ORIGINAL ARTICLE

MicroRNA-335 inhibits the growth, chemo-sensitivity, and metastasis of human breast cancer cells by targeting MAP3K2

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Summary

Purpose: The molecular mechanisms of breast cancer (BC) are relatively unknown and the metastatic process is highly complicated. In this context, the impact of miR-335 on different biological functions was evaluated.

Methods: qRT-PCR was carried out to analyze the expression of miR-335 in three different BC cell lines and normal epithelial breast cell line. Cell proliferation, survival, and viability of BC cells were estimated by MTT assay, colony formation assay, and DAPI staining methods. Target identification of miR-335 was made through online bioinformatics and validated through the correlation of gene expression, western blotting experiments, and luciferase reporter assay. Cell migration and invasion were analyzed through transwell chamber assay.

Results: miR-335 was downregulated in BC cells and had

inhibitory effect on cell growth, which was manifested as decline in cell survival and loss of cancer cell viability. Further, the chemo-sensitivity of BC cells to paclitaxel and doxorubicin was seen to be enhanced under miR-335 overexpression. miR-335 also inhibited the migration and invasion of cancer cells. MAP3K2 was shown to be the target gene of miR-335 and the silencing of MAP3K2 was seen to mimic the growth inhibitory effect of miR-335. The overexpression of MAP3K2 reversed the growth inhibition in miR-335 mimics-transfected BC cells.

Conclusion: miR-335 has growth inhibitory effect against BC and negatively regulates the cell migration and invasion along with enhancement of chemo-sensitivity of cancer cells.

Key words: breast cancer, metastasis, cell proliferation, migration, invasion, chemo-sensitivity.

Introduction

Human breast cancer (BC) is the most common type of cancer detected in women, causing the largest number of cancer-related deaths among females, excluding lung cancer [1,2]. Genetically, the BC is highly heterogeneous and comprises distinct subtypes which differ considerably not only in histological characteristics but also in their behavior towards the clinical assessments [3,4]. Although the employment of recent advancements in chemo- and radio-therapeutic measures along with hormonal treatment strategies have enhanced the overall survival of BC, the responses of patients with similar type of BC show differences, posing a serious concern, thus demanding the exploration of alternative anticancer treatments [5,6]. Also, the progression of BC, particularly the cancer metastasis, is very complicated and identification of the underlying molecular mechanism is yet to be fully elucidated [7]. Researchers have proved that there is an active involvement of an important class of RNA molecules, namely the micro RNAs (miRs), in most of the human cancers [8-10]. miRs are small

non-coding regulatory RNAs, with usually 20-22 nucleotides in length, which act at post-transcriptional level and repress their target genes by binding to mRNA untranslated (3' and 5') regions [11-13]. Almost 60% of eukaryotic genes are believed to be regulated by miRs [14]. miRs are generally repressed in human cancers but it is not true for all cases [15]. They act as tumor suppressors or oncogenes to regulate the tumorigenesis and progression of human cancers as well as playing a significant role in metastasis to surrounding tissues [16-18]. Studies have revealed the dysregulation of miRs in BC [19,20]. In the current study, when the expression of miR-335 was analyzed and compared in the human BC cell lines versus epithelial breast cells, a significant transcript repression of miR-335 was observed in the entire cancer cell lines involvement in BC. Further characterization revealed that miR-335 has an inhibitory role against BC growth and proliferation which was shown to be modulated through MAP3K2 signal. Interestingly, the overexpression of miR-335 increased the chemo-sensitivity of BC cells to paclitaxel and doxorubicin treatments. Summing up, the study indicates the possibility of miR-335 as a prognostic biomarker and signifies its therapeutic potential in human BC.

Methods

Culture and transfection of cancer cells

Three different human BC cell lines (BT20, Hs578T and MDA-MB231) and a normal breast epithelial cell line MCF-12A were obtained from ATCC, USA. As described previously, culturing and maintenance of cell lines were done using Dulbecco's modified Eagle medium (DMEM, Gibco, USA) with 10% fetal bovine serum (FBS) [21].

The cancer cells were stably transfected with the miR-335 constructs (miR-NC, miR-335 mimics and miR-335 inhibitor). For generating the silencing and overexpressing lines of MAP3K2, the cancer cells were transfected with si-MAP3K2 and pcDNA-MAP3K2, respectively, along with their respective negative controls (si-NC and pcDNA3.1 plasmid). For dual luciferase assay, wild-type (WT) 3'-UTR and mutated (MUT) 3'-UTR stretches of MAP3K2 mRNA were co-transfected with miR-NC or miR-335 mimics in Hs578T BC cells for 48h.

RNA isolation and qRT-PCR analysis

Total RNA was isolated from the cells with RNAiso reagent (Takara, Japan). The extracted RNA was given DNAse I (Thermo Fisher Scientific) treatment. cDNA synthesis was performed with the help of Primescript™ reverse transcription reagent (Takara, Japan). Quantitative Real Time-PCR (qRT-PCR) was performed on QuantStudio 3 Real Time- PCR system (Thermo Fisher Scientific) following the manufacturers' guidelines. The relative expression was normalized with human GADPH gene and $2^{-\Delta\Delta Ct}$ method was used to quantify the relative expression values. RT primers were synthesized through Primer3 v. 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/) online software.

MTT assay

The estimation of proliferation of BC cells was made through MTT assay. In brief, the Hs578T cancer cells were stably transfected with miR-NC, miR-335 mimics, or miR-335 inhibitor for 48h. Cells were also transfected with si-MAP3K2 and its negative control, si-NC. Co-transfection of pcDNA-MAP3K2 with miR-335 mimics was also done. Transfected cells were subsequently cultured in 96-well plates for 24, 28, 72, and 96h at 37°C and 5% CO_2. For chemosensitivity assessments, miR-335 cancer cells and non-transfected cells were administered 5 nM paclitaxel or 2.5 μM doxorubicin. Ten μl of Dulbecco's modified Eagle's medium (DMEM) from each well was replaced with 10μl of 5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Thermo Fisher Scientific) and the incubation continued for another 4h at 37°C. Then, 150μl of DMSO were added to each well for dissolving the formazan crystals. Subsequently, absorbance at 450nm was recorded with microplate photometer (BioTek, Synergy2, USA).

Analysis of cell survival and viability

Cell survival analysis of Hs578T cancer cells transfected with miR-NC, miR-335 mimics or miR-335 inhibitor for 48h was performed through colony formation assay. Cell suspension containing 6000 cells was added to each well of 6-well plates and cells were allowed to grow for a week. The cells were then harvested and washed three times with PBS. Four % paraformaldehyde was used to fix the cells for 20 min. Staining with 0.1% crystal violet was done and colony formation was observed.

For estimating the viability of transfected-Hs578T cancer cells, after fixing with paraformaldehyde, the cells were stained with fluorescent dye DAPI and examined for nuclear fluorescence under fluorescent microscope.

Migration and invasion assay

Transwell chamber with or without matrigel coating was used to assess respectively the invasion and migration of transfected-cancer cells. Briefly, 100μl cell culture containing 6000 cells was added to the upper chamber of transwell and lower chamber was added with 750μl of DMEM supplemented with 10% FBS. After 48h-incubation at 37°C/5%CO_2, cells from the surface of membrane's upper side were removed carefully with cotton swabs, while those sticked to the lower side of membrane were fixed with 70% ethyl alcohol and stained with 0.1% crystal violet. Light microscope (×100) was used for visualization of cells and photographs were taken. At least seven random fields were used for counting of migratory or invasive cells.

Bioinformatics for target identification and luciferase assay

TargetScanHuman7.2 (http://www.targetscan.org/vert_72/) was used to identify the targets of miR-335.

Target identification was further validated through assessment of base complementarity and binding energies performed through microRNA.org (http://34.236.212.39/microrna/home.do) and miRDB (http://www.mirdb.org/) online bioinformatics software tools.

Dual luciferase assay was performed for interactional study of miR-335 with 3'-UTR of MAP3K2. Here, the Hs578T cancer cells were co-transfected either with miR-NC and pGL3-wild type (WT)/mutated (MUT) 3' UTR stretches or miR-335 mimics and pGL3-wild type (WT)/mutated (MUT) 3' UTR of MAP3K2. Next, the measurement of luciferase activity was made through Dual Luciferase Reporter system (Promega Corporation) using *Renilla* luciferase for normalization.

Western blotting

RIPA lysis and extraction buffer (Thermo Fisher Scientific) were used to extract the total proteins from cancer cells. The protein concentration was estimated by Bradford method and from each sample, 45 µg of protein were separated on 12% SDS-PAGE gel and then blotted to PVDF membranes (0.45-µm, Millipor) with 110V for 1 h. Skimmed milk (5%) was used as the blocking solution. PVDF membrane was then incubated with *anti-MAP3K2* (1:500, Abcam) antibody at 4°C overnight. After, it was washed with TBS-T buffer for three times and the PVDF membrane was subsequently incubated with *goat anti-rabbit IgG* (1:3000, Abcam) at room temperature for 1 h. Finally, Efficient Chemiluminescence Western Blotting Substrate (Thermo Fisher Scientific) was used to visualize the protein bands. Human β-actin protein was used as a control in western blotting experiments.

Statistics

Each experiment was performed at least in triplicate. Standard deviation (SD) was calculated and final values were presented as mean±SD. Unpaired t-test was performed and p values ≤0.05 were taken as measure of statistical significance (represented as * or #).
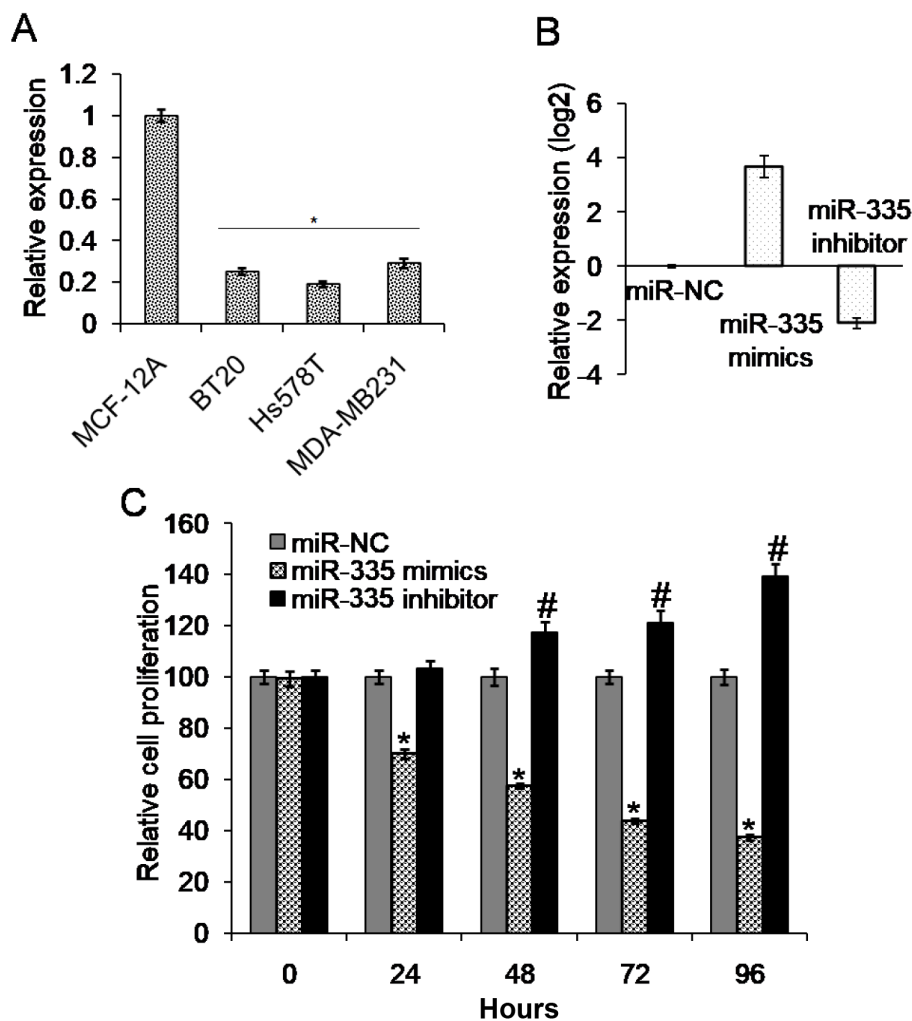
Figure 1. miR-335 negatively regulates breast cancer growth. **A:** Relative expression of miR-335 in breast cancer cell lines (BT20, Hs578T and MDA-MB231) and normal breast epithelial cells MCF12A. **B:** Relative expression of miR-335 in Hs578T cancer cells transfected with miR-NC, miR-335 mimics or miR-335 inhibitor. **C:** MTT assay for determination of cell growth in Hs578T cancer cells transfected with miR-NC, miR-335 mimics or miR-335 inhibitor. The experiments were performed in triplicate and expressed as mean±SD (*p<0.05 for miR-NC vs miR-335 mimics and #p<0.05 for miR-NC vs miR-335 inhibitor).

Results

## *miR-335 is downregulated in breast cancer cells*

qRT-PCR analysis was carried out for determining the relative expression levels of miR-335 in BC cell lines (BT20, Hs578T and MDA-MB231) and normal breast epithelial cell line (MCF-12A). miR-335 was seen to have significantly lower expression in all three cancer cell lines, being the lowest in Hs578T cell line (Figure 1A). This suggested a probable regulatory role of miR-335 in BC.

miR-335 inhibits the cancer cell growth by decreasing cell viability

To infer the regulatory role of miR-335 on BC growth, miR-335 mimics and miR-335 inhibitor together with miR-NC were transfected into Hs578T cancer cells for 48h and stable transfection was confirmed by RT-PCR (Figure 1B). MTT assay was performed following the culturing of transfected cells for 24, 48, 72 and 96h. The cell growth was significantly lower under miR-335 overexpression while it was significantly higher when miR-335
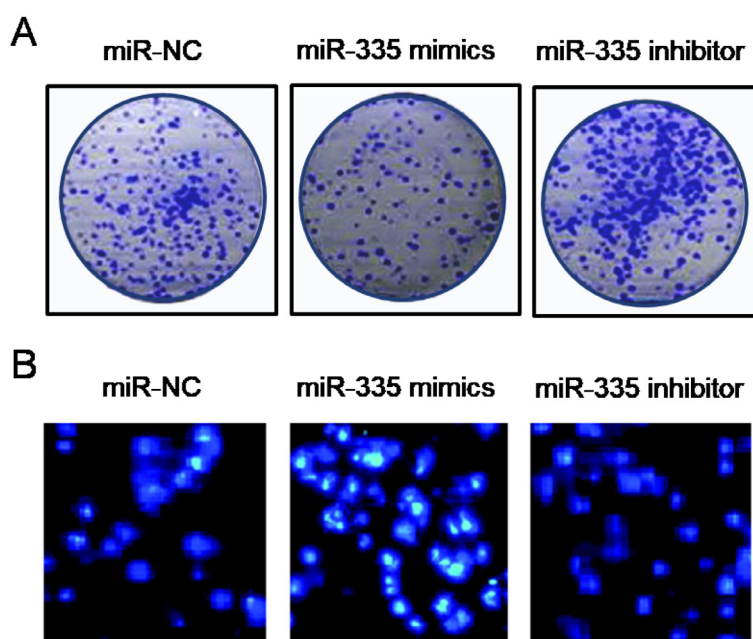
Figure 2. miR-335 decreases the breast cancer cell survival. **A:** Colony forming potential of Hs578T cancer cells transfected with miR-NC, miR-335 mimics or miR-335 inhibitor **B:** Fluorescent microscopic nuclear morphology assessment of DAPI-stained Hs578T cancer cells transfected with miR-NC, miR-335 mimics or miR-335 inhibitor showing miR-335 overexpression induces apoptosis in Hs5787 cancer cells. The experiments were performed in triplicate.
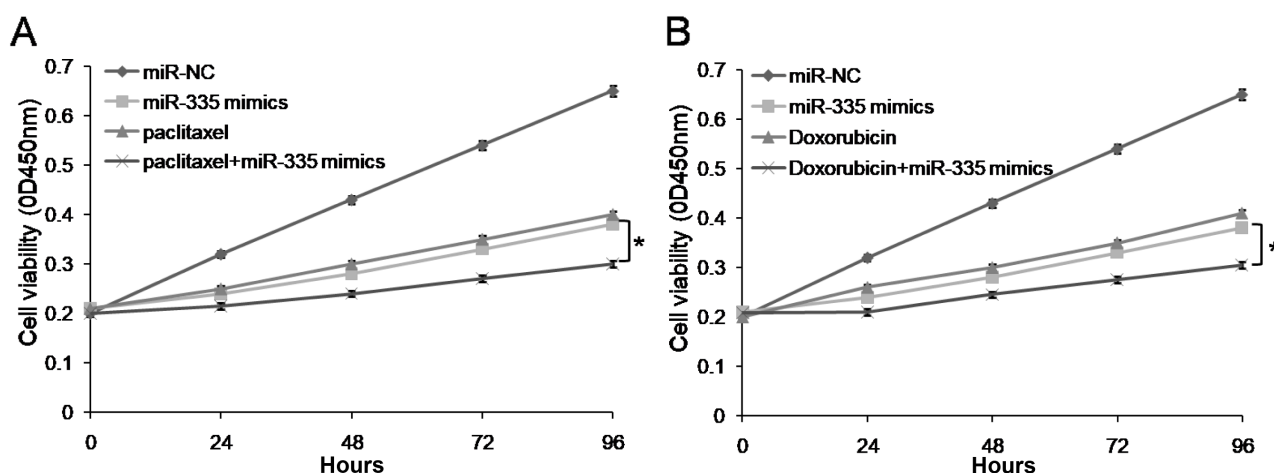
Figure 3. miR-335 enhances the chemo-sensitivity of breast cancer cells. Assessment of proliferation of Hs578T cancer cells transfected with miR-NC, miR-335 mimics, administered **A:** paclitaxel alone and paclitaxel plus miR-335 mimics transfection and **B:** doxorubicin alone and paclitaxel plus miR-335 mimics transfection. The experiments were performed in triplicate and expressed as mean±SD (*p<0.05).

inhibitor was transfected into the cancer cells (Figure 1C). Further, the colony formation assay showed that numbers of cancer cell colonies were decreased when miR-335 was overexpressed, while the colony number was increased significantly under miR-335 downregulation (Figure 2A). Again, the assessment of cell viability by DAPI staining revealed that transfection of miR-335 mimics considerably reduced the cancer cell viability. However, the cell viability was higher under miR-335 inhibitor transfection (Figure 2B). The results clearly indicate that miR-335 negatively regulates the growth of BC cells by decreasing their viability which may be due to its pro-apoptotic regulatory potential.

miR-335 overexpression enhances the chemosensitivity and restricts migration and invasion of Hs578T cells

The sensitivity of Hs578T cancer cells under miR-335 overexpression was determined for paclitaxel and doxorubicin. Assessment of proliferation rate was made through MTT assay for miR-NC and miR-335 mimics-transfected cells along with paclitaxel (5 nM) were administered untransfected and miR-335 mimics-transfected cancer cells. Cancer cell proliferation was lowest under miR-335 overexpression plus paclitaxel treatment and interestingly, it was significantly lower when compared to paclitaxel treatment alone (Figure 3A). Similar observation was made for doxorubicin administration (Figure 3B). Together, the results are indicative of that miR-335 has a potential to increase the chemosensitivity of BC cells to drug treatment and thus strongly advocate the application of chemotherapeutics against human BC.

Migratory and invasive potential of Hs578T cancer cells under miR-335 overexpression and downregulation was determined through transwell chamber assay using miR-NC transfected cells as negative control. Both the migration and invasion of cancer cells were decreased significantly under miR-335 overexpression but the downregulation
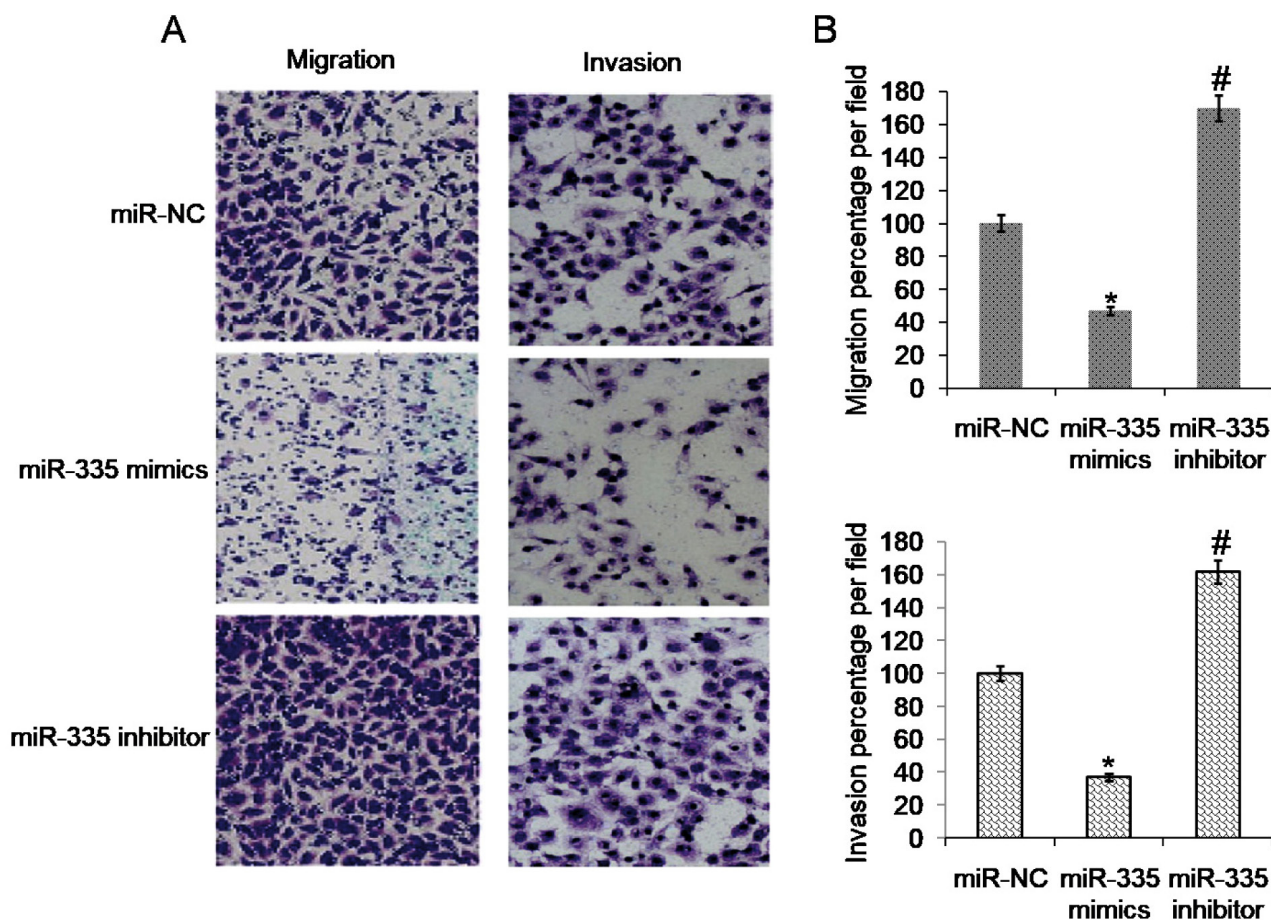
Figure 4. miR-335 reduces breast cancer cell migration and invasion. **A:** Transwell assay for analysis of migration and invasion of Hs578T cancer cells transfected with miR-NC, miR-335 mimics or miR-335 inhibitor showing miR-335 overexpression inhibits cell migration and invasion. Bar graphs showing the percentage of cell migration and invasion of Hs578 T cells. The experiments were performed in triplicate and expressed as mean±SD (*p<0.05 for miR-NC vs miR-335 mimics and #p<0.05 for miR-NC vs miR-335 inhibitor). **B:** Percentage of cell migration and invasion (p<0.05).

of miR-335 was seen to enhance the cell migration and invasion (Figure 4A). The migration and invasion of cancer cells was reduced below 50% with miR-335 overexpression and both parameters were increased by more 5-fold under miR-335 downregulation when compared with the miR-NC control-transfected cells (Figure 4B). Thus, it can be inferred that miR-335 negatively regulates the migration and invasion of BC cells, with a potential to restrict the cancer metastasis.
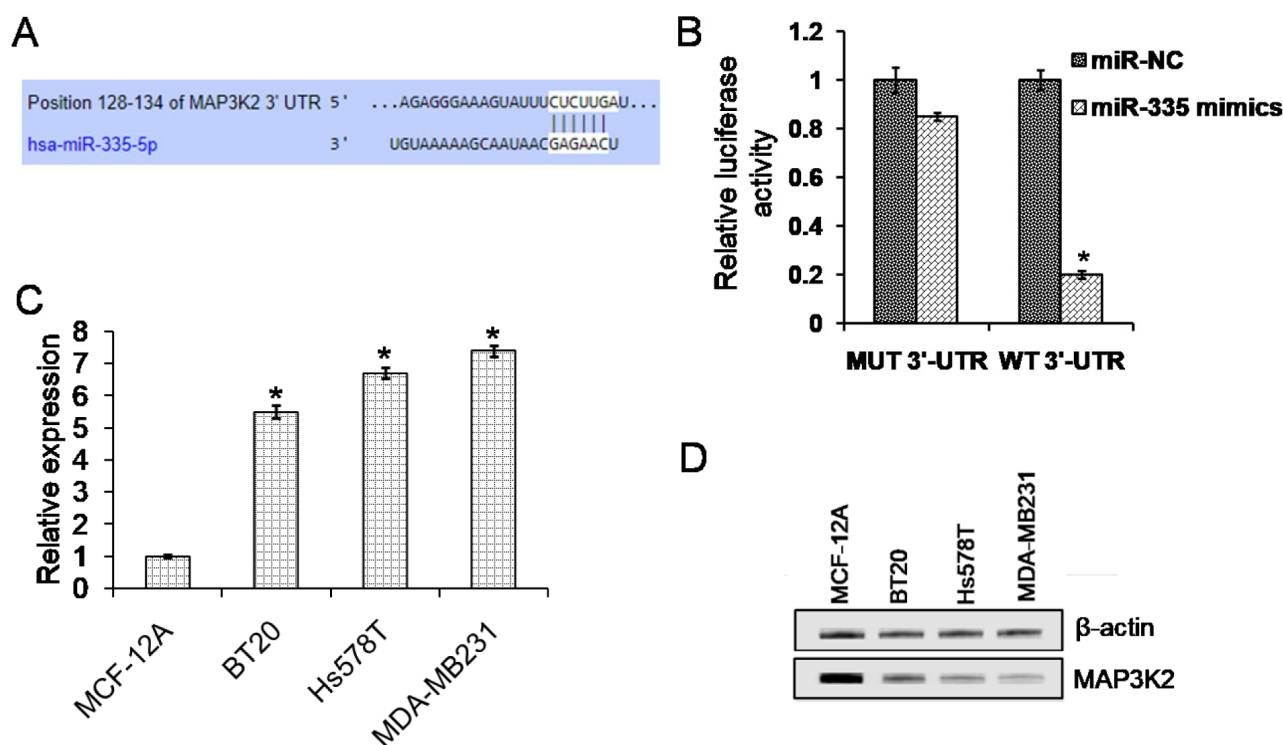
MAP3K2 is the target gene of miR-335

Taking into consideration the sequence complementarity and binding energy parameters, the online bioinformatics software tools predicted MAP3K2 as a target gene of miR-335. A 7-mer nucleotide sequence, CUCUUGA in 3'-UTR of MAP3K2 was identified as the complementary binding site of hsa-miR-335-5p (Figure 5A). To validate the target prediction, wild-type (WT) and mutated (MUT) 3'-UTR stretches of MAP3K2 were designed and their interaction with miR-335 was determined using dual luciferase reporter assay. Interaction of miR-335 with WT 3'-UTR of MAP3K2 was confirmed by a significant lower luciferase activity
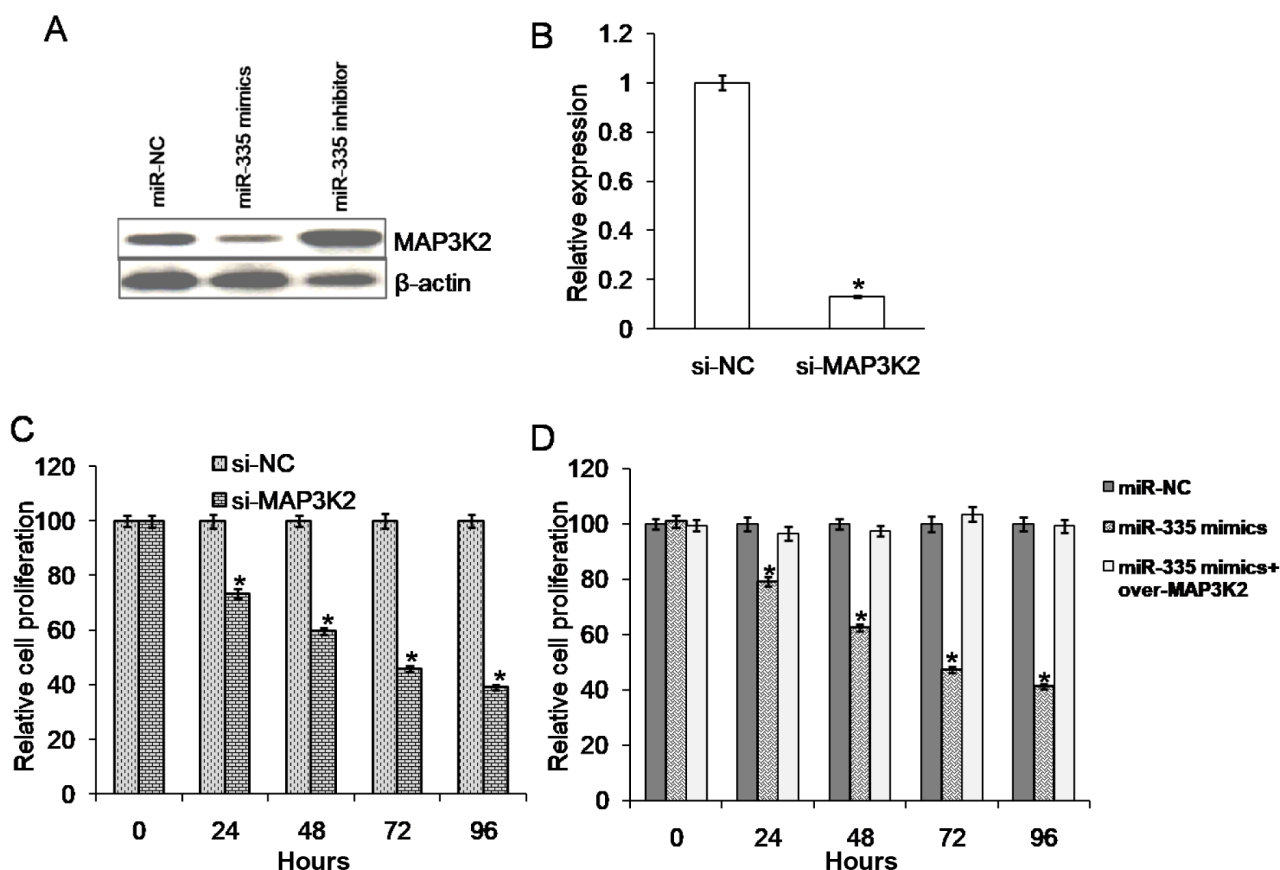
in cancer cells transfected with miR-335 mimics and pGL3-WT 3'-UTR (Figure 5B). The cancer cells exhibited very high luciferase activity when co-transfected with miR-mimics and pGL3-MUT 3'-UTR of MAP3K2. Further confirmation was drawn from the gene and protein expression studies of MAP3K2 in three different BC cell lines and normal epithelial cells whereby both cancer cell lines exhibited lower mRNA and protein levels (Figure 5C and D). The results were further supported by western blotting of MAP3K2 in miR-335 mimics and miR-335 inhibitor-transfected Hs578T cancer cells, which exhibited very low and high MAP3K2 protein levels, respectively when compared with those of miR-NC-transfected cancer cells (Figure 6A). Taken together, the results reveal that miR-335 represses MAP3K2 post-transcriptionally by interacting with its 3'-UTR in a sequence-specific manner.

MAP3K2 repeals the inhibitory effect of miR-335 on cancer cell growth

The knockdown of MAP3K2 gene in Hs578T cancer cells was performed through RNA interference by transfecting the cancer cells with si-

Figure 5. MAP3K2 is the target gene of miR-335. **A:** TargetScan analysis of miR-335 interaction with target gene, MAP3K2. **B:** Relative luciferase activity of Hs578T breast cancer cells co-transfected with miR-NC and pGL3-MUT 3'-UTR/pGL3-WT 3'-UTR or miR-335 mimics and pGL3-MUT 3'-UTR/pGL3-WT 3'-UTR. **C:** RT-PCR expression analysis of MAP3K2 in breast cancer cell lines (BT20, Hs578T and MDA-MB231) and normal breast epithelial cells, MCF12A. **D:** Western blotting of MAP3K2 in breast cancer cell lines (BT20, Hs578T and MDA-MB231) and normal breast epithelial cells MCF12A with human β-actin protein as control for normalization. The experiments were performed in triplicate and expressed as mean±SD (*p<0.05).

Figure 6. miR-335 regulates the breast cancer growth by targeting MAP3K2. **A:** Expression of MAP3K2 protein in Hs578T cancer cells transfected with miR-NC, miR-335 mimics or miR-335 inhibitor with human β-actin protein as normalization control. **B:** Relative gene expression of MAP3K2 in Hs578T breast cancer cells transfected with si-NC or si-MAP3K2. **C:** Relative proliferation (in percents) of Hs578T breast cancer cells transfected with si-NC and si-MAP3K2. **D:** Relative proliferation (in percents) of Hs578T breast cancer cells transfected with miR-NC, miR-335 mimics or mir-335 mimics+ pcDNA-MAP3K2. The experiments were performed in triplicate and expressed as mean±SD (*p<0.05

MAP3K2 RNAi construct. MAP3K2 knockdown or repression was confirmed by RT-PCR analysis and MAP3K2 relative expression was seen to be significantly lower compared to si-NC-transfected control cells (Figure 6B). The proliferation rate was also determined for si-MAP3K2-transfected cells using and compared (in percentage terms) with MAP3K2 silencing control cells. The si-MAP3K2 cancer cells exhibited significantly lower proliferation and effects were same as under miR-335 overexpression (Figure 6C). Again, when the pcDNA-MAP3K2 overexpression vector construct of MAP3K2 was co-transfected with miR-335 mimics into Hs578T BC cells, the anticancer growth effects of miR-335 overexpression were seen to be reversed and cancer cells exhibited the proliferation rates similar to those of miR-NC-transfected control cells (Figure 6D). Collectively, the results infer that the antiproliferative effects of miR-335 against breast cancer are modulated through translational repression of its target gene (i.e., MAP3K2) and the enrichment of MAP3K2 expression levels revert the anticancer growth effects of miR-335 in BC cells.

Discussion

The regulatory role of micro RNAs, discovered for the first time in *Caenorhabditis elegans* worm, has made lots of excitement in recent years. They are not only believed to regulate the overall development of animals but also are shown to possess profound role in fine designing of cellular fate and differentiation [22-24]. Disease development in animals is one of the prime aspects of animal biology falling under the regulation of this important group of regulatory RNAs. The dysregulation of micro RNAs has been shown to influence the onset and proliferation of almost all human cancers and researchers are actively involved in elucidating the role of micro RNAs in different human cancers to understand the molecular mechanics which lead to deviation of controlled cellular plan and initiate the development of cancer growth [25]. Studies on BC have enlightened the involvement of a number of micro RNAs in growth and development of this malignancy [26]. Micro RNA-335 (miR-335) has been implicated

as a prognostic factor in gastric cancer and was shown to be repressed in cancer tissues [27]. In yet another report, the epigenetic silencing of miR-335 was seen to be involved in hepatocellular carcinoma [28]. Here, we also report the downregulation of miR-335 in BC cells. A previous report has revealed that miR-335 is critical to BRCA 1 cascade in BC [29]. miR-335 has also been shown to be significant for the tumorigenesis of gastric cancer [30]. The results of this study also suggest similar type of regulation in BC and miR-335 was seen to negatively regulate the BC cell growth and its overexpression decreased the cancer cell viability. Paclitaxel and doxorubicin are some of the recommended drugs for treating BC according to NIH. When BC cells were administered paclitaxel and doxorubicin treatments along with miR-335 overexpression, the cancer cell growth was significantly affected and it was very low compared to the drug treatment only. Similar type of enhancement of chemosensitivity of BC towards cisplatin and docetaxel was noted when miR-27a was overexpressed in cancer cells [31]. Hence, our study explored miR having potential to increase the chemosensitivity of BC cells towards the administration of anticancer drug molecules. miR-335 was previously shown to inhibit the metastasis of lung cancer to bone tissue via the IGF-IR and RANKL pathways [32]. Further, it also suppressed the invasion of ovarian cancer cells by targeting Bcl-w [33]. Here we also depicted similar role of miR-335 and it was seen to considerably reduce the migration and invasion of Hs578T BC cells. miR-355 was shown to target mitogen-activated protein kinase kinase kinase 2 (MAP3K2) by *in silico* analysis. MAP3K2 is a protein kinase and belongs to serine/threonine kinase family and is involved in MAPK signaling pathway. MAPK signaling pathway is essential to normal cell processes like proliferation, growth, differentiation, migration and apoptotic cell death [34]. The mutations in MAPK signaling components are associated with cancer development [35,36]. MAP3K2 was found to be targeted by miR-186 and miR-520a-3p in lung cancer to exert their anticancer regulatory role [37,38]. The miR-335 targeting MAP3K2 was also seen to modulate the anticancer effects of miR-335 in this study as confirmed by knockdown and overexpression of MAP3K2 in BC cells. To sum up, the study explores the anticancer role of miR-335 against the BC proliferation together with its regulatory potential to enhance the chemosensitivity of cancer cells and to reduce the cancer metastasis through targeting MAP3K2.

Conclusion

In sum, the results of this study revealed miR-335 as a potential molecular marker and anticancer regulator of BC, with a key finding of chemosensitivity enhancement of cancer cells by miR-335. The study strongly advocates the employment of combinatorial administration of anticancer approaches in the management of BC and may thus have a leading role to conducting more such studies in human cancers in the future.

Conflict of interests

The authors declare no conflict of interests.

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