Original Article

Restoration of miR-451a-5p/miR-34a-5p could suppress the proliferation and migration of human breast cancer cells through Wnt/β- catenin and ERK/P-ERK signaling pathways

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ABSTRACT

MicroRNAs (miRNAs) are a class of small non-coding RNAs with a length of 21–25 nucleotides and play an essential role in the regulation of cancer initiation, development and progression. Breast cancer (BC) is the most commonly detected malignancy in women and one of the leading causes of death worldwide. In this study, the effects of transfection of microRNA-451a-5p and miR-34a-5p (tumor suppressors), individually and in combination on apoptosis, proliferation and migration of breast cancer cells *in vitro* were investigated. For this study, malignant breast cancer cells (MDA-MB-231) were transfected with the miR-451a-5p and miR-34a-5p mimics. Subsequently cytotoxicity, apoptosis, proliferation, migration protein and gene expression of caspase-3, caspase-8, MMP9, ROCK, vimentin and c-Myc of the cancer cells were analyzed by MTT, flow cytometry, q-RT-PCR (expression level of caspase-3, caspase-8, MMP9, ROCK, vimentin and c-Myc genes), wound healing and Western blot assays. The results showed that miR-34a-5p and miR-451a-5p could additionally induce apoptosis and cell cycle arrest in the sub-G1phase, suppress proliferation and migration in breast cancer cells, and also decrease the expression of β- catenin and ERK/P-ERK proteins . The present data document that restoration of the tumor suppressor miR-451/miR-34 strongly induces programmed cell death *in vitro* and apparently inhibits cell proliferation and migration in human breast cancer cells. In summary, miR-451a and miR-34a play an important role in breast cancer cell proliferation and migration via the Wnt/β-catenin and ERK/P-ERK signaling pathways. Therefore, the simultaneous restoration of the presented tumor suppressor miRNAs can be proposed as a valuable and potential therapeutic strategy in the treatment of breast cancer. However, further studies should be useful.

Keywords: Apoptosis, Breast cancer, miR-451, miR-34, Migration.

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1. Introduction

Of all fatal cancers, breast cancer (BC) remains the most commonly detected malignancy in women and a leading cause of death worldwide (1). Currently, several BC treatment options are invasive, expensive, and ineffective, especially for triple-negative breast cancer (TNBC). TNBC is an exclusively offensive subtype characterized by extremely high metastatic spread, progression, poor prognosis, drug resistance and a lack of obvious therapeutic alternatives (2). The identification of novel molecular biomarkers, therapeutic approaches and understanding of molecular pathogenesis would therefore be of great benefit in improving clinical care and early detection of BC (3,4). Recent evidence suggests that epigenetic alterations, such as DNA methylation, histone modifications, chromatin remodeling and non-coding RNAs may play an important role in cancer development (5). Non-coding RNAs (ncRNAs) are known to be involved in cancer by regulating various signaling pathways (6). MiRNAs are small ncRNAs with a length of almost 22 nucleotides that can act as negative modulators of specific target genes by interacting with the3′UTR (3' untranslated region). Dysregulation of miRNAs triggers a variety of human diseases (7), and these tiny molecules may be useful as potential diagnostic biomarkers, prognostic tools, and promising therapeutic targets (7,8). Numerous studies have identified the various functions of miRNAs in the pathogenesis of most, if not all, human malignancies by being involved in cell proliferation, angiogenesis, invasion and metabolism by targeting oncogenes or tumor suppressor genes (9). The miR-451 family consists of two major members, including hsa-miR-451a and hsa-miR-451b, which are located on chromosome 17 in the human genome and are involved in tumorigenesis and/or tumor progression, including BC (10). Interestingly, the three members of the miR-34 family, miR-34a, miR-34b and miR-34c, are encoded by two different genes. The miR-34a gene is located on chromosome 1, while the miR-34b and miR-34c homologs are found on chromosome 11. miR-34a plays a tumor-suppressive role in BC, making this miRNA a valuable diagnostic, prognostic and therapeutic tool (11). Therefore, the main objective of this study was to investigate the effects of miR-451a-5p, miR-34a-5p and the combination of miR-451a-5p/miR-34a-5p on growth inhibition and apoptosis induction in BC cells *in vitro*.

2. Materials and Methods

2.1. Cell culture

The human BC cell lines (MDA-MB-231, MDA-MB-468, MCF-7 and SKBR3) were purchased from the National Cell Bank, Pasteur Institute, Tehran, Iran. All cells were routinely maintained in RPMI 1640 (GIBCO, USA) with 10% FBS (GIBCO, USA) and 1% penicillin/streptomycin (GIBCO, USA) under standard conditions in a controlled humidified atmosphere of 5% CO2 and 37 °C. Since MDA-MB-231 cells showed the lowest expression levels of miR-451a-5p and miR-34a-5p, this metastatic BC cell line was selected for subsequent analysis.

2.2. Electroporation of miRNAs

The nucleotide sequence of miR-451a-5p (AAACCGUUACCAUUACUGAGUU) and miR-34a-5p (UGGCAGUGUCUUAGCUGGUUGU) was taken from http:// guanfiles.dcmb.med.umich.edu/mirmine. Subsequently, FITC-conjugated controls, miR-451a and miR-34a mimics (Microsynth AG, Switzerland) were transfected into MDA-MB-231 cells at two different concentrations of 50 and 100 pmol using electroporation technique via Gene Pulser Xcell (Bio-Rad, USA) at a voltage of 160 V for 25 ms. Subsequently,1×10^6 transfected BC cells were seeded into each well of six-well plates. After three different incubation times (24, 48 and 72 hours), the relative expression of miRNAs was assessed by qRT-PCR to determine the optimal time and dose. The MACSQuant 10 flow cytometer system (Miltenyi Biotec, Germany) was used to evaluate the efficacy of FITC-conjugated miRNA transfection.

2.3. Gene expression

The expression levels of selected genes including caspase -3, caspase -8, ROCK, MMP-9, vimentin and c-Myc, were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The online primer sequence design tool on the NCBI website was used to design the primers (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). In brief, total RNA was extracted from the cultured cells using TRIzol reagent (RiboEx Kit, GeneAll, South Korea) according to the manufacturer's recommendations. The quality and quantity of the isolated RNAs were analyzed by measuring the absorbance at 260 and 280 nm with NanoDrop (Thermo Scientific, USA). Complementary DNA (cDNA) was then synthesized using a commercial kit (Biofact, South Korea) according to the manufacturer's instructions. The miRNA Reverse Transcription Kit (BonMir, Iran) was used for cDNA synthesis to determine the expression levels of miR-451a-5p/-34a-5p. Reactions were performed using a qRT-PCR system with SYBR Premix Ex Taq (Biofact, South Korea) and a StepOne Plus real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, USA). GAPDH gene expression was used to normalize the expression of target genes, and U6 was selected as an internal endogenous control for miRNAs. The sequences of primers used in the present study are listed and shown in Table 1.

Table 1. Sequences of the primers used in the present study.

Gene Name	Nucletide sequeces (5′−3′)
c-Myc	For-AGGCTCTCCTTGCAGCTGCT Rev-AAGTTCTCCTCCTCGTCGCA
Rock	For-CTCCCTGTGTCAGACTGCTCTTT Rev-GGCCTTGCAACCTTGGTCTCTTC
Caspase-3	For-TGTCATCTCGCTCTGGTACG Rev-AAATGACCCCTTCATCACCA
Caspase-8	For-TGAAAAGCAAACCTCGGGGA Rev-TGAAGCTCTTCAAAGGTCGTG
MMP-9	For-TTGACAGCGACAAGAAGTGG Rev-GCCATTCACGTCGTCCTTAT
vimentin	For-AATCGTGTGGGATGCTACCT Rev-CAGGCAAAGCAGGAGTCCA
GAPDH	For-CAAGATCATCAGCAATGCCT Rev-GCCATCACGCCACAGTTTCC
U6	For-CTTCGGCAGCACATATACTAAAATTGG Rev-TCATCCTTGCGCAGGGG

2.4. Wound-healing assay (scratch test)

The wound-healing assay was performed to determine the effects of miR-451a and miR-34a on cellular migration. For this purpose, cells (15×10^4 cells per well) were seeded in 24-well plates and then incubated under standard conditions. Subsequently, wounds were created in monolayers of cells using a 200 µl pipette tip in the center of each well. Subsequently, the wells were photographed 0, 24 and 48 hours after wounding using an inverted microscope (Optika, Italy).

2.5. Cell viability assay

To understand the cytotoxic effect of the miRNA combination on BC cells, an MTT (3- (4, 5-dimethylthiazol-2yl) -2, 5-diphenyltetrazolium bromide) assay (Sigma-Aldrich, Germany) was performed. A total of MDA-MB-231 cells were seeded at a density of 12×10^3 per well in 96-well plates and incubated for 24 and 48 h after transfection. Subsequently, 50 µl MTT solution (2mg/ml) was added to the wells after the culture medium was removed. The cells were then incubated for an additional 4 h. After that, the culture medium containing MTT was discarded and 200 µl of DMSO (Sigma-Aldrich) was added to each well and kept at 37°C for another 30 min . The ocular density of each well was measured at 570 nm using a Sunrise ELISA reader (Tecan, Switzerland). All tests were performed in triplicate.

2.6. Quantitative apoptosis analyses

Annexin -V-FITC and propidium iodide (PI) double staining kit (Exbio, Czech Republic) was used to quantitatively assess the rate of induction of programmed cell death by miR-451a-5p/-34a-5p. After transfection of miRNAs, cells were seeded at a density of 1×10^6 per well in 6-well plates in four groups as follows: control, miR-451a-5p, miR-34a-5p, and miR-451a-5p/-34a-5p. After incubation at 37 °C for 24 and 48 hours, BC cells were detached from the plates with trypsin /EDTA solution, washed with PBS (3×), suspended in 500 µl binding buffer, and then treated with 5 µl FITC-conjugated annexin-V and 5 µl PI for 15 minutes on ice in the dark. The apoptosis rate of each experimental group was evaluated using a MACSQuant 10 flow cytometer system (Miltenyi Biotec, Germany), and the data presented were analyzed using FlowJo software version 7.6 (FlowJo, USA).

2.7. DAPI staining (a non-quantitative apoptosis assay)

To evaluate the quality of programmed cell death induction 4′, 6-diamidino-2-phenylindole (DAPI) staining was performed. In brief, 12×10^3 BC cells were seeded in a 96-well plate and categorized into control, miR-451a-5p, miR-34a-5p and miR-451a-5p/-34a-5p groups. After a 48-hour incubation, the transfected cells were washed with PBS (3×) and then fixed with paraformaldehyde for 2-4 hours. After another wash with PBS, 0.1% Triton X100 was added to each well and the treated cells were incubated for another 15 min. After another PBS wash, the cells were stained with 100 µl DAPI solution for 10 min at RT and in the dark conditions. Finally, 200 µl of PBS was added to each well for the last wash, and the apoptotic cells with fragmented chromatin were assessed using the Cytation 5 live imaging system (Biotech, USA).

2.8. Cell cycle analysis

In the current study, the flow cytometry assay was also used to detect the different phases of the cell cycle. In this way, BC cells were seeded at a density of 1×10^6 cells per well in 6-well plates, divided into the same groups as in previous assays, harvested with trypsin and washed with PBS. Subsequently, 1 ml of 75 % ethanol was added to each group for cell fixation, and the treated cells were incubated overnight at -20 °C. After a further PBS wash, the cells were incubated for 30 min at 37 °C in a phosphate buffer solution containing 1% RNase A. Then, the treated cells were stained with DAPI solution (0.1% DAPI (1 mg/ml), 0.1% Triton X100 in PBS) for 10 minutes in the dark. Finally, cell cycle arrest was evaluated using the MACSQuant 10 flow cytometer system, and data were analyzed using FlowJo software.

2.9. Immunoblotting analysis

Western blot analysis was performed to determine the effects of miR-451a-5p/-34a-5p on ERK1/2, p-ERK and β-catenin protein expression. In brief, following the recommended protocol, total protein was isolated using Radio Immuno Precipitation Assay (RIPA) buffer (Santa Cruz, USA). Subsequently, the isolated proteins were separated by SDS-PAGE electrophoresis and then transferred to a PVDF membrane (polyvinylidene difluoride) blotting (Roche, UK). PVDF membranes were blocked for 2 h in milk blocking buffer (2% nonfat dry milk in TBST) on a shaker at room temperature and then treated with monoclonal anti-ERK1/2 (1:300, sc-292838), p-ERK (1:300, sc-16981-R) and β-catenin (1:300, sc-7963) antibodies for 16-18 hours. The membranes were then incubated with mouse anti-rabbit IgG-HRP (1:1000, sc-2357) as a secondary antibody for 2 hours at RT. Finally, the membranes were placed in ECL prime Western blotting detection reagent (Amersham, UK) to visualize the protein bands. The signal intensity of each band was analyzed using ImageJ 1.62 software (National Institutes of Health, USA). All antibodies used in this study were purchased from Santa Cruz (USA), and β-actin (1:300, sc-47778) was used as a reference protein.

2.10. Statistical analysis

Each experiment was performed in triplicate. The mean value of replicates was determined for each experiment, which was used for further statistical analysis. Data were analyzed with GraphPad Prism 6 software using nonparametric one-way ANOVA and t-test. All data were normalized to the control values of the individual assays and are expressed as mean ± SD (standard deviation). The $p<0.05$ was considered statistically significant.

3. Results

3.1 Cell line selection, effective dosage, and ideal time determination

As shown in Figure 1A and B, both miR-451a-5p and miR-34a-5p were downregulated in BC cell lines, such as MDA-MB-468, MDA-MB-231, MCF-7 and SKBR3. Due to the remarkable downregulation of miR-451a-5p and miR-34a-5p in MDA-MB-231 compared to other BC cells, they were selected for further experiments. The MDA-MB-231 cells were successfully transfected with FITC-conjugated control miRNAs. The transfection efficiency of the BC cells was approximately 98.1%as measured by flow cytometry (Figure 1C). The effective dose and ideal time spans for miR-451a-5p and miR-34a-5p were determined by *qRT-PCR*. According to the qRT-PCR results (shown in Figures 1D and E), 100 pmol was considered the most effective concentration for both miRNAs ($P < 0.0001$). In addition, transfection was most effective after 24 h (for miR-451a-5p) and 48 h (for miR-34a-5p) ($P < 0.0001$) (Figure 1F and G).

3.2 miR-451a-5p and miR-34a-5p replacement effects notably on cell viability and apoptosis induction

As shown in Figure 2A, cell viability was reduced by individual replacement of miR-451a-5p and miR-34a-5p compared to the control group ($p< 0.0001$). In addition, the cell survival rate was significantly lower in the group of cells treated with the miR-451a/34a combination compared to the control group($p < 0.0001$). We then investigated the effects of co-treatment with miR-451a-5p and miR-34a-5p on the induction of programmed cell death. The results of DAPI staining and flow cytometry showed that transfection of miR-451a-5p and miR-34a-5p alone and/or in combination could significantly ($p < 0.0001$) decrease cell survival and increase apoptosis, resulting in nuclear fragmentation in the MDA-MB-231 breast cancer cell line (Figures 2B and C). Interestingly, upregulation of miR-451a-5p and miR-34a-5p affected the expression of apoptosis-related genes, including caspase-3 ($p < 0.001$) and caspase-8 ($p < 0.0001$). As shown in Figures 2D and E, significant overexpression of caspase-3 and caspase-8 was detected in the groups treated with either miRNAs or both compared with the control group.

3.3 miR-451a-5p and miR-34a-5p transfection reduced the breast cancer cell migration rate

The results of the wound-healing assay showed that restoration of miRNA-451a-5p and -34a-5p either alone or in combination could inhibit the motility and migration of MDA-MB-231 breast cancer cells. Compared to the negative controls, the co-treatment showed a significant inhibitory effect on cell migration after 48 h (Figure 3A).

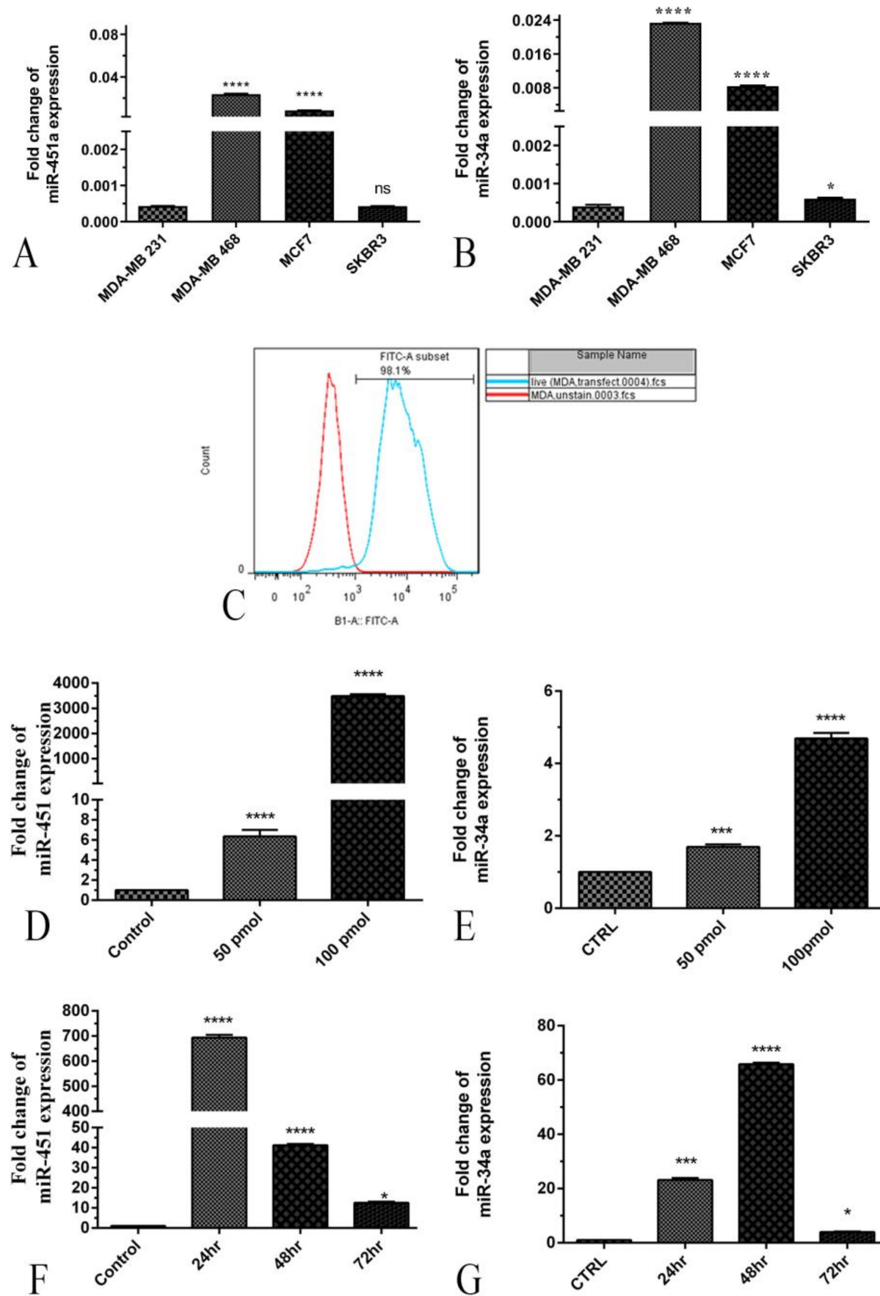

Figure 1: The relative expression levels of miR-451a-5p (A) and miR-34a-5p (B) in BC cell lines in comparison. (****P < 0.0001, *P < 0.05, ns = no significant). (C) Flow cytometry was used to assess the transfection efficacy. According to the flow cytometry results, the transfection rate of MDA-MB-231 cells was 98.1%. (D) The selected concentration of miR-451 mimic was 50 pmol (****P < 0.0001). (E) The selected concentration of miR-34a mimic was 100 pmol (****P < 0.0001). (F) The ideal time span of miR-451 mimic transfection to the MDA-MB-231 cell line (****P < 0.0001) was 24h. (G) The ideal time span of miR-34a mimic transfection to the MDA-MB-231 cell line was 48h (****P < 0.0001).

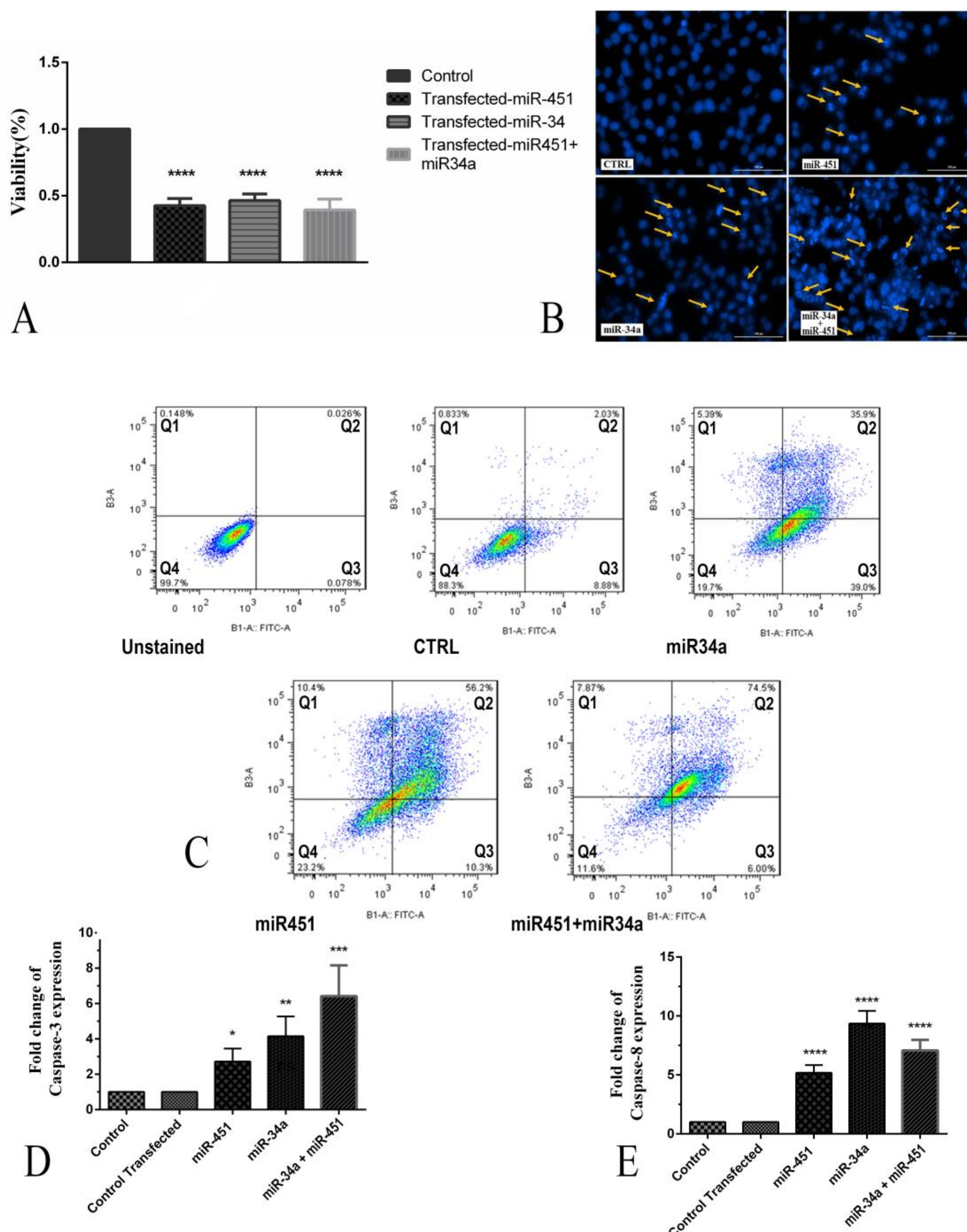

Figure 2: (A) miR-451 and miR34a individually and synergistically reduced the MDA-MB-231 cells proliferation by MTT assay. (B) DAPI staining was performed to detect chromatin fragmentation. (C) The ratio of apoptotic cells was determined by flow cytometry assay in MDA-MB-231 cells under various treatments. Simultaneous treatment with miR-451a-5p and -34a-5p synergistically enhanced the apoptosis of BC cells. A diagram can be divided into four regions that are defined as follows: the percentage of necrotic cells (Q1: PI/FITC +/-); the percentage of late apoptotic cells (Q2: PI/FITC +/+); the percentage of early apoptotic cells (Q3: PI/FITC -/+); the percentage of viable cells (Q4: PI/FITC -/-). The effects of upregulation of miRNA-451a-5p and -34a-5p alone and in combination with each other on the expression of caspase-3 (D) and caspase-8 (E).

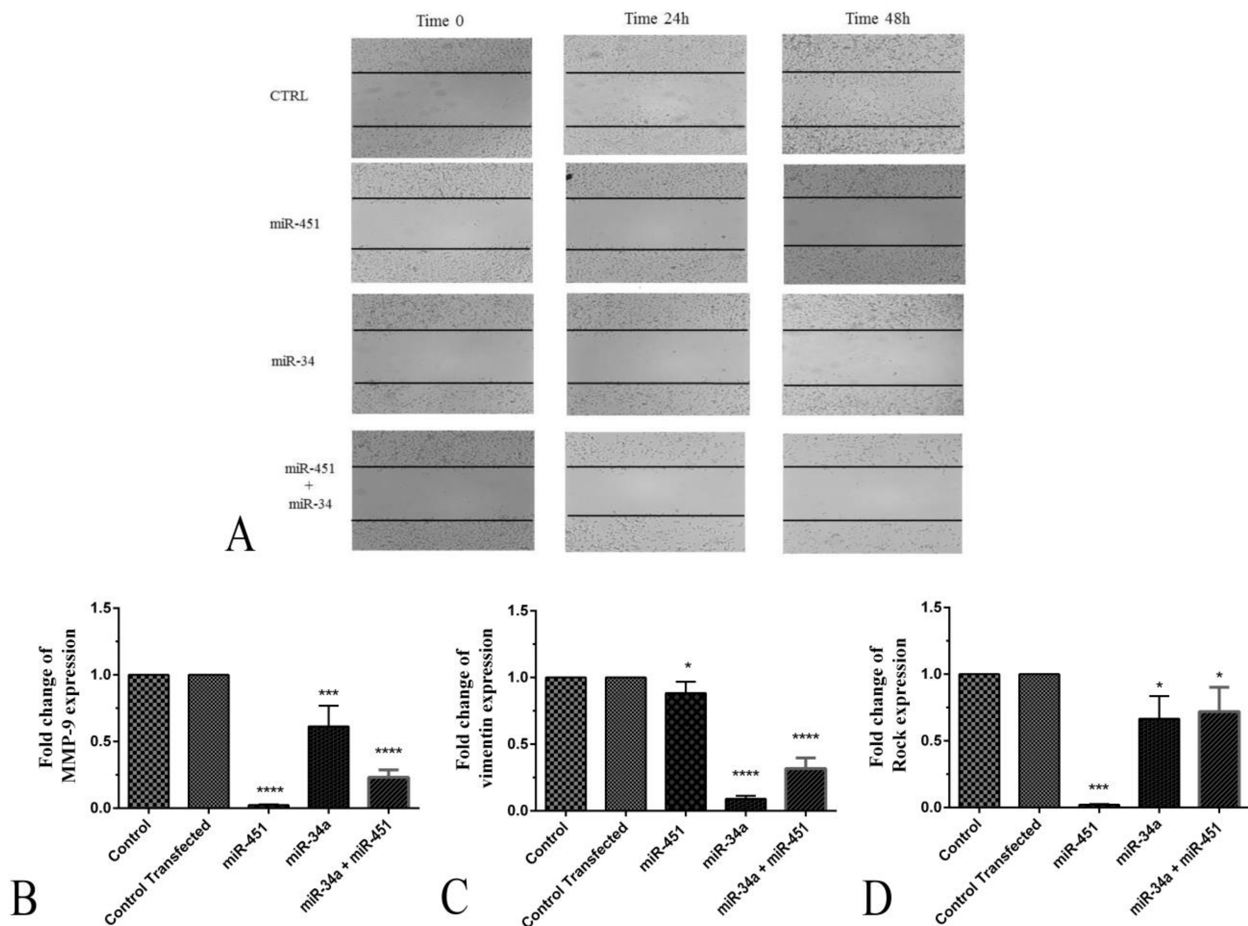

Figure 3: (A) migration ability of BC cells notably reduced in each treatment. (B) The effects of miR-451a-5p and -34a-5p transfection alone and in combination with each other on the expression of metastasis-associated genes, MMP9 (B), Vimentin (C), and ROCK (D) compared with the control group (*$p < 0.05$ ***$p < 0.001$, ****$p < 0.0001$).

According to the qRT-PCR results, the expression of metastasis-associated genes, MMP9, vimentin, and ROCK was significantly ($p<0.05$) decreased after transfection with miR-451a-5p or miR-34a-5p and the combination treatment compared with the control group (Figures 3B, C, and D).

3.4 miR-451a-5p and miR-34a-5p upregulation arrested the cell cycle in sub-G1-phase

The flow cytometry results showed that the restoration of miR-451a-5p and miR-34a-5p arrested the cell cycle in sub-G1phase. As shown in Figure 4A, the combination of miRNA-451a-5p/34a-5p increased the population of cells in the sub-G1phase from 0.106% to 69.6% compared to the control group. To confirm the cell cycle results, the expression of c-Myc , an important cell cycle promoter oncogene , was examined in MDA-MB-231 cells. The qRT-PCR results showed that treatment of MDA-MB-231 cells with miR-451a-5p or miR-34a-5p, and in combination significantly ($p < 0.0001$) down-regulated c-Myc mRNA expression levels compared to negative controls (Figure 4B).

3.5 miR-451a-5p/-34a-5p combination regulated Wnt/β-catenin pathway through decreasing the expression levels of β-catenin protein

According to the Western blot results (Figures 5A and B), miR-34a-5p transfection did not cause significant changes in β-catenin protein expression levels. However, miR-451a-5p alone or in combination with miR-34a-5p resulted in significant ($p<0.0001$) downregulation of β-catenin protein levels.

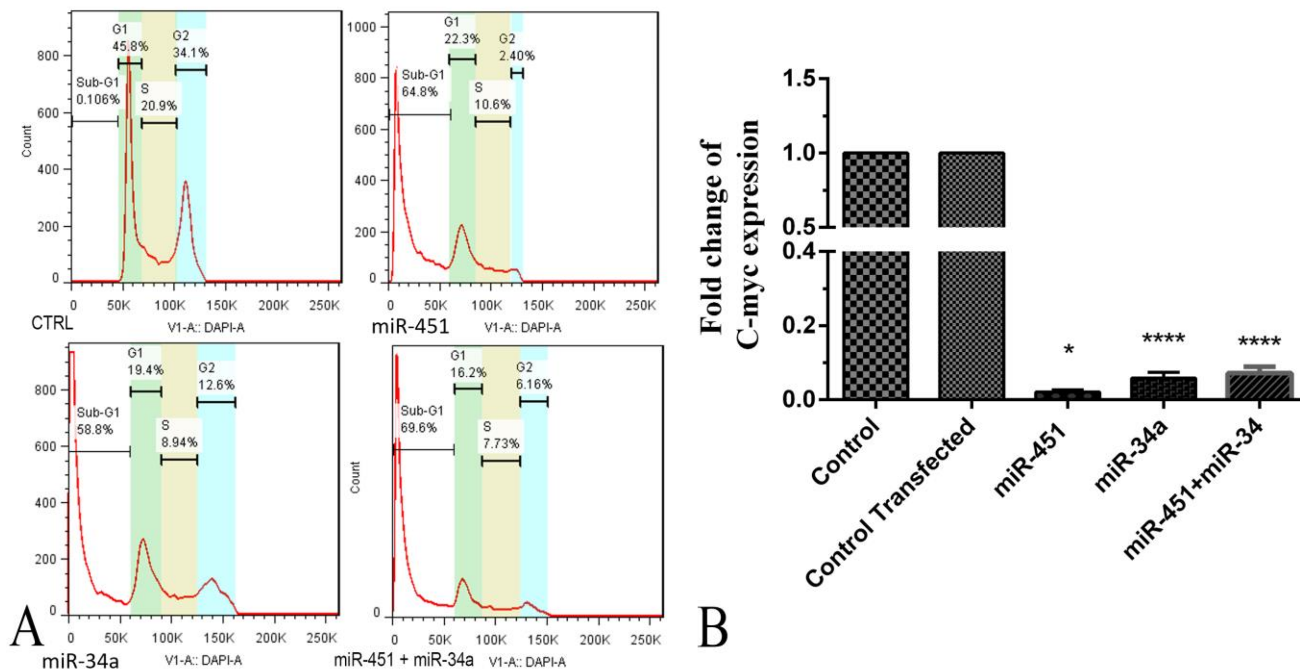

Figure 4: (A) Sub G1 arrest was observed by the FCM technique. (B) The impacts of miRNA-451a-5p/-34a-5p on the expression of c-Myc as a key cell cycle promoter.

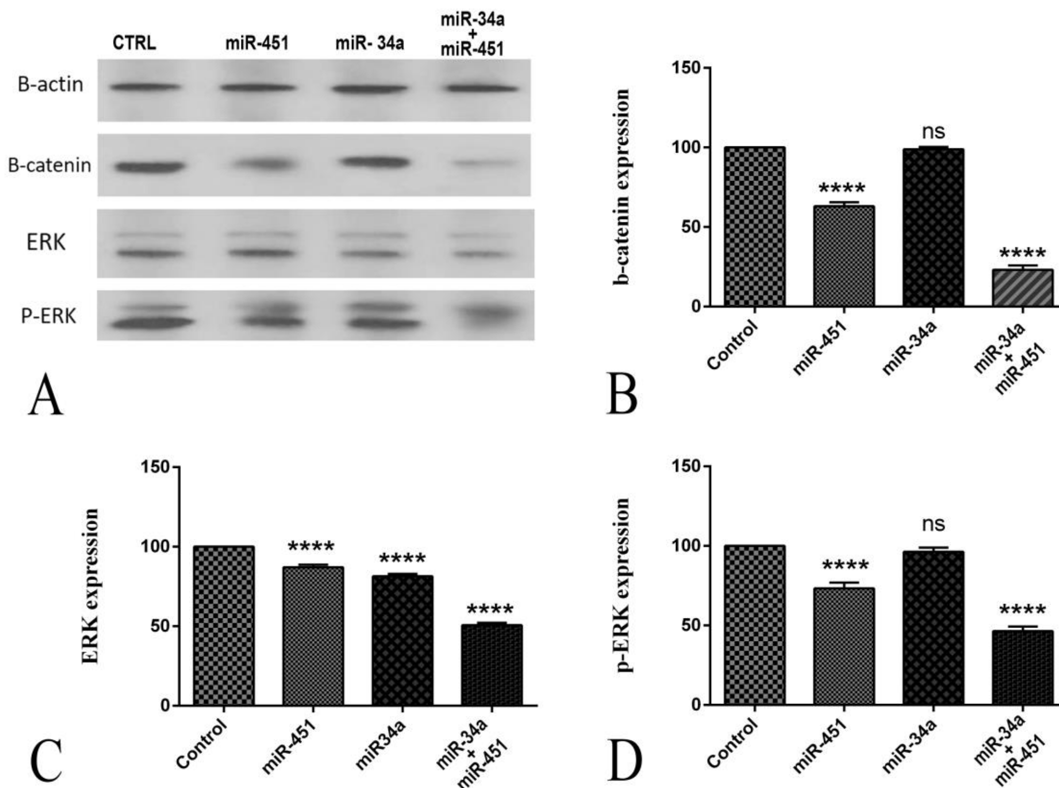

Figure 5: (A) miR-451a-5p and miR-34a-5p controlled Wnt/β-catenin pathway through downregulation of β-catenin protein in the MDA-MB-231 cells. Also, miR-451a-5p and -34a-5p modulated the expression of ERK and p-ERK in the MDA-MB-231 cell line. (B) There was no significant decrease in the expression of β-catenin in the miR-34a-5p transfected group. However, β-catenin levels were significantly downregulated in miR-451a-5p and combination groups. The immunoblotting of ERK (C) and p-ERK (D) proteins was evaluated in miR-451a-5p and -34a-5p individually and simultaneously transfected and untransfected (control) groups (****$p < 0.0001$).

3.6 Restoration of miR-451a-5p and miR-34a-5p suppressed the ERK signaling pathway in the MDA-MB-231cell line

The effects of miR-451a-5p/34a-5p on ERK/P-ERK activations in breast cancer cells were examined *in vitro* using the Western blot technique. The results showed that miR-451a-5p/-34a-5p regulates the expression of ERK and P-ERK in the MDA-MB-231 cell line. Overexpression of miR-451a-5p and -34a-5p led to downregulation of ERK and also phosphorylated protein expression compared to the control group (Figures 5A, C and D).

4. Discussion

In recent years, microRNA dysfunction has been identified as one of the major factors in the occurrence of cancer in humans. New evidence suggests that microRNAs play a crucial role in the development and progression of tumors. Moreover, these molecules are able to modulate the expression levels of various oncogenes and tumor suppressor genes, which are the most important markers of cancer biology (12). There is increasing evidence that miR-451 and miR-34 (separately) are particularly dysregulated in various human malignant tumors. They play an essential role in tumorigenesis and tumor development, especially as tumor suppressors. These miRs can also modulate cell proliferation, motility and migration through a variety of specific targets , such as c-Myc and MMP-9 (13,14). In this regard, recent papers have reported that the levels of miR-451 and miR-34 are reduced in human BC specimens, which has been associated with poor prognosis and lymph node metastasis. In the present study, the expression levels of miR-451a-5p and miR-34a-5p were low in BC cell lines, especially in MBA-MD-231. These results indicate that both microRNAs act as tumor suppressors in BC, which is consistent with previous studies (14). Over the past decade, miRNAs have been proposed as specific potential predictive, diagnostic and prognostic strategies for a variety of diseases including human cancer. Importantly, they are also used as a therapeutic tool in cancer by promoting or suppressing specific molecular pathways. Interestingly, it has been suggested that silencing an oncogenic miRNA or restoring a tumour -suppressive miRNA could be a beneficial antitumor therapy (15). Therefore, miRNA replacement therapy was first used as a treatment strategy for cancer in 2009(16). The aim of the present study was to investigate the restorative effects of miR-451a-5p and miR-34a-5p, individually and/or in combination, on breast cancer biology *in vitro* by transfecting them into MDA-MB-231 cells. Here, MTT assay, flow-cytometric analysis, DAPI staining, and the expression levels of caspase-3 and caspase-8 (by qRT-PCR) were used for the detection of apoptosis after the restoration of miR-451a and miR-34a and their combination in BC cells. After transfection, the viability of cancer cells was significantly reduced . Quantitative and qualitative apoptosis as well as caspase-3/8 expression levels were significantly increased in the treatment groups. These results showed that apoptosis was strongly increased after transfection of miR-451a and miR-34a, especially in the combination group. Caspases-3 and caspase-8 belong to the aspartate-specific cysteine proteases and contribute to extrinsic programmed cell death after external stimulation of death receptors as executive and initiator caspases, respectively. In this study, the expression levels of both caspases were significantly increased after restoration of miRNAs. These results are consistent with some other reports showing the strong anti-apoptotic effect of miR-34a and miR-451a in BC. In this context, it has been previously reported that overexpression of miR-184, miR-216a, and miR-145 as tumor suppressors in breast cancer cells, can induce apoptosis via activation of caspase-3/8 and subsequently inhibit cancer cell proliferation, invasion, and adhesion (17). In the present study, the results of the proliferation and migration analyses, including the wound healing assay and the expression levels of MMP9, vimentin, and ROCK genes, showed that the replacements of miR-451a, miR-34a and the miR-451a-34a-combination significantly inhibited the migration ability of the cancer cells, especially when applied alone. This is consistent with some publications in the literature. Higher expression of MMP-9, a member of zinc-dependent endopeptidases, has been mostly associated with breast cancer development and progression, especially in triple-negative and HER2-positive breast cancer (18,19), according to metastasis and the staging (18). The present results showed that MMP-9 was downregulated after miRNA transfection, particularly by miR-451. Vimentin has also been shown to be an indicator of pre-metastatic cancer cells undergoing epithelial to mesenchymal transition (EMT). Thus, as expected, its overexpression is associated with poor outcome in patients with solid cancers (20). Thus, several studies have demonstrated the contribution of miRNAs to the EMT process in BC cells through the expression of vimentin (20). The present findings are also consistent with recent studies linking overexpression of miR-34a to the downregulation of vimentin. Rho-associated protein kinases (ROCKs) are known to be essential managers of focal adhesion formation, cancer cell motility and invasion (21). Increasing evidence suggests that ROCK1 is downregulated by miR-193a and miR-340 in breast cancer (22). Strikingly high ROCK protein levels have

been found in breast cancer patients, which may be associated with tumor grade progression and poor overall survival. Previously, miR-340 and miR-148b have been reported to influence cell migration, invasion, metastasis, and cancer progression in breast cancer by affecting ROCK (22,23). Here, qRT-PCR was used to determine that the expression of ROCK was considerably reduced following the overexpression of miRNAs, particularly in the miR-451 group. In the current study, overexpression of miR-451a and miR-34a led to a significant cell cycle arrest in the sub-G1 phase, especially in the combination group. The upregulation of miR-451 and miR-34 prevented mitotic entry by inducing sub-G1 arrest and eliminating cells with impaired DNA. Furthermore, in this study, the expression of c-Myc , an important oncogene that promotes cell cycle-promoter , was significantly reduced in human breast cancer cells. In fact, c-Myc is a transcription factor found in the cell nucleus that is frequently dysregulated and overexpressed in many human cancers A previous study investigated c-Myc as a specific target of miR-451, which can regulate cancer cell proliferation and migration (13). These data demonstrate that overexpression of miR-451a and miR-34a, individually and/or combined, inhibits BC cell proliferation and causes cell cycle arrest in sub-G1-phase. The present Western blot analyses revealed that miR-34a-5p transfection did not significantly alter the expression of β-catenin protein. However, miR-451a-5p individually and/or combined with miR-34a significantly downregulated β-catenin protein levels. It was previously shown that overexpression of miR-451 can strongly inhibit the expression of some mesenchymal markers, such as β-catenin. The β- catenin is a multifunctional protein that acts as both a transcriptional co-modulator and an adaptor protein for intracellular adhesion. Remarkably, Wnt is the master regulator of β-catenin. Upon Wnt stimulation or genetic modification of Wnt components, β-catenin is deposited in the cytoplasm and then migrates to the nucleus. It can also trigger the transcription of some target genes such as c-Myc (24). In a similar study, it was reported that high expression of β-catenin (in the cytoplasm or nucleus) generally promotes tumorigenic characteristics and increases the proliferation and viability of cancer cells (25). Wnt/β-catenin signaling is prevalent in human cancers and is closely associated with tumor growth, malignancy, poor prognosis, and short survival (26). Recently, upregulation of miR-451 was shown to negatively modulate the Wnt/β-catenin signaling pathway via c-Myc of β-catenin, which is consistent with the results of the present study.In the current study, Western blot analyzed also showed that restoration of miR-451a/34a increased the protein expression levels of ERK and P-ERK (phosphorylated-ERK) in BC cells. ERK (extracellular signal-regulated kinase 1/2) belongs to the MAPK (mitogen-activated protein kinase) family, which functions in signaling pathways and transmits extracellular signals to intracellular receptors. The ERK cascade plays an essential role in various cellular processes, such as cell proliferation, adhesion, differentiation, migration and survival (27). Increased ERK expression has been already found in various human cancers such as BC (28). On the other hand, the expression of MMP-9 depends on the phosphorylation of ERK and the presence of P-ERK. Interestingly, the results of the present study suggest that miR-451a/34a reduces ERK and P-ERK expression and subsequently inhibits cell survival, proliferation and migration ability. Restoration of miR-451a/miR-34a reduced the activities of ERK/P-ERK, confirming that miR-451a and miR-34a play a remarkable role in RAS-RAF-ERK and related signaling pathways. This finding confirms the results of MTT assay flow cytometry (to determine apoptosis rate and cell cycle arrest), wound healing assay and qRT-PCR analyses. In summary, the present data document that restoration of the tumor suppressor miR-451/miR-34 strongly induces programmed cell death *in vitro* and significantly inhibits cell proliferation and migration in human breast cancer cells. However, there is a need for further studies on the co-involvement of tumor suppressive miR-451a and miR-34a in BC.

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Authors' Contribution

MKh and BB conceived and planned the experiments; FJA, EB, SN and ShR performed the experiments; all authors discussed the results and contributed to the final manuscript.

Ethics

Not applicable.

Conflict of Interest

The authors declare that they have no competing interests.

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Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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