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miR-451a-5p modulates breast cancer cell apoptosis, migration, and chemosensitivity to carboplatin through the PTEN pathway

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Abstract

Background: MicroRNAs (miRNAs) can play essential roles in the modulation of cancer cell growth, survival, and resistance to chemotherapy. Thus, we hypothesized that restoration of miR-451a-5p (a tumor suppressor) might affect sensitivity to chemotherapeutics in breast cancer cells.

Methods: For this purpose, malignant breast cancer cells (MDA-MB-231) were transfected with miR-451a-5p mimic and exposed with carboplatin. Then, the apoptotic rate was evaluated by flow cytometry and DAPI staining (apoptosis), q-RT-PCR (expression levels of caspase-3, caspase-8, MMP9, ROCK, vimentin, c-Myc genes). Moreover, the proliferation and migration of cancer cells were assessed by MTT (cell viability) and wound healing assay. The western blot assay was used for protein expression of PTEN, AKT, and P-AKT.

Results: Our findings demonstrated that a combination of miR-451a-5p restoration with carboplatin administration could additionally induce apoptosis, repress the proliferation and migration, and also increase PTEN protein expression with no significant alteration on the AKT/P-AKT protein expressions in the breast cancer cells. The present data was analyzed using GraphPad Prism 6 software by non-parametric one-way ANOVA and t-test.

Conclusion: In conclusion, it seems that overexpression of miR-451a can enhance the chemosensitivity of breast cancer cells to carboplatin therapy. Thus, it may shed new light on miR-451a management of breast cancer chemoresistance and may be a beneficial strategy for future cancer therapy. However, further studies, particularly in other signaling pathways, should be required.

Keywords: Breast cancer, miR-451a-5p, Carboplatin, Chemoresistance, Apoptosis.

Introduction

Breast cancer (BC) has presented the highest incidence and death among women malignancies worldwide.¹ Growing evidence proposed that epigenetic modifications, such as non-coding (nc) RNAs dysfunctions, can contribute a remarkable role in cancer development and treatment.² Among ncRNAs, microRNAs can play essential roles as tumor inducers or tumor suppressors in the pathophysiology of BC, such as tumor initiation, progression, angiogenesis, microenvironment, invasion, migration, and metastases.³ In this regard, deregulation of miR-451, which acts as a tumor suppressor, has been previously demonstrated in various human cancers like benign and malignant BC, gastric, colorectal, and lung cancers.⁴⁻⁸ The family of miR-451 includes two major members in the human genome consisting of hsa-miR-451a (which has two arms: miR-451a-3p and miR-451a-5p) and hsa-miR-451b, which are placed on chromosome 17 (chr17: 28861369-28861440 and 28861371-28861438).⁷ Of note, growing evidence has also highlighted the essential contribution of the miR-451 family in some vital biological processes like erythroid differentiation and maturation.⁷

Extensive research has been performed on cancer treatment in recent years. In this regard, nowadays, chemotherapy is the preferably and primary suggested treatment choice for patients with breast cancer, particularly in the triple-negative subtype.⁹ Although combined surgical and chemotherapy have decreased the mortality rate and increased the survival rate of BC, finding an effective treatment with fewer side effects remains a concern.¹⁰ On the other hand, the mostly emerging chemoresistance subsets a major obstacle for augmenting the effectuality of chemotherapeutic drugs associated with the clinical outcomes and prognosis of BC patients.¹¹ In this scene, novel therapeutic strategies to improve the therapeutic outcome are hence required. Thus, discovering molecular pathways and focusing on novel cancer treatment strategies like

microRNA replacement therapy have been interested in a large number of researchers in the basic medical sciences in the last decade.⁸

It was reported that ncRNAs could manage drug sensitivity and the emerging of cancer cell resistance towards platinum components, such as cisplatin and carboplatin.¹² Emerging evidence has proposed that silencing or knocking down a tumor inducer miRNA or restoring and replacement a tumor-suppressive miRNA could provide potential and valuable antitumor therapy.⁷ Carboplatin belongs to platinum complexes and is commonly an appropriate choice for breast cancer chemotherapy because of lower toxicity without affecting antitumor efficacy compared with the same drugs.¹³ Thus, the present study aimed to evaluate the restoration effect of miR-451a-5p to gather with carboplatin exposure on breast cancer cell proliferation and migration *in vitro*, which were assessed by MTT, qRT-PCR, flow cytometry, DAPI, scratch, and Western blot assays.

Materials and methods

Cell culture and transfection

Five malignant BC cell lines (MDA-MB-231, MDA-MB-468, MCF-7, SKBR3, and BT-474) were purchased from the National Cell Bank of Iran (Pasteur Institute, Iran) and they were cultured routinely in RPMI 1640 medium (GIBCO, USA), 10% fetal bovine serum (FBS, GIBCO, USA) and 100units/ml penicillin and 100µg/ml streptomycin in an incubator at 37°C with 95 % of humidity, and 5 % CO₂ atmosphere.

Considering that MDA-MB-231 cells displayed the lowest expression levels of miR-451, this BC cell line was selected for the following experiments. FITC-conjugated controls and miR-451a mimic (Microsynth AG, Switzerland) were electroporated into MDA-MB-231 cells at two

different doses (50 and 100 pmol) using a Gene Pulser Xcell (Bio-Rad, USA) according to the recommended protocols. Afterward, transfected BC cells were seeded into 6-well plates (1×10^6 each well). The relative expression of miR-451 was demonstrated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) after three different times of incubation (24, 48, and 72 hours) to choose the optimum time and concentration. Subsequently, MACSQuant 10 flow cytometer system (Miltenyi Biotec, Germany) was used to evaluate the efficacy of miRNA electroporation.

Cell viability assay

An MTT assay was carried out to evaluate cell viability. Indeed, it was done to assess the IC₅₀ for miR-451 and carboplatin. The cancer cells were firstly transfected with miR-451a-5p and then exposed to carboplatin. Briefly, the transfected BC cells and the negative control cells were independently seeded (2×10^3) into common 96-well plates. Then, the transfected cells were exposed to 24 ascending doses of carboplatin ranging from 0.1 to 6 $\mu\text{g/ml}$ (with 2.5 intervals) (Mylan, Saint-Priest, France) after 24 hours. Subsequently, 50 μl of prepared MTT (Sigma-Aldrich, Germany) solution was applied to wells and then incubated for 4 hours. The medium in each well was removed, and 200 μl DMSO (Sigma-Aldrich, Germany) was added to each well. The color was allowed to develop for 30 minutes at 37°C with gentle shaking. Finally, the absorbance values of each well were examined at 570 nm by a Sunrise ELISA reader (Tecan, Switzerland). All tests were conducted in triplicate.

RNA extraction and qRT-PCR analysis

Total RNA of the treated cells was isolated routinely using TRIzol reagent (RiboEx Kit, GeneAll, South Korea) and quantitated by measuring the absorbance at 260 and 280 nm with NanoDrop (Thermo Scientific, USA). Subsequently, cDNA Synthesis was carried out by Biofact cDNA

Synthesis kit (Biofact, South Korea) based on the manufacturer's instructions. To determine the expression levels of miR-451a-5p, the miRNA Reverse Transcription Kit (BonMir, Iran) was used. The expression levels of caspase-3, caspase-8, c-Myc, ROCK, MMP-9, and vimentin were then assessed by a qRT-PCR system with SYBR Premix Ex Taq (Biofact, South Korea) and a StepOne Plus RT-PCR system (Applied Biosystems, Thermo Fisher Scientific, USA). Relative expression levels of miRNA-451a-5p were normalized to U6, and GAPDH expression was considered as an internal loading control to normalize target gene expressions. The applied primer sequences are listed in Table 1.

Table 1. Primer sequences

Gene Name		Sequences
miR-451a-5p	Target sequence (5'–3')	AAACCGUUACCAUACUGAGUU
U6	Forward (5'–3')	CTTCGGCAGCACATATACTAAAATTGG
	Reverse (5'–3')	TCATCCTTGCGCAGGGG
GAPDH	Forward (5'–3')	CAAGATCATCAGCAATGCCT
	Reverse (5'–3')	GCCATCACGCCACAGTTTCC
Caspase-3	Forward (5'–3')	TGTCATCTCGCTCTGGTACG
	Reverse (5'–3')	AAATGACCCCTTCATCACCA
Caspase-8	Forward (5'–3')	TGAAAAGCAAACCTCGGGGA
	Reverse (5'–3')	TGAAGCTCTTCAAAGGTCGTG
c-Myc	Forward (5'–3')	AGGCTCTCCTTGCAGCTGCT
	Reverse (5'–3')	AAGTTCTCCTCCTCGTCGCA
Rock	Forward (5'–3')	CTCCCTGTGTCAGACTGCTCTTT
	Reverse (5'–3')	GGCCTTGCAACCTTGGTCTCTTC
MMP-9	Forward (5'–3')	TTGACAGCGACAAGAAGTGG
	Reverse (5'–3')	GCCATTCACGTCGTCCTTAT
Vimentin	Forward (5'–3')	AATCGTGTGGGATGCTACCT
	Reverse (5'–3')	CAGGCAAAGCAGGAGTCCA

Wound healing assay (scratch test) for migration and metastasis

MDA-MB-231 cells were commonly cultured in 24-well plates. Wounds were made in monolayers of cells using a 200 µl sterile pipette tip in the center of each well after incubation under normal conditions. The debris was washed by a serum-free medium. Then, from 0 to 48 hours after wounding, wells were observed with an inverted microscope (Optika, Italy).

Cell apoptosis analysis

The quantitative examination of cell apoptosis was detected by a routine apoptosis kit (annexin-V-FITC and propidium iodide (PI) double staining kit) (Exbio, Czech Republic) based on the manufacturer's guidelines and evaluated using a MACSQuant 10 flow cytometer system (Miltenyi Biotec, Germany) and FlowJo software version 7.6 (FlowJo, USA). In this way, there were four experiment groups comprising control, miR-451a-5p, carboplatin, and combined miR-451a-5p/carboplatin. Briefly, cells from each of the four groups were seeded at a density of 1×10^6 per well in usual 6-well plates, and after 24 and 48 hours of incubation at 37°C, they were detached from the plates using Trypsin/EDTA solution, washed with PBS (3×), suspended in 500 µL of binding buffer, and then treated with 5 µL of FITC-conjugated annexin-V and 5 µL of PI for 15 minutes on ice in the dark.

Apoptosis induction in experimental groups was also investigated using DAPI (4',6-diamidino-2-phenylindole) staining. Here, 12×10^3 MDA-MB-231 cells were routinely seeded in 96 well plates, incubated for 48 hours, and then washed with PBS (3×). After fixation with paraformaldehyde for 2-4 hours, the cells were washed by PBS again. Then, 0.1% Triton X100 was added to each well, and the plate was incubated for 15 minutes. After another PBS-washing, cells were stained with 100 µl of DAPI solution for 10 minutes at dark and room temperature. Finally, 200 µl of PBS was added to each well for the last wash, and the fragmented chromatin of apoptotic cells was observed in Cytation 5 live imaging system (Biotech, USA).

Western blot assay

Total protein of treated cells was extracted from BC cells using Radio Immuno Precipitation Assay buffer (Santa Cruz, USA), separated by SDS PAGE, and transferred onto polyvinylidene difluoride membranes (Roche, UK). After blocking with non-fat milk buffer for 2 hours, the membranes were incubated with anti AKT1 (1:300, sc-5298), P-AKT1 (1:300, sc-52940), PTEN (1:300, sc-7974), and β - actin (1:300, sc-47778) mouse monoclonal antibodies (Santa Cruz, USA) overnight at 4°C. Subsequently, the membranes were exposed to mouse anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:1000, sc-2357) for 2 hours at RT. To visualize the protein bands and assess the signal intensity of each band, ECL prime Western blotting detection reagent (Amersham, UK) and ImageJ 1.62 software (National Institutes of Health, USA) were used.

Statistical analysis

Each experiment was repeated three times. The present data were analyzed using GraphPad Prism 6 software by non-parametric one-way ANOVA and t-test, and the results were provided as the mean \pm standard deviation (SD).

Results

MiR-451a-5p was down-regulated in the examined BC cell lines

The expression levels of miR-451a-5p were evaluated in five human BC cell lines (including MDA-MB-231, MDA-MB-468, MCF-7, SKBR3, and BT-474 BC0 by qRT-PCR. As exhibited in Figure 1A, The MDA-MB-231 cell line was selected for further research since miR-451a-5p expression levels were considerably lower in MDA-MB-231 cells when compared to other BC cell lines. After transfecting control FITC-conjugated miRNA into MDA-MB-231 cells, the efficiency

of the cell transfection was assessed using FCM, and the results revealed that the amount of transfection was around 98.1%. Furthermore, according to the qRT-PCR results shown in Figures 1B and C, 50 pmol and 24 hours ($P < 0.0001$) were considered to be the most effective concentration and time span for miR-451a-5p.

Treatment by miR-451a-5p in combination with carboplatin significantly suppressed cell viability and induced programmed cell death in BC cells

To determine the effects of miR-451a-5p and carboplatin on cell proliferation or apoptosis, the MTT assay was done to find the IC50 value of carboplatin. As demonstrated in Figure 2A, a remarkable decrease in BC cell viability was found in all experimental groups. In more detail, our results demonstrated that the replacement of miR-451a-5p could reduce the IC50 value of carboplatin (IC50 before treatment = 3.19 $\mu\text{g/ml}$; IC50 after treatment = 2.77 $\mu\text{g/ml}$) in BC cells (Figure 2A). Thus, it was suggested that contemporary restoration of miR-451a-5p and application of carboplatin *in vitro* could decrease the cell proliferation in BC cells.

The qRT-PCR results demonstrated that caspase-3 and caspase-8 (apoptotic genes) were obviously upregulated after miR-451a-5p transfection and carboplatin treatment (Figures 2B). Besides, miR-451a-5p in combination with carboplatin led to nuclear fragmentation and apoptosis induction in MDA-MB-231 cells. According to the results of DAPI staining, a qualitative apoptosis test, the condensed and fragmented nucleus was mostly seen in the combination group rather than in other experimental groups (Figure 2C). Moreover, the results of the flow cytometry assay revealed that miR-451a-5p enhanced the apoptosis rate of MDA-MB-231 cells both alone and in combination with carboplatin (Figure 2D).

miR-451a-5p upregulation alongside carboplatin inhibited the BC cell proliferation and migration

Here, miR-451a-5p up-regulation alongside carboplatin treatment altered the expression levels of genes involved in metastasis and proliferation (MMP9, ROCK, c-Myc, and Vimentin), which were examined by qRT-PCR. The expression levels of evaluated genes, including MMP9 and Vimentin were significantly lower in miR-451a-5p and carboplatin combination group compared to other experimental groups (Figures 3A). However, the down-regulation of Rock was most significant in the miR-451a-5p transfected group. The relative expression of c-Myc, a proliferative gene, was decreased in all of the experimental groups, as illustrated in Figure 3A.

Moreover, to determine the impact of miR-451a-5p overexpression and carboplatin exposure, the wound-healing assay (scratch test) was conducted. The current findings indicated that mimic transfection of miRNA-451a-5p either alone or combined with carboplatin reduced BC cells migration rate compared to negative controls (Figure 3B).

MiR-451a-5p and carboplatin co-treatment altered PTEN protein expression level in MDA-MB-231 cell line

After miR-451a-5p mimic transfection and carboplatin treatment, Western blotting was used to assess the protein expression of PTEN, AKT, and p-AKT. As illustrated in Figure 4A-D, the expression levels of PTEN protein were noticeably increased in all experimental groups, which was more notable in the combination group. However, there were no remarkable alterations in AKT protein expression in the carboplatin and combination groups. The P-AKT protein expression was more significant in the combination, miR-451, and carboplatin groups, respectively.

Discussion

In the present study, using *in vitro* experimental system wherein cultured the malignant BC (MDA-MB-231) cell line was transfected with tumor suppressor miR-451a-5p and subsequently treated

with carboplatin individually or in combination, as expected, we observed that miR-451a acts as a promoter in chemosensitivity in BC cells. Of note, the impact of miR-451a-5p restoration on enhancing chemosensitivity was in agreement with our initial hypothesis. Surprisingly, upregulation of miR-451a in combination with carboplatin treatment significantly induced apoptosis and inhibited migration to gather with more notable alterations in caspases-3/8, ROCK, and vimentin gene expression levels. Moreover, the expression levels of caspase-3, MMP9, and c-Myc showed additional changes in the combination group. Importantly, the expression level of PTEN protein notably increased, which notably did not show remarkable alteration in AKT/P-AKT protein expression. Probably, carboplatin chemoresistance of BC upon miR-451 restoration might not manage via the PTEN/AKT/P-AKT pathway.

It was previously reported that the levels of miR-451a are reduced in human breast cancer specimens, which was related to the weak prognosis and lymph node metastases.⁵ Here, firstly, we indicated that the expression level of miR-451a-5p is low in the examined breast cancer cell lines, particularly in the MBA-MD-231, which was selected for further analyses. These findings confirmed the tumor suppressor role of miR-451a in BC, which was inconsistent with the previous investigations.^{5,14}

In recent years, growing evidence demonstrated that miRNAs modulate a large verity of pathological features of cancer cells, such as growth, proliferation, programmed cell death, motility, and chemosensitivity, through direct and specific targeting genes.^{15,16} To date, chemotherapy is frequently the preferably and primary selected treatment option for patients with breast cancer.⁹ In the last decade, treatment of various human solid tumors by carboplatin exhibited better tolerability and less toxicity, particularly nephrotoxicity, when compared with cisplatin.¹⁷ On the other hand, it has been suggested that restoring a tumor-suppressive or silencing a tumor

inducer miRNA might represent an effective cancer therapy.⁷ In this line, systemic administration of miR-451 is recently proposed as an effective method for lung cancer treatment through STAT3 regulation.⁶ Regarding no study has presented whether miR-451a-5p contributes to the management of chemotherapy-based platinum in BC, the current study was conducted.

Platinum complex, such as carboplatin, are cytotoxic DNA-damaging compounds that result in cell apoptosis.¹³ Here, functional assays, including MTT assay, flow cytometry, DAPI staining, and qRT-PCR (caspase-3 and caspase-8), were used for the identification of apoptosis upon restoration of miR-451a and carboplatin exposure and their combination in BC cells. The viability of the cancer cells notably decreased upon miRNA transfection. The apoptotic rate was strongly increased and reached 77.3%, apoptotic cells increased by DAPI staining, and the expression levels of caspase-3/8 were remarkably raised in the treated groups, particularly in the combination group. Of note, we observed caspases-3/8 could be functional targets of miR-451a in BC cells. Caspases-3 and caspase-8 contribute to the extrinsic programmed cell death pathway following the external promotion of the death receptors as executioner and initiator caspases, respectively.¹⁸ Similar results have been reported that miR-451 upregulation increased resistance of BC cells to paclitaxel through Bcl-2 expression.¹⁹ Recently, another study demonstrated that miR-451 could sensitize chemotherapy resistance of lung cancer cells to cisplatin by Mcl-1 regulation.²⁰ Here, it seems that carboplatin modulates programmed cell death through upregulation of caspase3/8. More importantly, we found overexpression of miR-451a-5p can increase the chemosensitivity of BC cells to carboplatin by induction of apoptosis.

Here, using a scratch test and q-RT-PCR assay (expression levels of c-Myc, MMP9, vimentin, and ROCK genes), we observed that the replacements of miR-451a-5p and carboplatin administration remarkably inhibited the migration ability of the cancer cells, especially in the combination group,

with more notable alterations in ROCK and vimentin expression levels. All evaluated genes are strongly involved in the malignant behavior of BC. In more detail, MMP9 involves in tumor metastasis and staging.²¹ Vimentin has also been reported as an indicator for pre-metastatic cancer cells, which is related to unfavorable outcomes in patients with solid cancer.²² ROCKs contribute to cancer cell motility and invasion.²³ c-Myc is a major proto-oncogene, which is mostly upregulated in many human malignancies.²⁴ A previous study investigated the c-Myc as a specific target of miR-451, which can regulate the proliferation and migration of cancer cells.²⁵ Our findings are close to those of others, which strongly indicated that re-expression of miR-451 could be a helpful option for the treatment of chemoresistance by targeting c-Myc in lung cancer patients.²⁶ In BC cells *in vitro*, overexpression of miR-451 significantly enhanced its sensitivity to doxorubicin and tamoxifen therapies independently.^{4,27,28} Our data is consistent with these researches. The present findings identified that c-Myc, MMP9, vimentin, and ROCK could act as functional targets of miR-451a, which are able to enhance the chemosensitivity of BC cells to carboplatin through inhibition of migration ability.

Here, western blot analyses indicated that although the combination of miR-451a/carboplatin could raise the protein expression levels of PTEN and P-AKT (phosphorylated-AKT), there was no remarkable increase in the expression levels of AKT. In the current study, it seems that the miR-451/carboplatin combination might impact the PTEN/AKT/P-AKT signaling pathway, and PTEN and P-AKT modulate cancer cell proliferation through other molecular pathways. PTEN (Phosphatase and tensin homologue deleted on chromosome ten) is an important tumor suppressor in a variety of human tumors such as breast cancer and takes a considerable role in the management of proliferation and programmed cell death.²⁹ On the other hand, PTEN could be functionally targeted by a large number of ncRNAs like miRNAs. In this connection, it was previously reported

that miR-21 induced epithelial to mesenchymal transition (EMT) associated with gemcitabine resistance through the PTEN/AKT pathway in BC.³⁰ In addition, the cervical cancer cell sensitivity was considerably reduced by miR-221 to gefitinib via the PTEN/PI3K/AKT signaling.³¹ Similar studies have been demonstrated that upregulation of miR-451 raised the sensitivity of lung cancer cells to chemotherapy (cisplatin and docetaxel) via the AKT and c-Myc pathways independently.^{26,32} Here, more importantly, it seems that miR-451a/carboplatin could impact the PTEN. But, they do not modulate the chemosensitivity of BC cells to carboplatin through PTEN/AKT pathway.

Conclusion

To date, dysregulation of miRNAs has been proposed as one of the discovered molecular mechanisms that contributed to chemoresistance in cancer cells. Here, the influence of a tumor suppressor miRNA (miR-451a-5p) on the platinum drug (such as carboplatin) efficacy in BC cells was discussed. It was concluded that miR-451a-5p functions as a tumor suppressor and has a significant role in increasing the chemosensitivity of BC cells to carboplatin by apoptosis induction and migration repression *in vitro*, which were progressed by targeting caspases-3/8, c-Myc, MMP9, vimentin, and ROCK. It may be one of the probable mechanisms that chemotherapy combined with a miRNA is considerably preferable to chemotherapy alone. Thus, it may shed new light on miR-451a management of BC chemoresistance and may be a valuable strategy for upcoming cancer therapy. In the present study, the lack of *in vivo* evidence is a major limitation. Therefore, for a better understanding of the impact of carboplatin with ncRNAs like miRNAs, further complementary studies would be required.

Abbreviations

EMT: Epithelial-mesenchymal transition; BC: Breast cancer; FCM: Flow cytometry; miR: microRNA; PTEN: Phosphatase and tensin homologue deleted on chromosome ten; TNBC: Triple-negative breast cancer.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MKh, ASH, and BB conceived and planned the experiments; FJA, EB, SN, and HE carried out the experiments; all authors discussed the results and contributed to the final manuscript.

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Ethics approval and consent to participate

Not applicable

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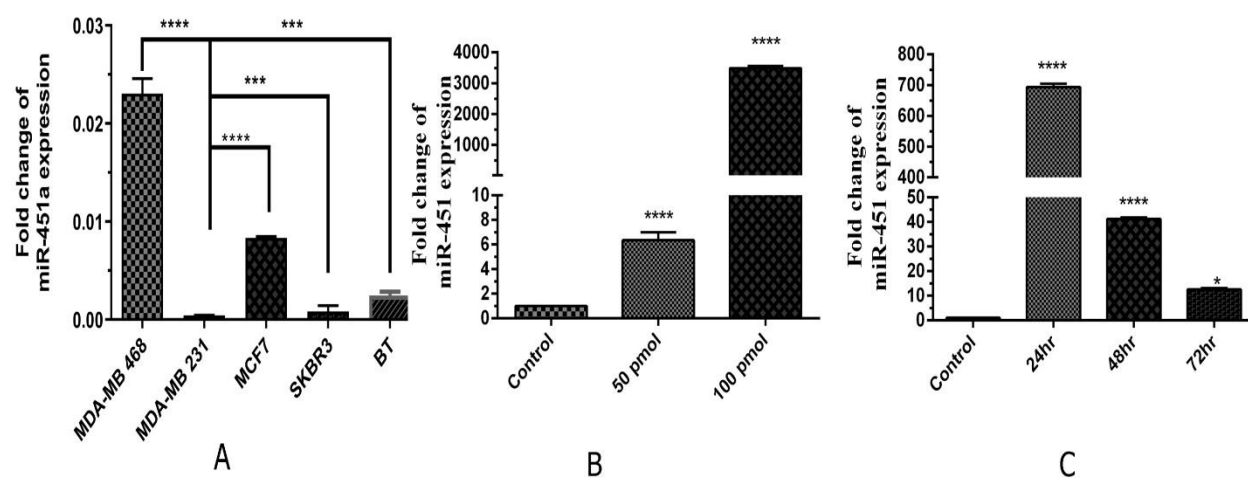
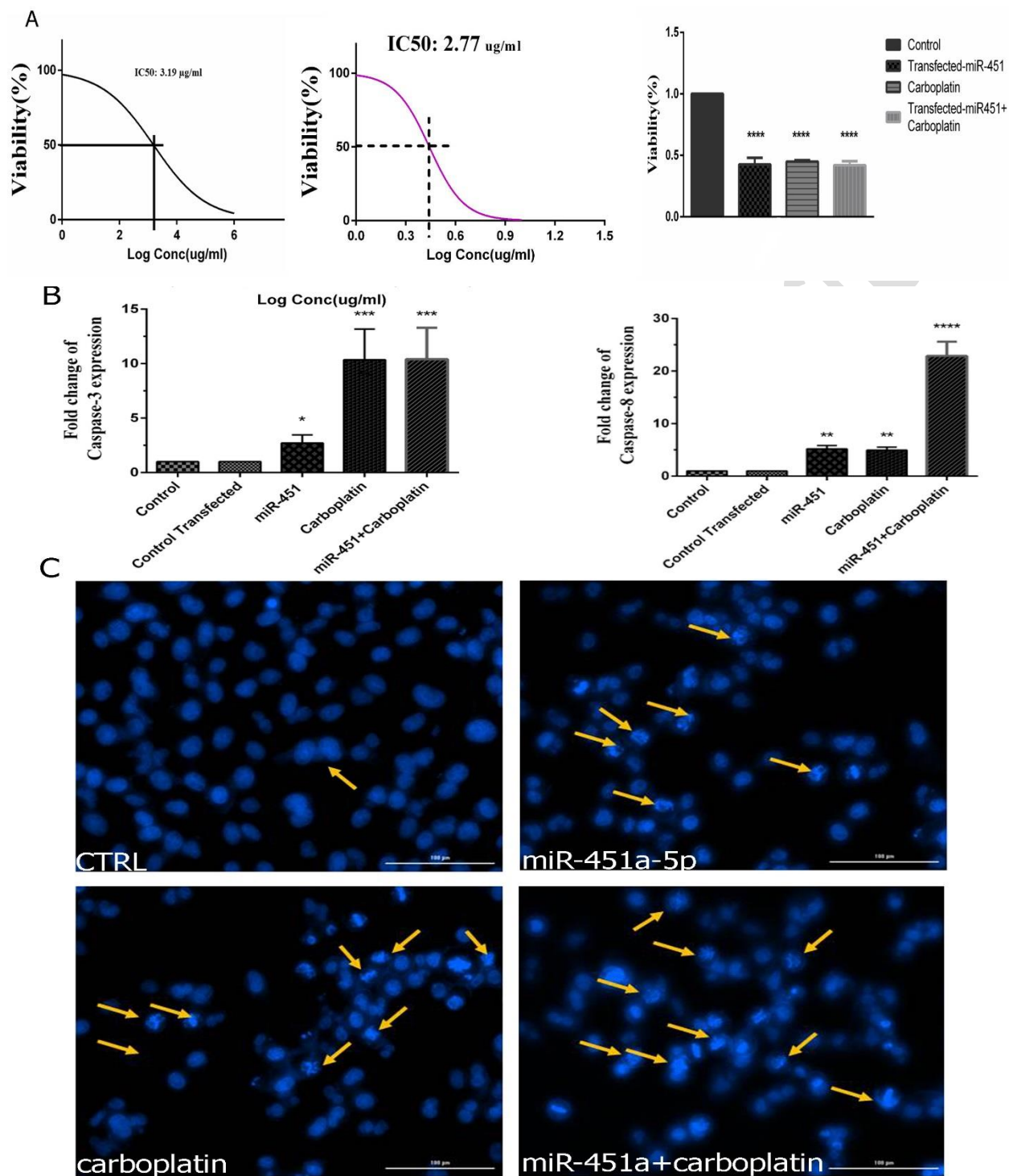


Figure 1: (A) miR-451a-5p expression levels in BC cell lines. (B) The selected concentration of miR-451 mimic was 50 pmol (**** $P < 0.0001$). (C) Ideal time span of miR-451 mimic transfection to MDA-MB-231 cell line (**** $P < 0.0001$) was 24h.



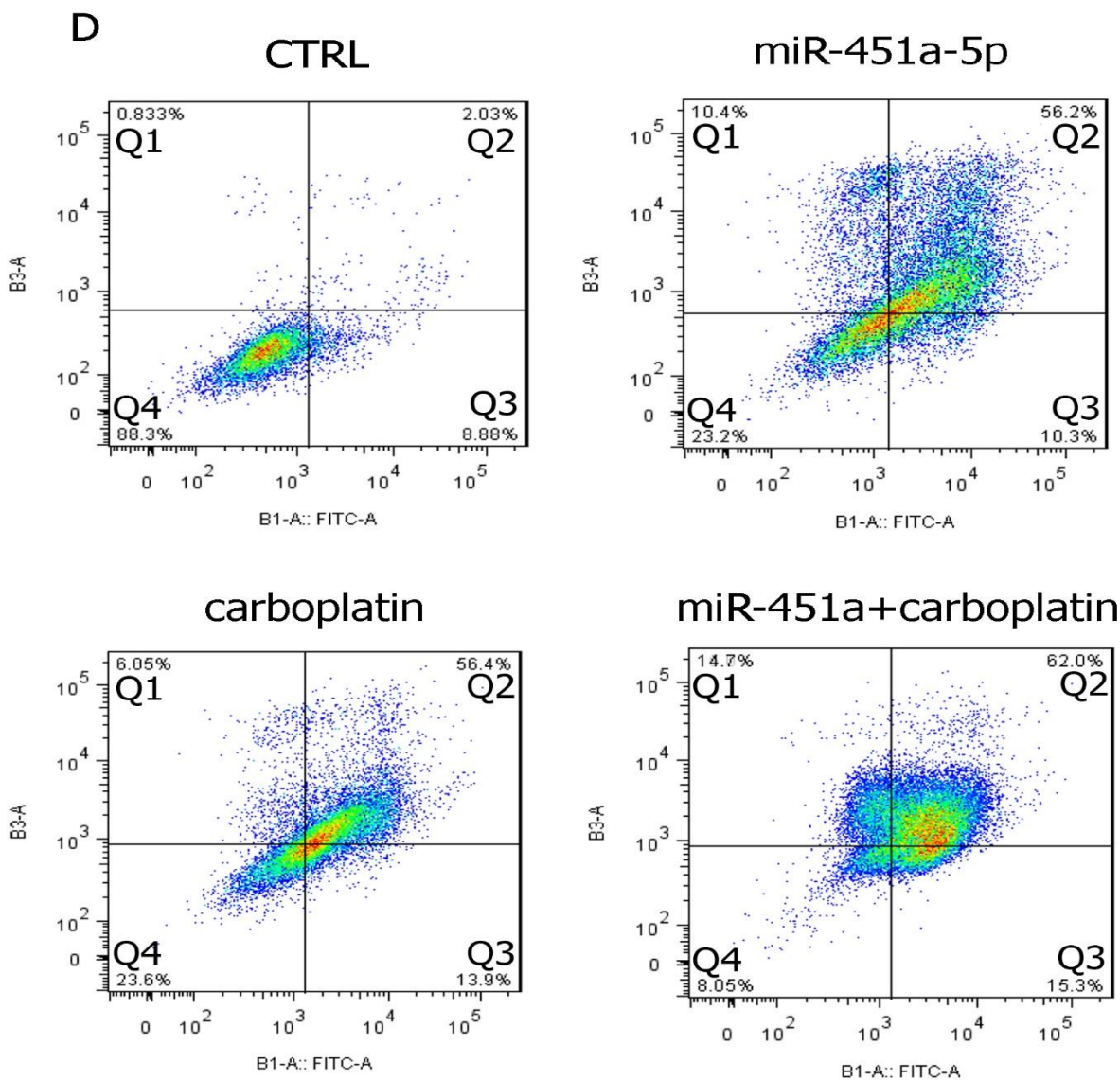


Figure 2: (A) The MTT assay was done to find the IC₅₀ value of carboplatin before (IC₅₀ = 3.19 µg/ml) and after (IC₅₀ = 2.77 µg/ml) treatment. Also, miR-451a-5p and carboplatin individually and synergistically reduced the MDA-MB-231 cell proliferation.

(B) The effect of miRNA-451a-5p and carboplatin alone and in combination with each other on the expression levels of caspase-3 and caspase-8. (C) DAPI staining was performed to detect nucleus fragmentation.

(D) The ratio of apoptotic cells was determined by FCM assay in MDA-MB-231 cells under various treatments. 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 -/-). (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

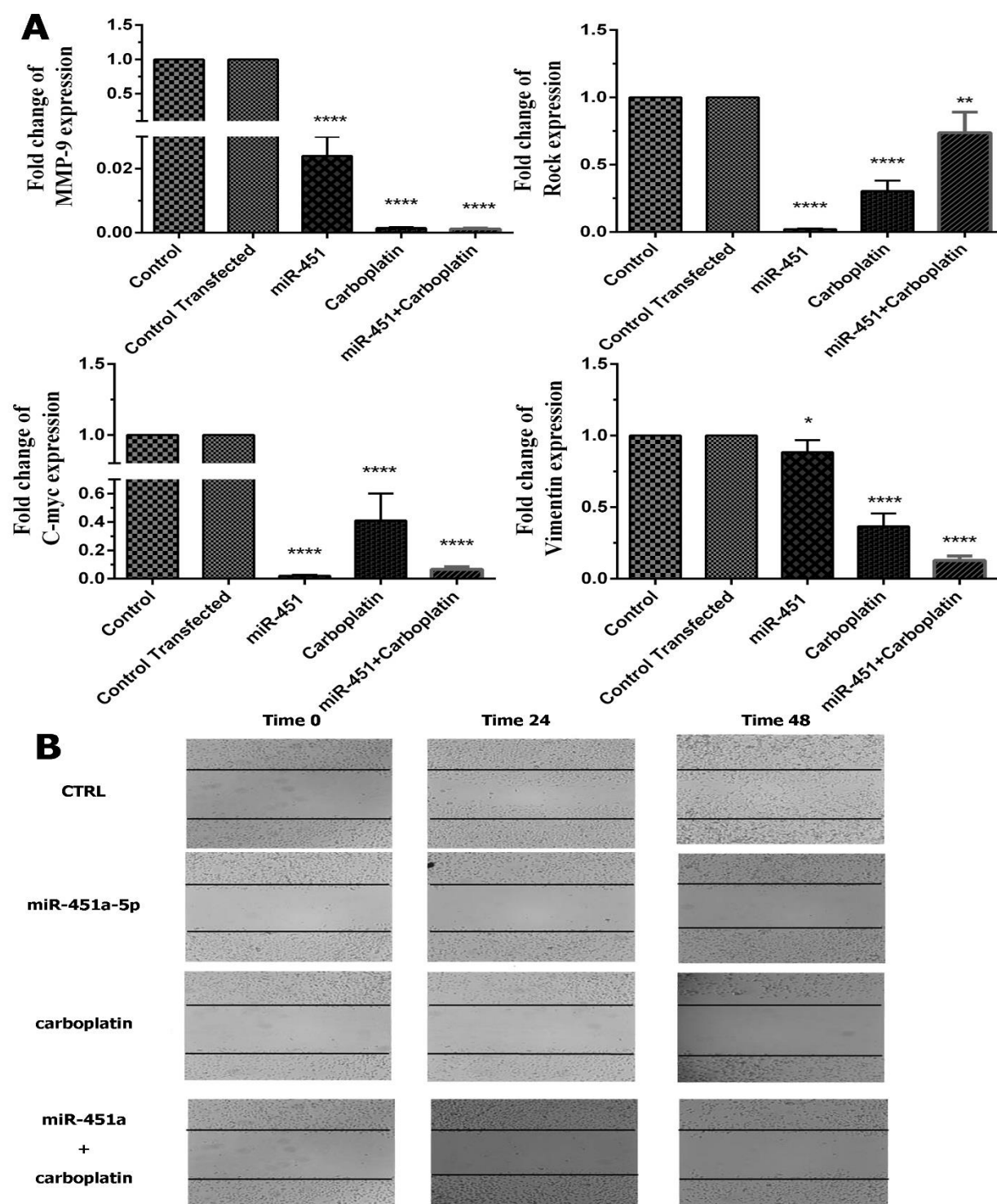


Figure 3: (A) The effect of miRNA-451a-5p and carboplatin alone and in combination with each other on the expression levels of MMP9, ROCK, c-Myc, and Vimentin, (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). (B) A scratch test exhibited that the migration ability of cancer cells decreased upon restoration of miR-451a and carboplatin treatment.

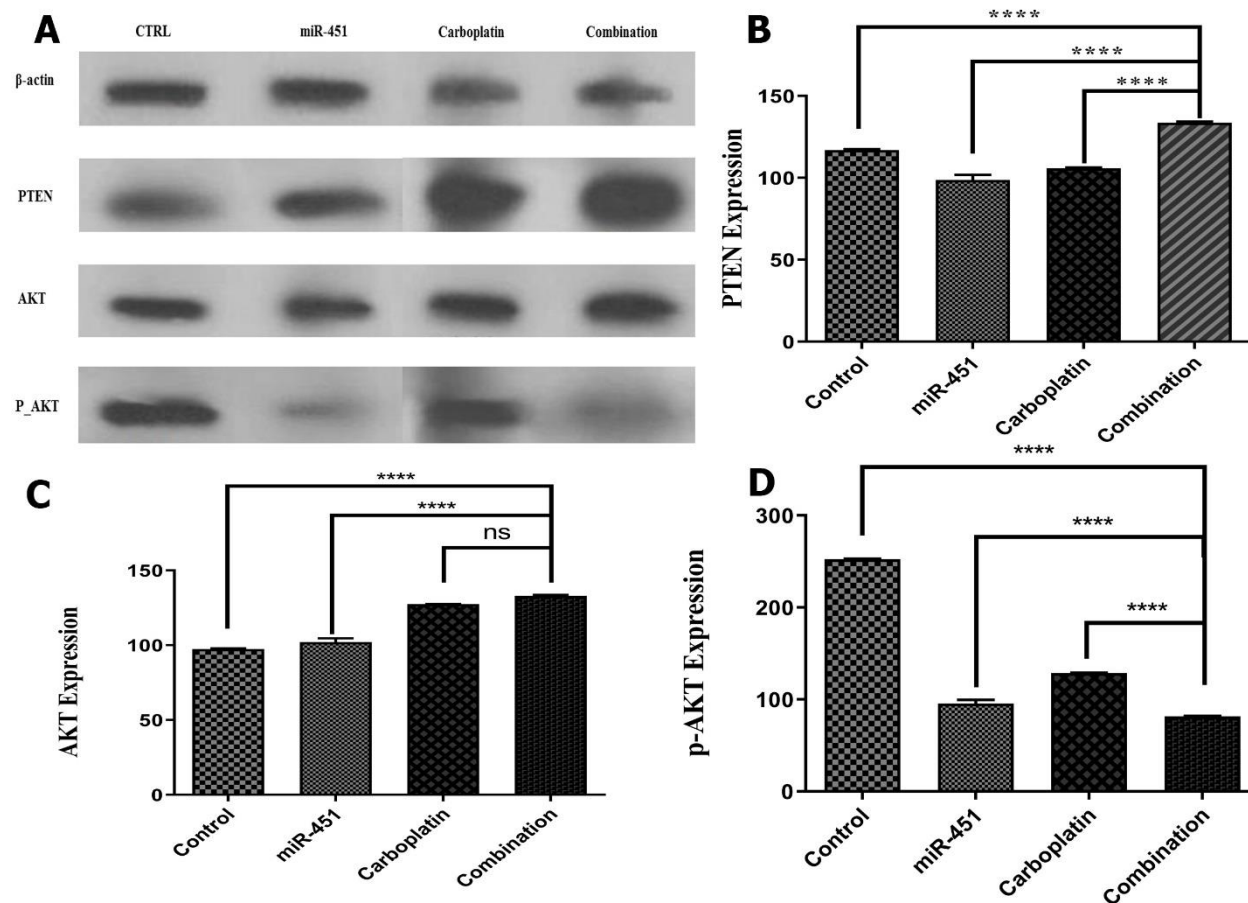


Figure 4: (A) Western blot was used to assess the protein expression of PTEN, AKT, and p-AKT. (B) The expression levels of PTEN protein were noticeably increased in all experimental groups, which was more notable in the combination group. (C) There was a significant difference in AKT protein expression in the miR-451 transfection and control groups. However, there were no remarkable alterations in AKT protein expression in the carboplatin and combination groups. (D) The P-AKT protein expression was more significant in the combination, miR-451, and carboplatin groups, respectively. (**** $p < 0.0001$).