

## ORIGINAL ARTICLE

# MicroRNA-204 inhibits the proliferation and metastasis of breast cancer cells by targeting PI3K/AKT pathway

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## Summary

**Purpose:** Breast cancer is one of the deadliest malignancies in women. Lack of biomarkers and the unavailability of reliable therapeutic targets are main hurdles in the treatment of breast cancer. The present study was therefore designed to assess the role and therapeutic potential of miR-204 in the treatment of breast cancer.

**Methods:** The expression of miR-204 was checked by qRT-PCR. The transfections were performed by Lipofectamine 2000 reagent. Cell viability was determined by WST-1 colorimetric assay. The effect of miR-204 was evaluated on the breast cancer metastasis by cell migration and invasion transwell assays. Immunoblotting was used to determine the protein expression in breast cancer cells.

**Results:** The results revealed that the expression of miR-204 was downregulated in all the tested breast cancer cell lines. Overexpression of miR-204 in the MCF7 breast cancer

cell line suppressed the proliferation of these cells by triggering apoptotic cell death and G2/M cell cycle arrest. Furthermore, miR-204 overexpression inhibited the migration and invasion of the MCF7 breast cancer cells. Bioinformatic analysis revealed PTEN to be the target of miR-204. Since, PTEN regulates the PI3K/AKT signalling pathway, the effect of miR-204 overexpression was also assessed on this pathway and showed that miR-204 overexpression inhibits the expression of p-AKT and p-PI3K significantly in MCF7 breast cancer cells.

**Conclusion:** We conclude that miR-204 regulates the proliferation and metastasis of the breast cancer cells and as such may prove to be an important therapeutic target.

**Key words:** apoptosis, breast cancer, cell cycle arrest, metastasis, microRNA

## Introduction

Breast cancer is the prevalently detected cancer among women and causes significant mortality worldwide [1]. It is the most common cause of death in females, with more than 0.4 million deaths annually [2]. Reportedly, breast cancer constitutes around 14% of all the cancer-related deaths in women and its incidence is still on the increase [3]. The main obstacles for the treatment of breast cancer include late diagnosis, lack of reliable biomarkers and therapeutic targets and the limited availability of the efficient drugs [4,5]. MicroRNAs (miRs) are

very small non-coding RNA molecules that play vital roles in humans [6]. They have a number of roles which include but are not limited to cell division and transcription. The miRs have been reported to be involved in the onset of several diseases such as cancer, and the expression of most of the miRs has been reported to be dysregulated in cancer [7]. Their expression in cancer cells is either downregulated or upregulated and therefore miRs are being considered as important therapeutic targets for the management of several types of cancers. miR-

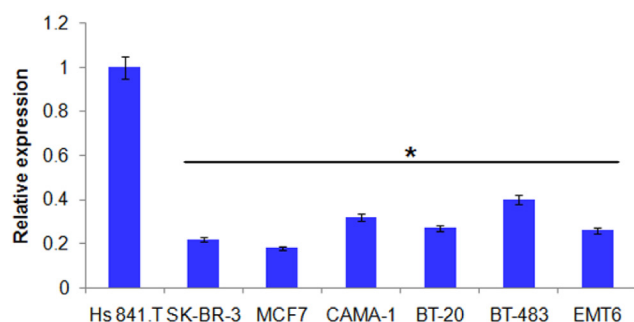
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204 has been shown to be dysregulated in several types of cancer cells. For instance the expression of miR-204 has been reported to be downregulated in hepatocellular carcinoma [8]. Moreover, miR-204 has been shown to inhibit the proliferation of cancer cells like in prostate and gastric cancers [9,10]. However, the role of miR-204 has not been fully investigated in breast cancer. This study was therefore designed to explore the role and therapeutic potential of miR-204 in breast cancer.

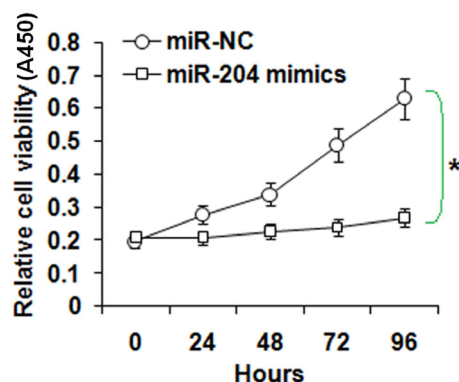
## Methods

### Cell lines and culturing conditions

Breast cancer cell lines MCF7, CAMA-1 SK-BR-3, BT-20, BT-483 and EMT6, and normal breast cell line Hs 841.T were procured from American Type Culture Collection. All of these cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin), and 2 mM glutamine. The cells were cultured in an incubator (Thermo Scientific, Waltham, Mass, USA) at 37°C with 98% humidity and 5% CO<sub>2</sub>.



**Figure 1.** Expression of miR-204 in human breast cancer cell lines and one normal breast cancer cell line. The experiments were carried out in triplicate and expressed as mean  $\pm$  SD (\* $p < 0.05$ ).



**Figure 2.** Cell viability assay showing that miR-204 overexpression inhibits the viability of MCF7 breast cancer cells. The experiments were carried out in triplicate and expressed as mean  $\pm$  SD (\* $p < 0.05$ ).

### RNA isolation, cDNA synthesis and quantitative real-time PCR

The total RNA was extracted from the breast cancer cells with the help of RNeasy Kits (Qiagen, Hilden, Germany). To reverse-transcribe the cDNA, the Omniscript RT (Qiagen) was employed using 1 µg of the extracted RNA. The cDNA was then used as template for quantitative real-time PCR (qRT-PCR) with the help of Taq PCR Master Mix Kit (Qiagen) according to the instructions of the manufacturer.

### Transfections

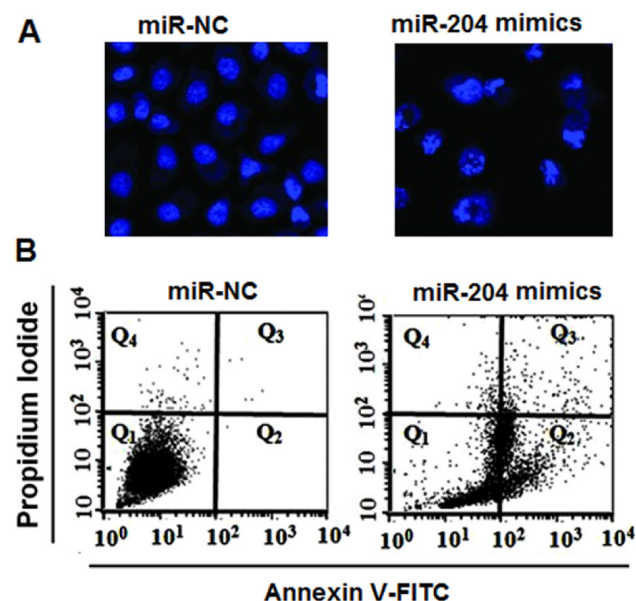
As the breast cancer MCF7 cells reached 80% confluence, they were transfected with miR-negative control (NC), miR-204 mimics (10 pmol, Shanghai Gene Pharma, China) with the help of Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) as per the manufacturer's instructions.

### Cell viability

The cell viability of the breast cancer cells was assessed by WST-1 colorimetric assay. Briefly, the breast cancer cells were seeded in 96-well plates at the density of  $2 \times 10^5$  cells/well. The cells were then incubated with WST-1 at 37°C for 4 h. The absorbance at 450 nm was then taken by a microplate reader at different time intervals (0, 12, 24, 48 and 96 h) to determine the viability of breast cancer cells.

### Apoptosis assay

The nuclear morphology of the MCF7 breast cancer cells was assessed by fluorescence microscopy after subjecting the cells to cell-permeable Hoechst 33342 dye. Ten fields with 100 cells/field were randomly selected for



**Figure 3.** Overexpression of miR-204 in MCF7 cells triggers apoptosis as indicated by **A:** Hoechst staining and **B:** Annexin V/PI staining. The experiments were carried out in triplicate.

estimation of the cells with condensed nuclei. Annexin-V/propidium iodide (PI) double staining was used for the determination of the percentage of the apoptotic breast cancer cells as described previously [11].

#### Cell migration and invasion

The capacity of the MCF7 breast cancer cells to migrate and invade was determined by transwell assay as previously described [12].

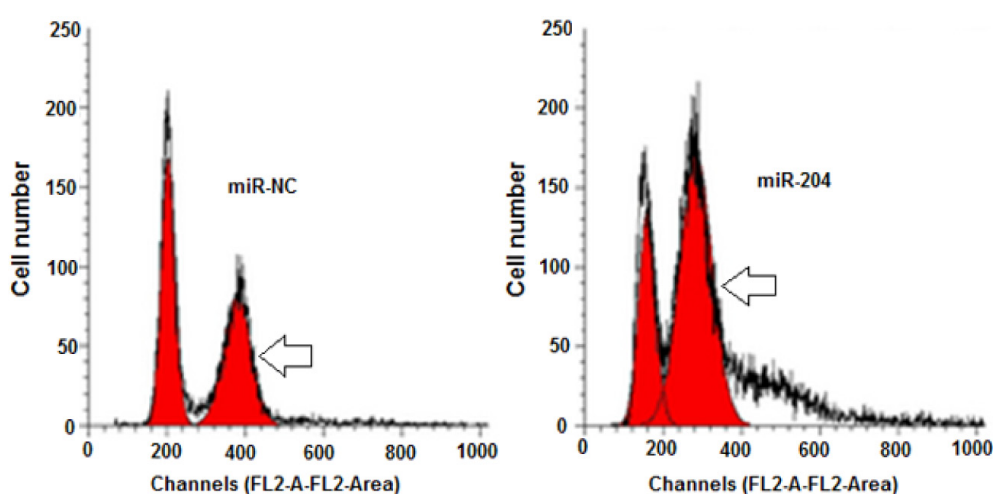
#### Western blot analysis

The breast cancer MCF7 cells were lysed with ice-cold hypotonic buffer. After estimating the protein concentrations in each of the cell extracts, the samples

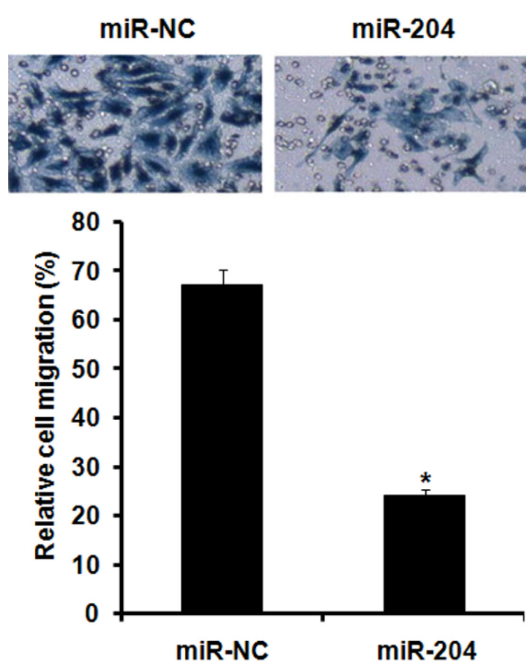
containing the proteins were loaded and separated on SDS-PAGE. This was followed by transference to a nitrocellulose membrane and incubation with the primary antibody (1:1000) for 24 h at 4°C. Thereafter, the membrane was incubated with HRP-conjugated secondary antibody (1:1000) for at 24°C for about 1 h. The visualisation of the proteins was carried out by enhanced chemi-luminescence reagent.

#### Statistics

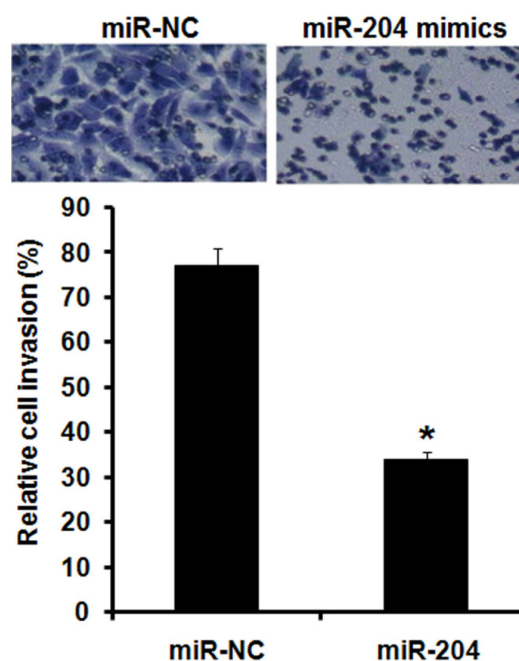
The experiments were performed in triplicate and presented as mean  $\pm$  SD. Student's t-test with the Graph-Pad prism 7 software were used for statistical analyses.  $P < 0.05$  was taken as statistically significant.



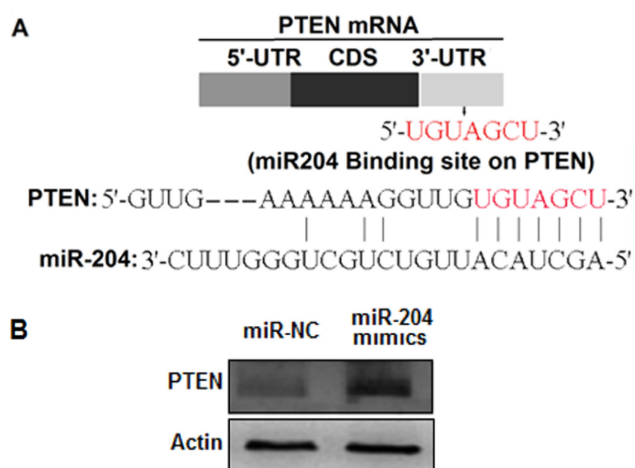
**Figure 4.** Overexpression of miR-204 triggers apoptosis and G2/M cell cycle arrest in MCF7 breast cancer cells. Arrows show the G2/M phase cells. The experiments were carried out in triplicate.



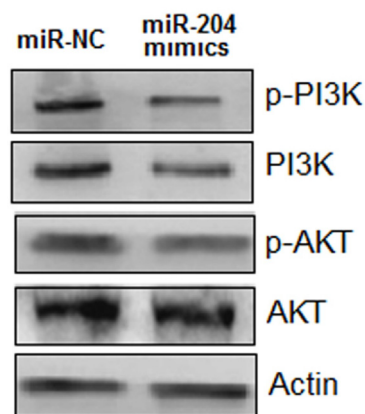
**Figure 5.** Overexpression of miR-204 inhibits the migration of MCF7 breast cancer cells. The experiments were carried out in triplicate and expressed as mean  $\pm$  SD (\* $p < 0.05$ ).



**Figure 6.** Overexpression of miR-204 inhibits the invasion of MCF7 breast cancer cells. The experiments were carried out in triplicate and expressed as mean  $\pm$  SD (\* $p < 0.05$ ).



**Figure 7. A:** MiR-204 targets PTEN as indicated by bioinformatic analysis. **B:** Western blotting showing that overexpression of miR-204 causes overexpression of PTEN. The experiments were carried out in triplicate.



**Figure 8.** Overexpression of miR-204 causes inhibition of the PI3K/AKT signalling pathway as indicated by western blotting. The experiments were carried out in triplicate.

## Results

### *miR-204 is downregulated in human breast cancer cell lines*

The expression of miR-204 was examined in 6 breast cancer cell lines (MCF7, CAMA-1, SK-BR-3, BT-20, BT-483, EMT6) and one normal breast cell line (Hs 841.T) by quantitative RT-PCR. The expression of miR-204 was considerably downregulated in all the breast cancer cells, been 2.5 to 5 fold lower in breast cancer cells, in comparison to the normal Hs 841.T (Figure 1). The lowest was observed in the MCF7 breast cancer cell line and as such this cell line was selected for further experimentation.

### *Overexpression of miR-204 inhibits the proliferation of MCF7 breast cancer cells*

miR-204 overexpression inhibited the proliferation of breast cancer MCF7 cells (Figure 2). For

the investigation of the underlying mechanism for miR-204, miR-NC and miR-204 mimics-transfected MCF-7 cells were subjected to Hoechst 33342 and annexin V/PI staining. The results showed that overexpression of miR-204 triggers apoptotic cell death in MCF7 breast cancer cells (Figure 3). In addition, cell cycle analysis revealed that miR-204 overexpression induced the G2/M cell cycle arrest of the breast cancer cells (Figure 4).

### *Overexpression of miR-204 inhibits the metastasis of human MCF7 breast cancer cells*

The effect of miR-204 on the metastasis of breast cancer cells was examined by the cell migration and invasion assays. The results revealed that miR-204 overexpression caused considerable inhibition of the migration of MCF7 breast cancer cells. Similar results were obtained with cell invasion assay (Figure 5). Taken together, these results indicate that miR-204 regulates the metastasis of breast cancer cells (Figure 6).

### *MiR-204 targets PI3/AKT signalling pathway in MCF7 breast cancer cells*

To find out the potential target of miR-204, TargetScan online software was used and PTEN was identified as the potential target of miR-204. In addition, it was observed that overexpression of miR-204 caused significant upregulation in the expression of PTEN (Figure 7A-B). Since PTEN is the negative regulator of PI3K/AKT signalling pathway, the effects of miR-204 overexpression caused significant reduction in the expression of p-AKT and p-PI3K (Figure 8).

## Discussion

Breast cancer is one of the deadly malignancies, responsible for considerable mortality in women across the globe [13]. It is one of the frequently detected cancers in women and is mostly diagnosed at advanced stages due to the unavailability of reliable biomarkers. Also, the currently available chemotherapeutic regimens are associated with a number of side effects. Furthermore, reliable therapeutic targets that could be used for the treatment of breast cancer are limited [14]. MiRs, being involved in a wide array of biological functions are being considered prospective therapeutic targets that could be utilised in the treatment of different types of cancers [15]. MiR-204 is an important miR that has been reported to regulate the proliferation of several types of cancers [8]. However, the role of miR-204 has not been established in breast cancer. Therefore, the present study focussed on the inves-



tigation of the role of miR-204 and exploration of its therapeutic potential. In this study, it was observed that miR-204 was considerably downregulated in all the breast cancer cells as compared to the normal cells. Our results are well supported by the studies carried out previously wherein the expression of miR-204 has been found to be dysregulated in several types of cancers. For example, the expression of miR-204 is downregulated in renal cell carcinoma [16]. The present study revealed that miR-204 was overexpressed in breast cancer MCF-7 cells and its overexpression caused significant reduction in the proliferation of the breast cancer cells. Furthermore, the effects of miR-204 on the proliferation of the breast cancer cells were found to be due to the induction of apoptosis and G2/M cell cycle arrest. Apoptosis and cell cycle arrest are important mechanisms by which many of the anti-cancer agents exert their effects. While as apoptosis eliminates the cancer cells completely, cell cycle arrest prevents the cells to complete their division [17,18]. Hence, both these mechanisms caused decline in the viability of the MCF7 breast cancer cells. Metastasis is an important factor that determines the prognosis of cancers [19]. In this study we evaluated the impact of miR-204 overexpression on MCF7 breast cancer cells by cell migration and invasion assays and it was observed that miR-204 overexpression suppresses the MCF7 migration and invasion, indicating that miR-204 regulates

the metastasis of the breast cancer cells. MiRs exert their effects by targeting different genes. Bioinformatic analysis revealed that miR-204 targets PTEN. Western blotting analysis showed that overexpression of miR-204 leads to enhanced expression of PTEN. Since PTEN is a negative regulator of PI3K/AKT [20], we checked the effect of miR-204 on the PI3K/AKT signalling pathway and observed that miR-204 caused significant reduction in the expression of p-PI3K and p-AKT. Taken together, miR-204 inhibits the proliferation and metastasis of the breast cancer cells and deserves further research efforts.

## Conclusion

In conclusion, miR-204 is significantly downregulated in the breast cancer cells and its overexpression in breast cancer cells inhibits their proliferation by triggering apoptosis and G2/M cell cycle arrest. Besides, miR-204 overexpression inhibits breast cancer cell metastasis by targeting PI3K/AKT signalling pathway. Given these results, further evaluation of this molecule *in vivo* will help establish miR-204 as the therapeutic target for the treatment of breast cancer.

## Conflict of interests

The authors declare no conflict of interests.

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