (mimic NC), as well as miR-106a-5p inhibitors and its negative control (inhibitor NC) (Invitrogen, USA), were transfected into breast cancer cells. These cells were used for further studies.

RNA extraction and qRT-PCR

Total RNA was extracted from transfected MDA-MB-231 and MCF7 breast cancer cells using Trizol Reagent (Invitrogen, USA). Next, 1 μg of total RNA was used to synthesize the complementary DNA templates using a Reverse Transcription Kit (Qiagen, USA), according to manufacturer instructions. Expression levels of miR-106a-5p were measured by real-time quantitative PCR. U6 was used as the endogenous control. RT-PCR conditions were as follows: 95°C for 3 minutes, 36 cycles at 95°C for 15 seconds, 60°C for 55 seconds, and 72°C for 30 seconds, with a dissociation stage. Sequences of primers were as follows: miR-106a-5p forward: 5’-GCCTGAGGGGCAGAGAGC-3’ and reverse 5’-CCACGTGTCGTGGAGTC-3’. All independent experiments were repeated three times in triplicate.

Cell viability assays

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Roche, USA) was performed to detect cell viability. Briefly, transfected MCF-7 cells and MDA-MB-231 cells were placed in 96-well plates at density of 5 × 10³ cells/well and grown in a 100 µl/well culture medium. After 24 hours of culturing at 37°C in a humidified incubator, the medium was replaced with a culture medium containing different concentrations of 5-flurouracil (0, 20, 40, 60 μM). To determine the combination of hyperbaric oxygen (HBO2) and 5-flurouracil-induced inhibitory effects of miR-106a-5p on proliferation of breast cancer cells, cells were incubated at 37°C in a hyperbaric chamber containing 95% O₂ and 5% CO₂ at 1 atmosphere absolute for 90 minutes. Every 48 hours, 20 µl MTT solution was added to the medium and incubated with the cells for 4 hours. The supernatant was then removed and DMSO was added to dissolve the crystal with gentle agitation. Absorbance values were determined at 570 nm using a microplate reader (BioTek, Germany). All independent experiments were repeated three times in triplicate.

Apoptosis assays

Caspase 3/7-activity assays were conducted to determine the apoptosis activity of breast cancer cells with or without 5-flurouracil treatment, using a Caspase-Glo assay kit (Promega, USA).
Briefly, transfected breast cancer cells (2 × 10⁵/well) were seeded in 24-well plates and cultured overnight at 37°C in a humidified incubator. Breast cancer cells were then treated with different concentrations of 5-flourouracil (0, 20, 40, 60 μM) for 48 hours in DMEM culture medium. Next, 100 μl of Caspase-Glo reagent was added to each well and the cells were incubated at room temperature for 2 hours with gentle agitation. Luminescence was detected with a plate-reading luminometer (Thermo Labsystems, USA) with parameters of 1-minute lag time and 0.5 second/well read time. All independent experiments were repeated three times in triplicate.

Cell migration and invasion assays

To examine the effects of miR-106a-5p on migration and invasion of breast cancer cells, Transwell assays were performed in 24-well chambers pre-coated without (migration) or with (invasion) Matrigel (BD Bioscience, USA). Briefly, breast cancer cells were suspended in serum free DMEM. Afterward, 200 μl of cell suspension was added into the upper chamber at a density of 5 × 10⁴/well and 500 μl of DMEM with 10% FBS was added into the lower chamber. Breast cancer cells were cultured in a 5% CO₂ humidified incubator at 37°C for 18 hours. Non-invading breast cancer cells were removed gently. Invaded cells were stained and counted under a light microscope at five random fields. All independent experiments were repeated three times in triplicate.

Western blot analysis

Transfected breast cancer cells were lysed with RIPA buffer (Abcam, USA). Protein concentrations were measured using a BCA kit (ThermoFisher Scientific, USA), according to manufacturer protocol. A total of 30 μg of protein was separated by SDS-PAGE in a 10% gel, then transferred to PVDF membranes (Sigma, USA). The membranes were incubated with non-fat powdered milk in PBS for one hour at room temperature, with gentle agitation. Primary antibodies were added and incubated with the membranes overnight at 4°C, with gentle agitation. The membranes were then incubated with a secondary antibody for 1 hour at room temperature, with gentle agitation. Enhanced chemiluminescence reagent was used to develop the signal. GAPDH was used as the loading control.

Statistical analysis

Statistical calculations were conducted using SPSS 18.0 statistical software (IBM Corp, USA). Differences were analyzed using one-way analysis of variance (Tukey, ANOVA). P values <0.05 indicate statistical significance.

Results

miR-106a-5p improved proliferation of breast cancer cells and induced 5-flourouracil resistance in a dose-dependent manner

To examine the effects of miR-106a-5p on proliferation of breast cancer cells with or without 5-flourouracil treatment, miR-106a-5p mimics or miR-106a-5p inhibitors were transfected to MCF-7 and MDA-MB-231 breast cancer cells using Lipofectamine 2000. Proliferation of breast cancer cells was examined using MTT assays. Scrambled negative control RNAs were used as negative control. As shown in Figures 1A and 3A, miR-106a-5p expression levels were significantly increased in both MCF-7 and MDA-MB-231 breast cancer cells after transfection with miR-106a-5p mimics. Overexpression of miR-106a-5p promoted proliferation of MCF-7 (Figure 1B) and MDA-MB-231 cells (Figure 3B), compared with breast cancer cells transfected with negative control scrambled RNAs (p<0.05). Additionally, survival rates of MCF-7 (Figure 1C-E) and MDA-MB-231 cells (Figure 3C-E) were increased after overexpression of miR-106a-5p at presence of 5-flourouracil, compared to the negative control group (p<0.05). Interestingly, miR-106a-5p also improved the proliferation of MCF-7 (Figure 1F) and MDA-MB-231 cells (Figure 3F) when the breast cancer cells were cultured in a hyperbaric chamber.

Moreover, miR-106a-5p expression levels were significantly decreased in both MCF-7 (Figure 2A) and MDA-MB-231 (Figure 4A) cells after transfection with miR-106a-5p inhibitors. Downregulation of miR-106a-5p decreased proliferation of MCF-7 (Figure 2B) and MDA-MB-231 (Figure 4B), compared with breast cancer cells transfected with negative control scrambled RNAs. Furthermore, survival rates of MCF-7 (Figure 2C-E) and MDA-MB-231 (Figure 4C-E) were decreased after downregulation of miR-106a-5p in the presence of 5-flourouracil, compared to the negative control group (P<0.05). Additionally, miR-106a-5p inhibited the prolif-
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miR-106a-5p inhibited 5-flourouracil-induced apoptosis of breast cancer cells

Assessing the effects of miR-106a-5p on 5-flourouracil-induced apoptosis, transfected breast cancer cells were cultured with different doses of 5-flourouracil. The 5-flourouracil-induced apoptosis was analyzed by caspase 3/7 activity. Up-regulation of miR-106a-5p inhibited 5-flourouracil-induced apoptosis by decreasing the caspase 3/7 activities in MCF-7 cells (Figure 5A) and MDA-MB-231 cells (Figure 5B). Western blotting was used to further evaluate expression of apoptotic related proteins of transfected breast cancer cells in the presence of 5-flourouracil. As shown in Figure 5C, 5D, overex-
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Expression of miR-106a-5p decreased BAX expression, while Bcl-2 expression was increased in both MCF-7 cells and MDA-MB-231 cells.

miR-106a-5p altered migration and invasion of breast cancer cells

Evaluating the function of miR-106a-5p on migration and invasion of breast cancer cells, MCF-7 and MDA-MB-231 breast cancer cells were seeded in chambers with or without Matrigel. Migration and invasion were examined using Transwell migration and invasion assays. Upregulation of miR-106a-5p enhanced migration and invasion of MCF-7 cells (Figure 6A, 6B) and MDA-MB-231 cells (Figure 6E, 6F). In contrast, downregulation of miR-106a-5p decreased migration and invasion of MCF-7 cells (Figure 6C, 6D) and MDA-MB-231 cells (Figure 6G, 6H).

Figure 2. Downregulation of miR-106a-5p decreased proliferation and survival of MCF-7 cells. A. Expression levels of miR-106a-5p in MCF-7 breast cancer cells after transfection with miR-106a-5p inhibitors; B. Proliferation of MCF-7 cells after downregulation of miR-106a-5p; C-E. Proliferation of MCF-7 cells transfected with miR-106a-5p inhibitors in the presence of different doses of 5-fluorouracil (20, 40, and 60 μM); F. Proliferation of MCF-7 cells transfected with miR-106a-5p inhibitors when the cells were cultured in HBO.
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miR-106a-5p altered expression of EMT markers and activated AKT pathways in breast cancer cells

To further examine the roles of miR-106a-5p in cell proliferation, migration, and invasion in breast cancer cells, multiple signaling pathways were analyzed via Western blotting. E-cadherin expression was decreased and expression levels of N-cadherin, Snail, and VIMENTIN were significantly increased in both MCF-7 cells and MDA-MB-231 cells after upregulation of miR-106a-5p (Figure 7A-C). However, downregulation of miR-106a-5p increased expression levels of E-cadherin and decreased expression levels of N-cadherin, Snail, and VIMENTIN in both MCF-7 cells and MDA-MB-231 breast cancer cells (Figure 8A-C). Interestingly, miR-106a-5p inhibited PTEN expression and increased p-AKT expression in MCF-7 cells and MDA-MB-231 breast cancer cells (Figure 7D-F). Moreover, downregulation of miR-106a-5p increas-
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Growing evidence has suggested that miRNAs play important roles in tumor development and progression [19]. Recent studies have demonstrated that miR-106a-5p can act as either an oncogene or tumor suppressor gene, regulating multiple biological processes in different types of cancer. Pan et al. reported that miR-106a-5p inhibited migration and invasion of renal cell carcinoma by targeting PAX5 both in vitro and in vivo [20]. Further studies have shown that miR-106a-5p decreased the proliferation and migration of astrocytoma cells and improved apoptosis by directly targeting PASTK [21]. Zhi et al. showed that expression levels of miR-106a-5p were significantly increased in astrocytoma patients and the upregulation of

![Figure 4](https://example.com/figure4.png)

Figure 4. Downregulation of miR-106a-5p decreased proliferation and survival of MDA-MB-231 cells. A. Expression levels of miR-106a-5p in MDA-MB-231 breast cancer cells after transfection with miR-106a-5p inhibitors; B. Proliferation of MDA-MB-231 cells after downregulation of miR-106a-5p; C-E. Proliferation of MDA-MB-231 cells transfected with miR-106a-5p inhibitors in the presence of different doses of 5-fluorouracil (20, 40, and 60 μM); F. Proliferation of MDA-MB-231 cells transfected with miR-106a-5p inhibitors when the cells were cultured in HBO.
miR-106a-5p was associated with poor outcomes [22]. In addition, Li et al. found that expression levels of miR-106-5p were significantly increased in breast cancer patients, compared to healthy controls, in both plasma and serum [23]. In the current study, upregulation of miR-106a-5p improved the proliferation of breast cancer cells and downregulation of miR-106a-5p inhibited proliferation of breast cancer cells. These findings indicate that miR-106a-5p plays an essential role in proliferation of breast cancer cells. Moreover, 5-fluorouracil is one of the most effective chemotherapy agents. It has been used to treat different types of cancers, including colon cancer, pancreatic cancer, and breast cancer. In recent decades, 5-fluorouracil alone or combination with other chemotherapy agents has significantly improved survival rates, both in patients with metastatic breast cancer and in those that have undergone surgery. However, recurrence, due to chemoresistance during treatment, is a major problem in breast cancer patients with 5-fluorouracil-based chemotherapy. Resistance to 5-fluorouracil-based chemotherapy involves many signaling pathways, such as apoptotic pathways, cell proliferation, and DNA damage repair. Great effort has been made to increase the sensitivity of 5-fluorouracil. Kurokawa et al. reported that miR-19b is upregulated in colon cancer cells in response to 5-fluorouracil-based chemotherapy [24]. Further studies have shown that miR-320a might regulate 5-fluorouracil resistance by targeting PDCD4. Zhang et al. demonstrated that miR-587 inhibits 5-fluorouracil-induced apoptosis in colorectal cancer by regulating PPP2R1B [25]. In the current study, upregulation of miR-106a-5p enhanced survival of breast cancer cells after 5-fluorouracil treatment. Downregulation of miR-106a-5p inhibited survival of breast cancer cells after 5-fluorouracil treatment. Current results indicate that miR-106a-5p induces 5-fluorouracil resistance of breast cancer cells. Further studies have indicated that expression levels of miR-106a-5p were associated with the 5-fluorouracil induced apoptosis in both MCF-7 and MDA-MB-231 breast cancer cells. These results sug-
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Figure 6. Effects of miR-106a-5p on migration and invasion of MCF-7 cells and MDA-MB-231 breast cancer cells. A. Upregulation of miR-106a-5p increased migration of MCF-7 breast cancer cells; B. Upregulation of miR-106a-5p increased invasion of MCF-7 breast cancer cells; C. Downregulation of miR-106a-5p decreased migration of MCF-7 breast cancer cells; D. Downregulation of miR-106a-5p decreased invasion of MCF-7 cells; E. Upregulation of miR-106a-5p increased migration of MDA-MB-231 breast cancer cells; F. Upregulation of miR-106a-5p increased invasion of MDA-MB-231 cells; G. Downregulation of miR-106a-5p decreased migration of MDA-MB-231 breast cancer cells; H. Downregulation of miR-106a-5p decreased invasion of MDA-MB-231 cells.

Figure 7. miR-106a-5p altered EMT protein expression and activated AKT expression in breast cancer cells. (A) Expression of EMT proteins in MCF-7 cells and MDA-MB-231 breast cancer cells after transfection with miR-106a-5p mimics. Relative expression levels of EMT proteins on MCF-7 cells (B) and MDA-MB-231 cells (C) after transfection with miR-106a-5p mimic by densitometric analysis; (D) Expression levels of p-AKT, AKT, and PTEN in MCF-7 breast cancer cells and MDA-MB-231 breast cancer cells after transfection with miR-106a-5p mimics. Relative expression levels of proteins on MCF-7 cells (E) and MDA-MB-231 cells (F) after transfection with miR-106a-5p mimics by densitometric analysis.
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suggest that miR-106a-5p might affect chemosensitivity of breast cancer cells to 5-fluorouracil therapy.

Epithelial–mesenchymal transition (EMT) is a biological process in which epithelial cells gain mesenchymal features by multiple signaling pathway involvement. It has been shown that EMT plays important roles in progression, invasion, and metastasis of cancer cells, including breast cancer. According to analysis of circulating tumor cells (CTCs) from breast cancer patients, Yu et al. found that both single cell and cluster of CTCs can undergo mesenchymal transformation in the bloodstream [26]. Additional studies have indicated that CTCs from

Figure 8. Downregulation of miR-106a-5p altered EMT protein expression and inhibited AKT expression in breast cancer cells. (A) Expression of EMT proteins in MCF-7 cells and MDA-MB-231 breast cancer cells after transfection with miR-106a-5p inhibitors. Relative expression levels of EMT proteins on MCF-7 cells (B) and MDA-MB-231 cells (C) after transfection with miR-106a-5p inhibitor by densitometric analysis; (D) Expression levels of p-AKT, AKT, and PTEN in MCF-7 breast cancer cells and MDA-MB-231 breast cancer cells after transfection with miR-106a-5p inhibitors. Relative expression levels of proteins on MCF-7 cells (E) and MDA-MB-231 cells (F) after transfection with miR-106a-5p inhibitors by densitometric analysis.
advanced breast cancer patients expressed both epithelial and mesenchymal markers [27]. Other studies have provided evidence that EMT contributes to resistance to chemotherapy in breast cancer by increasing multidrug resistance protein 1 (MDR1) expression [28, 29]. More studies have demonstrated that aberrant expression of miRNAs regulates cancer invasion and metastasis by induction of EMT program in breast cancer. For example, overexpression of miR-373 and miR-520c increased invasion and migration of breast cancer cells by inhibiting CD44 expression [30]. The current study found that miR-106a-5p played critical roles in invasion and migration of breast cancer cells by regulation of EMT. These findings suggest that expression levels of miR-106a-5p are associated with breast cancer progression. Other results have suggested that miR-106a-5p changed expression levels of Phosphatase and Tensin homolog (PTEN) and p-AKT in breast cancer cells. PTEN is a well-known tumor suppressor that negatively regulates PI2K/AKT signaling. Lack of expression of PTEN improves cancer cell survival, while decreasing proliferation and apoptosis in different cancers. PTEN loss has been associated with poor outcomes in breast cancer patients [31]. Recent studies have provided evidence that miRNAs can regulate expression of PTEN in breast cancer. For instance, Chai et al. reported that miRNA-498 inhibited PTEN expression levels in triple negative breast cancer cells by directly targeting the 3’ untranslated region of PTEN mRNA [32]. Up-regulation of miR-106b and miR-93 decreased expression levels of PTEN in breast cancer cells [33]. These results suggest that miR-106a-5p might regulate proliferation and chemosensitivity of breast cancer cells by targeting AKT signaling pathways.

**Conclusion**

miR-106a-5p plays important roles in tumorigenesis and disease progression of breast cancer. Present results indicate that miR-106a-5p could act as a potential biomarker, predicting chemosensitivity and prognosis in breast cancer.

**Disclosure of conflict of interest**

None.

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**References**

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