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ORIGINAL ARTICLE

MicroRNA-155 regulates the proliferation and metastasis of human breast cancers by targeting MAPK7

Jian-Hua Liu, Yong Yang, Qi Song, Jie-Bao Li

Department of Breast and Thyroid Surgery, Wuhan No.1 Hospital (Wuhan Chinese & Western Medicine Hospital), Wuhan, Hubei, 430022, China.

Summary

Purpose: *Breast cancer causes significant mortality in women world over. The lack of efficient and reliable biomarkers and therapeutic targets impedes the treatment of breast cancer. Herein, the role and therapeutic potential of miR-155 was investigated in different breast cancer cell lines .*

Methods: *Cell viability was determined by WST-1 and colony formation assays. Transfections were performed by Lipofectamine 2000 reagent. Cell cycle analysis was carried out by flow cytometry and apoptosis was detected by AO (acridine orange)/EB (ethidium bromide) staining. Cell migration and cell invasion were determined by wound healing assay. RNA and protein expressions were determined by qRT-PCR and western blotting, respectively.*

Results: *miR-155 was significantly upregulated in all the breast cancer cells. Suppression of miR-155 in SK-BR-3 cells inhibited the growth and colony formation. The inhibition of SK-BR-3 cell proliferation was found to trigger apoptotic cell death and cell cycle arrest. Induction of apoptosis was also accompanied with enhancement of cytochrome c, Bax caspase 3, 8 and 9 and inhibition of Bcl-2. Besides, suppression of miR-155 resulted in the decrease of cell migration and invasion. Bioinformatic analysis revealed MAPK7 to be the potential target of miR-155. The MAPK7 expression was also upregulated in all the breast cancer cells and suppression of miR-155 resulted in its downregulation.*

Conclusion: *Taken together, miR-155 may prove essential in the management of breast cancer.*

Key words: *microRNA, proliferation, apoptosis, cell cycle*

Introduction

Being one of the most devastating and prevalent types of cancer in females, breast cancer causes considerable morbidity and mortality [1]. It has been reported that breast cancer causes 0.4 million deaths annually accounting for 14% of all the cancer-related mortality world over [2]. Although low grade breast cancers are treated with surgery followed by chemotherapy, the overall survival rate for advanced-stage metastatic cancers is still unsatisfactory. In addition, late diagnosis, development of chemoresistance and lack of safe and viable drug options are significant obstacles in the treatment of breast cancer [3]. Hence, the development of reliable biomarkers, identification of therapeutic targets and efficient drugs are urgently required. Over the last few decades a group of small non-coding RNAs called microRNAs (miRs) have gained enormous attention as therapeutic targets/agents [4]. Owing to the role of miRs in a wide array of cellular processes, they are believed to act as essential therapeutic targets/agents [5]. Accumulating evidence indicates the aberrant expression of several of the miRs in cancer tissues and restoration of normal expression of these miRs has been shown to impede the growth of cancer [6]. The miR-155 has been shown to play a vital role in the progression and development of several cancer types and it has been reported to be highly overexpressed

Corresponding author: Yong Yang, MD. Department of Breast and Thyroid Surgery, Wuhan no.1 Hospital (Wuhan Chinese & Western Medicine Hospital), Wuhan, no.215 Zhongshan Ave, Qiaokou District, Hubei, 430022, China.
Tel/Fax: +86 27 8585 5900, Email:AlistadLhos@yahoo.com
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in Burkitt's lymphoma in children [7]. The miR-155 has also been shown to control the growth and invasion of hepatocellular carcinoma [8]. In yet another study, it has been reported to control the survival and chemosensitivity of breast cancer by altering the FOXO3a expression [9]. This study, therefore, was designed to investigate the role and the therapeutic implications of miR-155 in breast cancer. Moreover, the mitogen activated protein kinase 7 (MAPK7) was investigated for the first time as the possible target of miR-155 in breast cancer.

Methods

Cell lines and culture conditions

Breast cancer cell lines EMT6, BT-20, MCF7, CAMA-1 SK-BR-3 and normal breast cell line Hs 841.T were procured from American Type Culture Collection. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) having 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% fetal bovine serum (FBS).

Quantitative RT-PCR

The RNA was isolated from the breast cancer cell lines by TRIzol reagent and then transcribed into cDNA using RevertAid cDNA synthesis kit. The expression of miR-155 was determined by qRT-PCR as described previously [10].

Transfections

As the breast cancer SK-BR-3 cells reached 80% confluence, they were transfected with miR-NC and miR-155 inhibitor (10 pmol, Shanghai GenePharma, China) with the help of Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA).

Cell viability

The viability of breast cancer cells was examined by WST-1 assay. In brief, the breast cancer cells were cultured at a density of 2.5×10^5 cells/well in 96-well plates and transfected with appropriated miR-155 inhibitor constructs. This was followed by incubation of the breast cancer cells with WST-1 for 3 h at 37°C and the proliferation rate was determined by taking the absorbance at 450 nm using a spectrophotometer. Colony formation assay was performed as previously described [10].

Apoptosis assay

For propidium iodide (PI) staining, the breast cancer SK-BR-3 cells (0.6×10^6) were grown in 6-well plates. Following an incubation of around 12 h, the SK-BR-3 cells were subjected to transfection with miR-155 inhibitor, using Lipofectamin 2000 (Invitrogen, Thermo Fisher Scientific Inc.) and then were incubated for 24 h at 37°C. About 25 µl of cell culture were put onto a glass slide and stained with PI. The slides were then cover-slipped and examined with fluorescent microscope.

Cell cycle analysis

The distribution of the breast cancer SK-BR-3 cells in different cycle phases was performed by flow cytometry after PI staining, following the method reported by Hua et al [11]. In brief, the transfected breast cancer cells were grown in 6-well plates for 24h at 37°C and then collected, washed with phosphate buffered saline (PBS) and fixed in 70% ethanol. After overnight incubation at 4°C, the cells were subjected to PI staining and flow cytometry.

Cell migration and invasion

The cell migration potential of the SK-BR-3 cells was checked by wound healing assay as previously described [11]. The cell invasion assay was performed as described by Wang et al. [12].

Western blot analysis

The SK-BR-3 cells were firstly subjected to washing with ice-cold PBS and suspended in a lysis buffer at 4°C and then shifted to 95°C. Thereafter, the protein content of each cell extract was checked by Bradford assay. About 40 µg of protein was loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then subjected to treatment with tris-buffered saline (TBS) and exposed to primary antibodies at 4°C. Then, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent.

Statistics

Data are shown as mean ± SD. Statistical analyses were done using Students *t*-test with GraphPad prism 7 software. Values of $p<0.05$ were taken as indicative of significant difference.

Results

Expression analysis of miR-155 in breast cancer cells

The miR-155 expression was examined in 5 different breast cancer cell lines as well as in one

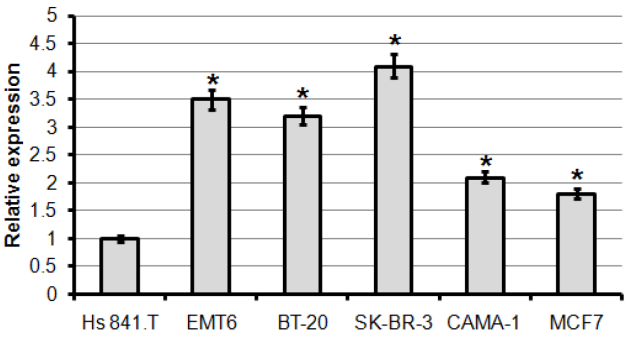

Figure 1. Expression of miR-155 in one normal and five different breast cancer cell lines as determined by qRT-PCR analysis. The experiments were performed in triplicate and the values represent mean ± SD (*$p<0.05$).

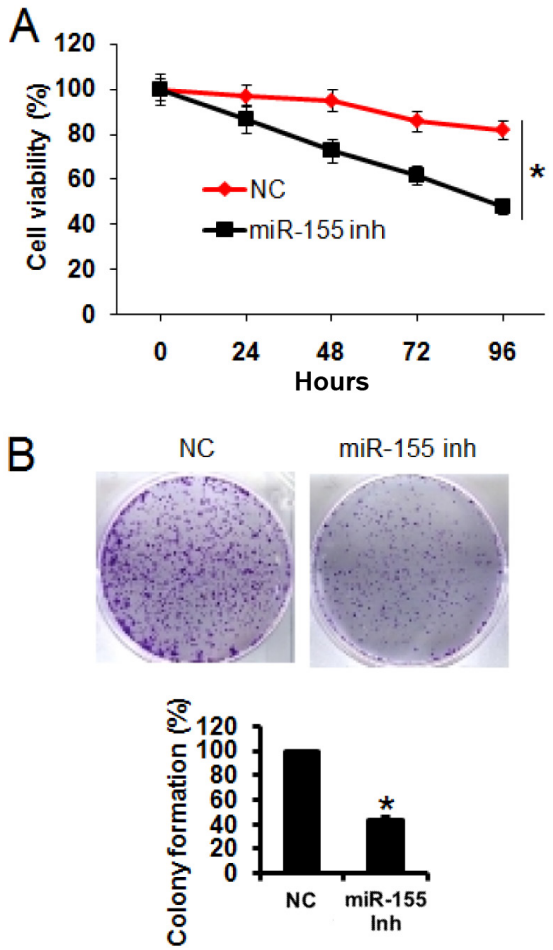

Figure 2. Transfection of SK-BR-3 cells with miR-155 inhibitor inhibits cell viability **(A)** and colony formation **(B)**. The experiments were performed in triplicate and the values represent mean ± SD (*p<0.05) (NC: negative control, miR-155 Inh: miR-155 inhibitor).

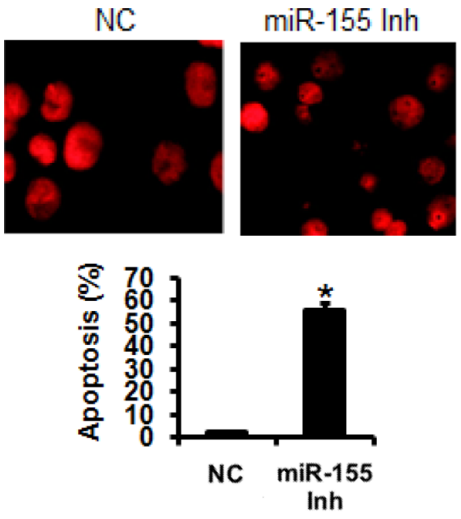

Figure 3. miR-155 inhibitor, showing induction of apoptosis in SK-BR-3 cells as depicted by the propidium iodide staining. The experiments were performed in triplicate and data are shown as mean ± SD (*p<0.05) (NC: negative control, miR-155 Inh: miR-155 inhibitor).

normal cancer cell line by qRT-PCR. It was found that the expression of miR-155 was considerably upregulated in all the 5 breast cancer cell lines relative to the normal cell line. The fold upregulation of miR-155 in the breast cancer cell lines ranged from 1.8 to 4.1 relative to the expression of miR-155 in the normal cell line (Figure 1). The lowest upregulation was observed in the MCF7 cell line with 1.8 fold upregulation and the highest fold upregulation of 4.1 was observed in the SK-BR-3 cell line.

Suppression of miR-155 inhibits growth of SK-BR-3 cells

Since the miR-155 expression was considerably upregulated in the breast cancer cells, we attempted to know the effect of miR-155 suppression on the growth of SK-BR-3 cells. The results revealed that miR-155 suppression resulted in significant decrease in the proliferation of the SK-BR-3 cells time-dependently (Figure 2A). Additionally, miR-155 suppression caused inhibition of the colony development potential of the SK-BR-3 cells (Figure 2B).

Suppression of miR-155 triggers apoptosis and cell cycle arrest in the SK-BR-3 cells

Next, we sought to know about the mechanism behind the inhibition of SK-BR-3 breast cancer cell proliferation prompted by miR-155 suppression.

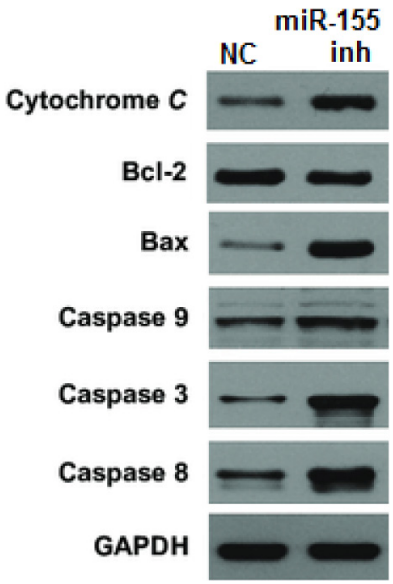

Figure 4. miR-155 inhibits the expression of NC apoptosis related proteins as depicted by western blot analysis. The Figure shows that inhibition of miR-153 expression increased the expression of Bax, Caspase-3, Caspase-8 and Caspase-9 and decreased the expression of Bcl-2. The experiments were performed in triplicate (NC: negative control, miR-155 Inh: miR-155 inhibitor).

Therefore, the SK-BR-3 cells were subjected to PI staining and it was found that the miR-155 suppression induced apoptotic cell death of the SK-BR-3 cells (Figure 3) which was accompanied with upregulation of Bax and caspase 3 and downregulation of Bcl-2 (Figure 4). Cell cycle analysis revealed that suppression of miR-155 expression also triggered arrest of the breast cancer SK-BR-3 cells at the G0/G1 check point of the cell cycle (Figure 5). The percentage of G0/G1 phase cells was 67.31% in the miR-155 suppressed SK-BR-3 cells in comparison to 32.22% in the negative control cells (NC).

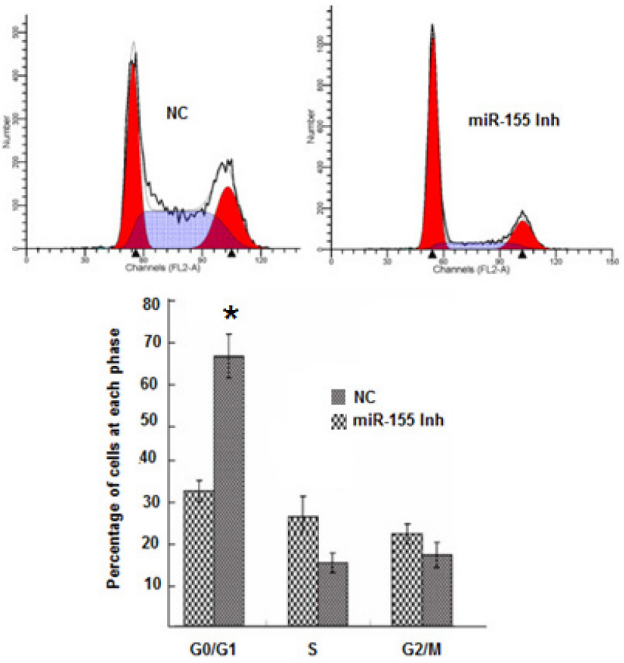

Figure 5. Suppression of miR-155 expression triggers G0/G1 cell cycle arrest of the SK-BR-3 cells as depicted by flow cytometry analysis. The experiments were performed in triplicate and data represent mean±SD (*p<0.05). (NC: negative control, miR-155 Inh: miR-155 inhibitor).

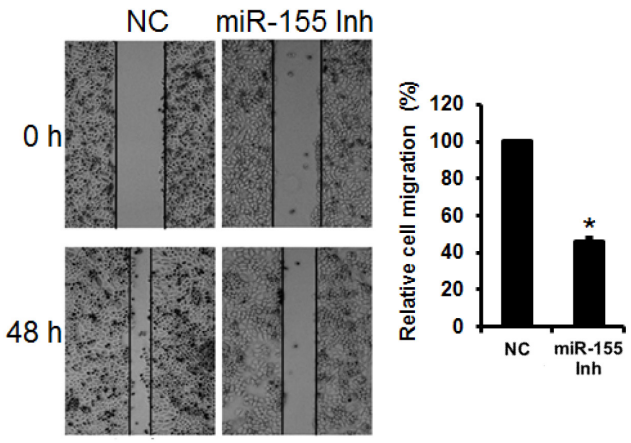

Figure 6. miR-155 suppression inhibits the migration of the SK-BR-3 cells as depicted by the wound healing assay. The experiments were repeated thrice and data represent mean ± SD (*p<0.05) (NC: negative control, miR-155 Inh: miR-155 inhibitor).

Suppression of miR-155 inhibits metastasis of the SK-BR-3 cells

The effects of miR-155 suppression were also investigated on the metastatic potential of SK-BR-3 cells. The results of the wound healing assay showed that suppression of miR-155 resulted in significant inhibition of cell migration (Figure 6). In addition, the cell invasion assay also revealed that miR-155 inhibits the invasive potential of the SK-BR-3 cells (Figure 7).

miR-155 targets MAPK1 in breast cancer cells

To decipher the miR-155 target, TargetScan analysis was performed. The results revealed MAPK1 to be the potential target of miR-155 (Figure 8). To further validate it, the expression of MAPK7 was also investigated in the breast cancer cells. It was found that MAPK7 expression was

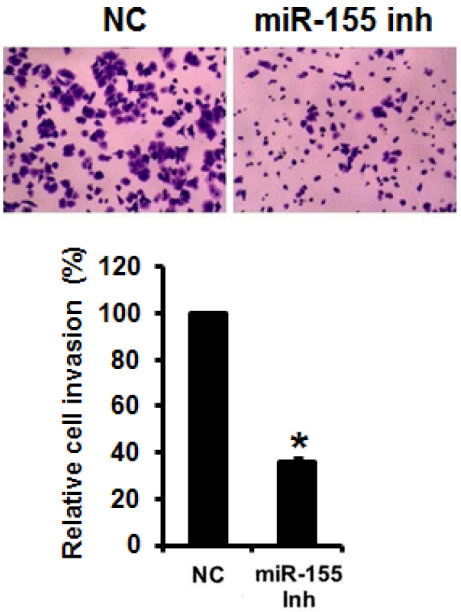

Figure 7. miR-155 suppression inhibits the invasion of the SK-BR-3 cells as depicted by the Boyden chamber assay. The experiments were repeated in triplicate and data represent mean ± SD. (*p<0.05) (NC: negative control, miR-155 Inh: miR-155 inhibitor).

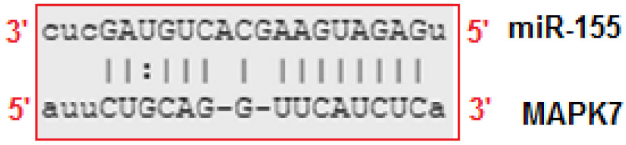

Figure 8. Identification of MAPK7 as the target of miR-155. The analysis was performed online by TargetScan software using default parameters.

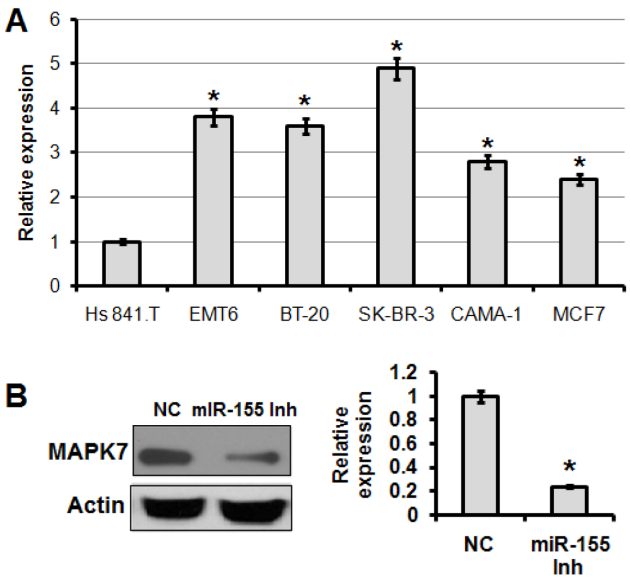

Figure 9. A: Expression of MAPK7 in one normal and 5 different cancer cell lines as depicted qRT-PCR analysis. **B:** Western blot showing significant suppression of MAPK7 expression upon inhibition of miR-155 expression. The experiments were repeated in triplicate and data represent mean±SD. (*p<0.05) (NC: negative control, miR-155 Inh: miR-155 inhibitor).

considerably upregulated in all the breast cancer cells with fold upregulation of up to 4.8 (Figure 9A). Further the results of the western blot analysis indicated that suppression of miR-155 expression caused considerable downregulation of the MAPK7 expression (Figure 9B).

Discussion

Breast cancer is the second principal reason for cancer-related mortality in women worldwide [13]. The treatment for breast cancer is mainly impeded by its late diagnosis, lack of reliable biomarkers and therapeutic targets [2]. Owing to the wide array of roles of miRs, such for instance cell proliferation, they are considered extremely important therapeutic targets/agents [14]. Herein, the miR-155 expression was examined in 5 different breast cancer cell lines as well as in one normal breast cell line. It was found that miR-155 expression was considerably upregulated in all the breast cancer cell lines with maximum expression in the SK-BR-3 cells. These results are also in good concordance with previous investigations. For example, miR-155 expression was been reported to be considerably upregulated in the Burkitt's lymphoma, hepatocellular carcinoma and pancreatic adenocarcinoma [7,8,15]. Next, to unveil the role of miR-155 in breast cancer, the miR-155 expression was suppressed and it was found that its suppression caused inhibition of SK-BR-3 cell proliferation and also inhibited their colony formation potential. In hepatocellular carcinoma [8], the high expression of miR-155 has been reported to inhibit proliferation, further supporting our results. The underlying reason for the miR-155 suppression triggered inhibition of cell proliferation was investigated by PI staining and it was found that its suppression activated the apoptotic death of SK-BR-3 cells which was associated with increase of cytochrome C, Bax, caspase-3, 8 and 9 and decline of Bcl-2 expression. Apoptosis is a vital process that enables to maintain the tissues homeostasis contributing to the proliferation of the normal cells and elimination of the abnormal, harmful or cancer cells [16]. The induction of apoptosis is an imperative mechanism through which several of the anticancer agents act [17]. Besides apoptosis, some anticancer agents also block the cell division at different checkpoints [18]. Herein, we found that miR-155 suppression resulted in the arrest of the SK-BR-3 cells at the G0/G1 phase of the cell cycle. The suppression of miR-155 also inhibited the metastasis of the SK-BR-3 breast cancer cells, suggesting that miR-155 suppression may also play a role in inhibiting the metastasis of breast cancers *in vivo*. Approximately 30% of genes in humans are controlled by miRs and each miR acts by targeting other genes [19]. Herein, MAPK7 was found to be the potential target of miR-155. MAPK7 has been reported to play essential role in the proliferation of cancer cells and suppression of miR-155 resulted in inhibition of the MAPK7 expression [20].

Conclusion

Taken together, we conclude that miR-155 is considerably overexpressed in the breast cancer cells and its suppression halts breast cancer cell growth by triggering apoptosis. Besides, miR-155 suppression caused arrest of breast cancer cells at G0/G1 phase of the cell cycle and also inhibited their migration and invasion. Hence, miR-155 may prove beneficial in the therapeutic management of breast cancer.

Conflict of interests

The authors declare no conflict of interests.

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