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ORIGINAL ARTICLE

MicroRNA-1 regulates the growth and chemosensitivity of breast cancer cells by targeting MEK/ERK pathway

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Summary

Purpose: *The microRNAs (miRs) control a vast number of biological and cellular processes. miR-1 has been implicated in the development and progression in different types of cancers. Nonetheless, the function and therapeutic implications of miR-1 have not been studied in breast cancer. This study was undertaken to study the role of miR-1 in human breast cancer cells.*

Methods: *MBA-MD-231 breast cancer line and the normal MB-157 cell line were mainly used in this research. Expression analysis was performed by qRT-PCR. Cell viability was determined by MTT assay and apoptosis was detected by acridine orange (AO)/ethidium bromide (EB) and DAPI staining. Transwell assay was used for cell migration and invasion and western blot analysis was used to determine the protein expression.*

Results: *miR-1 was significantly but aberrantly suppressed in breast cancer cells relative to the MB-157 normal cells. Overexpression of miR-1 in MBA-MD-231 suppressed their proliferation dose-dependently. The inhibition of MBA-MD-231 cell proliferation was found to be due to induction of apoptosis. The apoptotic cell percentage was 37.1% in miR-1 mimics transfected in comparison to 3.7% in miR-negative control (NC) transfected cells. Additionally, miR-1 also suppressed the chemosensitivity of the MBA-MD-1 breast cancer cells to cisplatin. Transwell assay showed that miR-1 overexpression suppressed the migration and invasion of the MBA-MD-231 cells. The results clearly showed that overexpression of miR-1 suppressed the phosphorylation of MEK and ERK.*

Conclusion: *miR-1 acts as a tumor suppressor and may exhibit therapeutic implications in the treatment of breast cancer.*

Key words: *microRNA, apoptosis, cell viability, metastasis, chemosensitivity*

Introduction

The microRNAs (miRs) include a large group of endogenous RNA molecules of around 19 nucleotides that are not translated into proteins. The function of these miRs is to control the expression of genes post-transcriptionally [1]. Studies have shown that the processes such as proliferation, cell death, autophagy, necrosis, differentiation and others are regulated by miRs [2]. Additionally, it has been revealed that most of the miRs are dysregulated in cancer tissues and hence might play a role in the development of cancers in humans [3]. miR-1 has been shown to act as tumor suppressor in different cancers. For instance, the proliferation of esophageal cancer is inhibited by miR-1 via post-transcriptional inhibition of downregulation cyclin D1, MET, as well as CDK4 [4]. MiR-1 has also been shown to suppress the growth of breast cancer cells and to be a prospective biomarker for the detection and progression of breast cancer [5]. The high expression of miR-1 has been shown to be linked with poor hepatocellular cancer prognosis [6]. Over the years, globally breast cancer incidence has increased substantially and has become one serious health issues in women [7]. Across the globe, more

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than 1.3 billion breast cancer cases and approximately half million deaths are attributed to breast cancer each year. In China alone, more than 0.45 million new breast cancer cases and 0.13 million deaths were reported to be due to breast cancer [8]. The heterogeneous nature, diagnosis at advanced stage, unreliable molecular markers and inefficient treatment regimens makes it very difficult to manage this disease [9]. This study was designed to investigate the function and evaluate the therapeutic potential of miR-1 in breast cancer hoping that will pave the way for the exploration of miR-1 in the treatment of breast cancer and deserves further research studies

Methods

Cell viability assessment

Cells were seeded in 96-well plates (3000 cells/well) and exposed to miR-NC or miR-1 mimics at particular concentrations and the cell viability was evaluated by the MTT kit (Roche Diagnosis, Indianapolis, IND, USA).

Expression analysis

The transfected MBA-MD-231 breast cancer cells were lysed and the RNA was extracted by RNeasy Kit. Following RNA extraction, the Omniscript RT kit was used to synthesize the cDNA from 1 µg of RNA. Thereafter, qRT-PCR was employed to determine the expression using the Taq PCR Master Mix Kit (Qiagen, Hilden, Germany) as per the guidelines of the manufacturer.

Apoptosis assay

To unveil any evidence of apoptosis induction in transfected MBA-MD-231 cells, around 6 µL of the transfected MBA-MD-231 cells were treated with 2.5 µL of AO/EB or DAPI solution. The cells were then instantly assessed by fluorescent microscopy and photographs of the MBA-MD cells were taken by Olympus BX41 fluorescence microscope. The annexin V-FITC/PI assay was done as per the methodology described previously [10].

Transwell assay

Transwell migration and invasionsystem was utilised for evaluation of the metastatic capabilities of transfected MBA-MD-231 cells. In case of invasion, the bottom chamber was covered with Matrigel and then incubation was carried at 37°C for 25 min. Approximately 2.5×10^4 MBA-MD-231 cells in 100 µL serum-free Dulbecco's Modified Eagle's medium (DMEM) were cultured in the transwell system upper chamber. Subsequently, 250 µL DMEM with 10% FBS were transferred into the lower chamber of the transwell system. DMEM acted as the chemo-attracting substance for the MBA-MD-231 cells. After 24 h of incubation, the upper chamber was removed and wiping of the cells was performed. The cells that had already invaded to the lower block were made visible by staining with crystal violet and subsequently assessment was done using inverted phase contrast microscopy. In case of cell migration, the Matrigel was not used but the methodology was the same.

Western blotting

Transfected MBA-MD-231 cells were subjected to mechanical lysis with ice-cold hypotonic buffer contain-

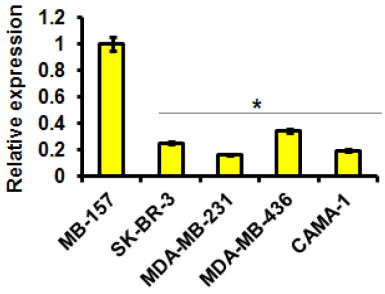

Figure 1. Expression of miR-1 in normal and breast cancer cell lines. The experiments were performed in triplicate and shown as mean ±SD (*p<0.05).

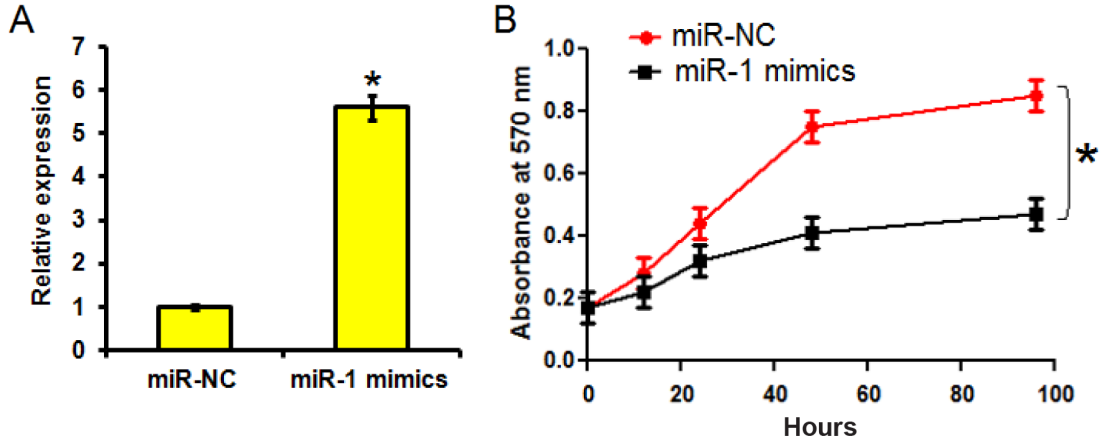

Figure 2. A: Expression of miR-1 in miR-NC and miR-1 mimics transfected MBA-MD-231 cells (*p<0.05). **B:** Cell viability of miR-NC and miR-1 mimics transfected MBA-MD-231 cells. The experiments were performed in triplicate and shown as mean ±SD (*p<0.05).

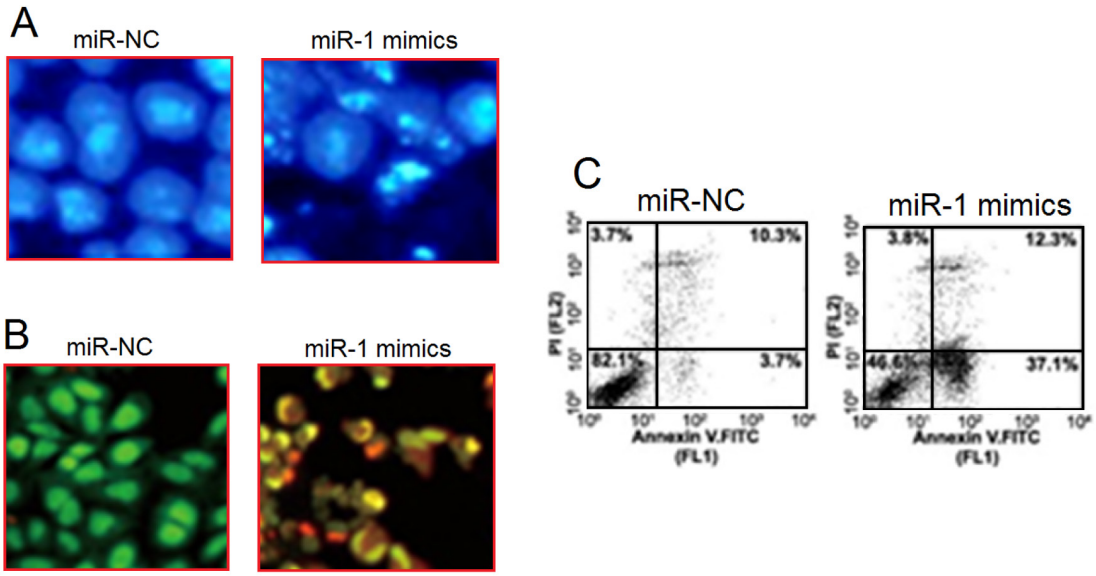

Figure 3. A: DAPI staining, **B:** AO/EB staining, and **C:** Annexin-V/PI staining of miR-NC and miR-1 mimics transfected MBA-MD-231 cells. The Figure depicts that miR-1 overexpression induces apoptosis in human breast MBA-MD-231 cells. The experiments were performed in triplicate.

ing protease inhibitors. The protein content of the MBA-MD-231 cell lysates were evaluated by bicinchoninic acid (BCA) assay. Similar quantities of the proteins from each sample were run on SDS-PAGE. After transferring the gels to the nitrocellulose membranes, the membranes were treated with primary antibodies for 55 min for at 23°C. This was followed by incubation with secondary antibody. The visualisation of the bands was carried out by chemi-luminescence reagent.

Statistics

Three independent experiments were performed to confirm the present data. The values are shown as mean ± SD. Student's t-test was used for comparisons between two samples. P<0.05 was taken as statistically significant.

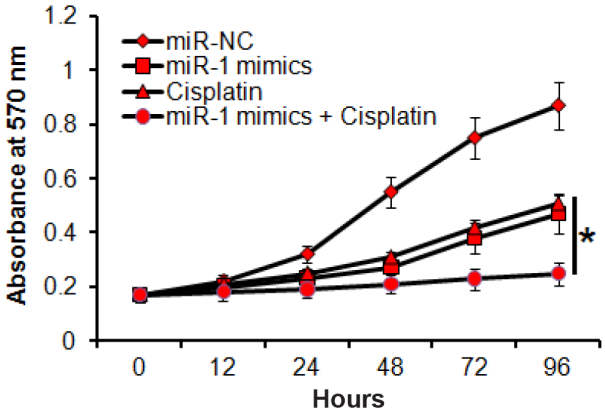

Figure 4. miR-1 overexpression enhances the chemosensitivity of MBA-MD-231 breast cancer cells. The experiments were performed in triplicate and shown as mean ±SD (*p<0.05).

Results

miR-1 is downregulated breast cancer cells

The expression analysis was performed for examining the relative expression levels of miR-1 in breast cancer cell lines (SK-BR-3, MDA-MB-436, MDA-MB-231 and CAMA-1) and normal MB-157breast cells. The miR-1 expression was up to 5.6-fold lower in breast cancer cell lines. The miR-1 had significantly lower expression in all three cancer cell lines, being lowest in MDA-MB-231 cell line (Figure 1), suggesting a probable regulatory role of miR-1 in breast cancer.

miR-1induced apoptosis inMDA-MB-231 cells

To unveil the function of miR-1 in the regulation of breast cancer growth, miR-1 mimics and miR-NC were transfected into MDA-MB-231 cancer cells for 48 h and stable transfection was confirmed by RT-PCR (Figure 2A). The MTT assay showedthat following the culturing of transfected cells for 0, 12, 24, 48 and 96 h at all the time points, the cell growth was significantly lowered under miR-1 overexpression (Figure 2B). Assessment of nuclear morphology by DAPI and AO/EB staining revealed that transfection of miR-1 mimics induced changes in the nuclear morphology of the MDA-MB-231 cells, suggestive of apoptosis (Figure 3A and 3B). To confirm apoptosis, annexin V/PI staining assay was performed which revealed increase in the apoptotic cell percentage. The apoptotic cell percentage was 37.1% in miR-1mimics transfected in comparison to 3.7% in miR-NC transfected cells (Figure 3C).

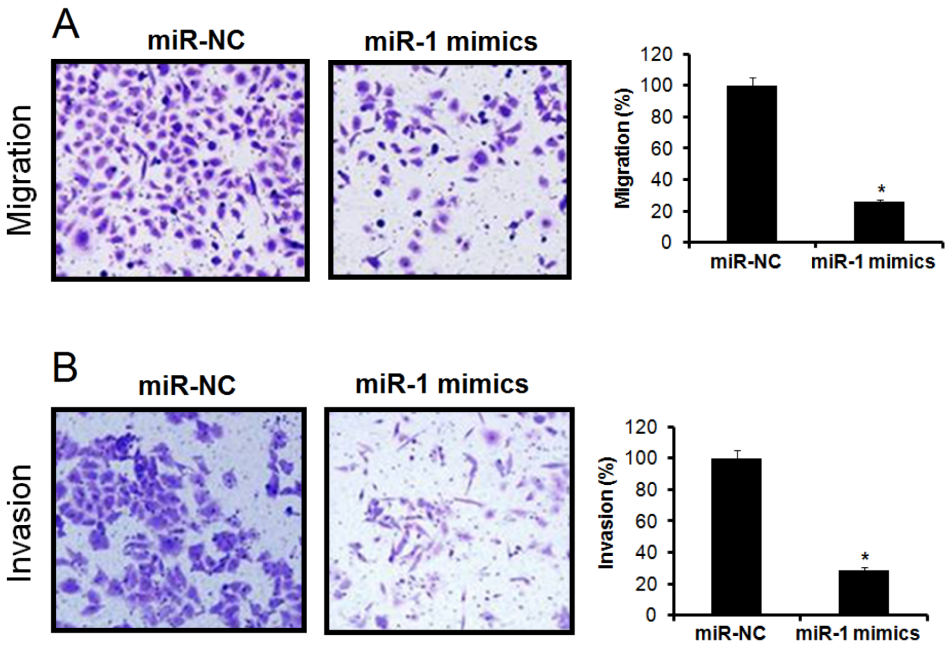

Figure 5. A: Cell migration and **B:** Invasion of miR-NC and miR-1 mimics transfected MBA-MD-231 cells. The Figure shows that miR-1 overexpression inhibits the migration and invasion of human MBA-MD-231 breast cancer cells. The experiments were performed in triplicate and shown as mean ±SD (*p<0.05).

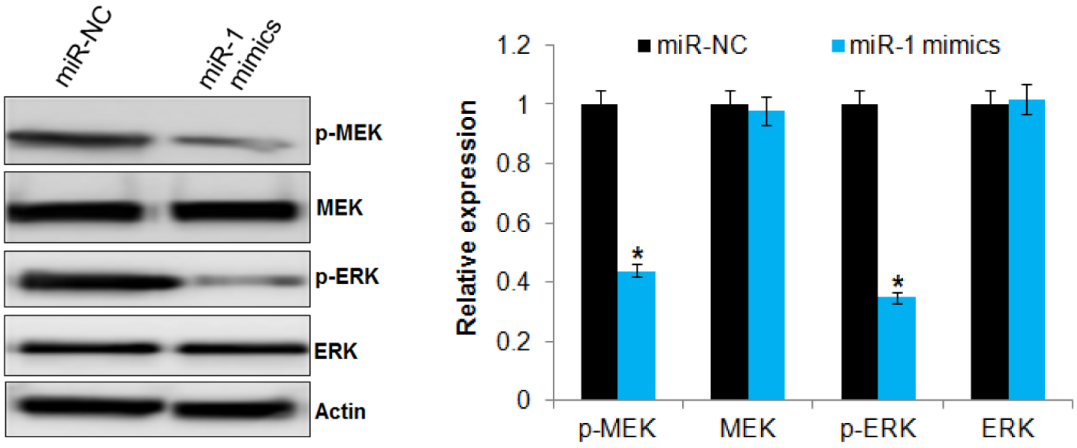

Figure 6. Western blot analysis showing that overexpression of miR-1 blocks the phosphorylation of MEK and ERK.The experiments were performed in triplicate and shown as mean ±SD (*p<0.05).

miR-1 regulates chemosensitivity of MDA-MB-231 cells

The miR-1 overexpression effects on the chemosensitivity of MDA-MB-231 cells was determined by CCK8 assay for cisplatin. The analysis of the proliferation rate was made through MTT assay for miR-NC transfected, miR-1 mimics transfected cells, miR-1 mimics transfected and cisplatin (0.5 μM) treated plus miR-1 mimics transfected cancer cells. Cancer cell proliferation was lowest under miR-1overexpression plus cisplatin as compared to cisplatin or miR-1 overexpression separately (Figure 4). Together, the results are indicative that miR-1 has a potential to increase the chemosensitivity of breast cancer cells to drug treatment and thus strongly advocating the application of combinatorial molecular and chemotherapeutics against human breast cancer.

miR-1suppressed the metastasis of the MDA-MB-231 cells

The effects of overexpression of miR-1 were examined on the metastatic potential of the MDA-MB-231 cells by transwell assay. The results showed that migration and invasion of cancer cells decreased significantly under miR-1 overexpression (Figure 5A and B). The migration and invasion of cancer cells were reduced by 74% and 71% under miR-1 overexpression.

miR-1 effects on the MEK/ERK signalling pathway

The effects of miR-1 overexpression were also examined on the MEK/ERK signalling pathway in MDA-MB-231 cells. The results clearly showed that overexpression of miR-1 suppressed the phosphorylation of MEK and ERK. Nonetheless, no visible change was observed on the total MEK and ERK protein content.

Discussion

MiRs have been extensively studied since their involvement in the development and progression of cancer was discovered. A vast number of miRs have been shown to either suppress or promote the growth of different types of cancers [11,12]. Herein, we explored the function of miR-1 in breast cancer. The results showed that miR-1 is significantly and aberrantly suppressed in breast cancer cells. A previous study had also shown that miR-1 is overexpressed in breast cancer and may have prospective implications as biomarker for this disease [5]. In the current study we found significant suppression of miR-1 in breast cancer cells relative to the normal cells. In addition, enhancement of miR-1 in breast cancer cells suppressed their proliferation rate. The inhibition of cancer cell proliferation by miR-1 has also been revealed in previous studies. For instance, post-transcriptional suppression of G6PD by miR-1 inhibited the growth and progression of cervical cancer [13]. The inhibition of breast cancer growth by miR-1 was found to be due to the promotion of apoptosis. This is consistent with a previous investigation wherein miR-1 has been shown to trigger apoptotic cell death of human cancer cells [14]. This study also revealed that miR-1 remarkably enhanced the chemosensitivity of the breast cancer cells. Additionally, the transwell assay showed that the migration and invasion of the breast cancer cells were significantly suppressed, indicating that miR-1 may also suppress metastasis of cancer cells *in vivo*. The results of this study were also in agreement with a study wherein miR-1 has been shown to suppress the migration and invasion of the esophageal squamous cell carcinoma [15]. The MEK/ERK signalling pathway has been reported to be remarkably activated in cancer cells [16]. Herein, we found that miR-1 blocks the MEK/ERK signalling pathway suggesting that miR-1 may exhibit therapeutic implications in breast cancer.

Conclusion

In conclusion, miR-1 is significantly and aberrantly suppressed in breast cancer cells. Overexpression of miR-1 inhibits the proliferation and metastasis of breast cancer cells by modulating the MEK/ERK signalling pathway. Taken all together, miR-1 may have therapeutic implications in breast cancer treatment and warrants further research endeavours.

Conflict of interests

The authors declare no conflict of interests.

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