# MiR-138-5p improves the chemosensitivity of MDA-MB-231 breast cancer cell line to paclitaxel

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**Research Article**

**Keywords:** Breast Cancer, miR-138-5p, Paclitaxel, Chemosensitivity, Combination Therapy

**Posted Date:** June 5th, 2023

**DOI:** https://doi.org/10.21203/rs.3.rs-2984483/v1



**Version of Record:** A version of this preprint was published at Molecular Biology Reports on August 24th, 2023. See the published version at https://doi.org/10.1007/s11033-023-08711-y.

# Abstract

### Background

Chemotherapy is a predominant strategy for breast cancer (BC) treatment and paclitaxel (PTX) has been known as a conventional chemotherapeutic drug. However, insensitivity of BC cells to PTX limits the anti-tumor effects of this agent. MicroRNAs are closely related to BC which are suggested as therapeutic factors in the combination therapy of BC. We examined the possible efficacy of miR-138-5p restoration in combination with PTX to impove BC treatment.

### Methods and Results

The human breast cancer cell line MDA-MB-231 was transfected with miR-138-5p mimics and treated with PTX, in a combined or separate manner. The MTT assay was accomplished to determine inhibitory doses of PTX. Annexin V/PI assay and DAPI staining were applied to evaluate apoptosis. Flow cytometry was applied to determine cells arrested in different phases of the cell-cycle. Expression levels of molecular factors involved in cell migration, proliferation, apoptosis, and cell cycle were determined via western blotting and qRT-PCR.

MiR-138-5p combined with PTX suppressed cell migration via modulating MMP2, E-cadherin, and vimentin and sustained colony formation and proliferation by downregulation of the PI3K/AKT pathway. qRT-PCR showed that miR-138-5p increases BC chemosensitivity to PTX by regulating the apoptosis factors, including Bcl-2, Bax, Caspase 3, and Caspase 9. Moreover, miR-138-5p restoration and paclitaxel therapy combined arrest the cells in the sub-$G_1$ and $G_1$ phases of cell cycle by regulating p21, CCND1, and CDK4.

### Conclusions

Restored miR-138-5p intensified the chemosensitivity of MDA-MB-231 cell line to PTX, and the combination of miR-138-5p with PTX might represent a novel approach in BC treatment.


# 1. Introduction

Breast cancer (BC) is the most common cancer in women and remains a global problem of public health [1]. Chemotherapy is predominantly used for breast cancer. There are several classes of chemo-therapeutics, including alkylating agents, antimetabolites, immunological elements, hormonal components, and mitotic deprivation [2]. Paclitaxel (PTX), approved by the FDA (Food and Drug Administration) in 1994, is an anticancer drug that is performed in BC treatment [3]. PTX, as an anti-mitotic drug, is frequently used in the treatment of metastatic breast cancer as first-line therapy that causes mitotic arrest by restaining dynamics of mitotic microtubules [4], which leads to cell death during mitotic arrest or exit mitosis without cell division forming tetraploid cells [5–7]. PTX causes creating a 4C DNA peak in flowcytometry, which is called '$G_2$/M' arrest and also initiates different signaling pathways

leading to apoptosis [8]. However, high concentrations of PTX could result in chemoresistance in BC patients [9]. The resistance of BC cells to chemotherapeutics like PTX may be result from disequilibrium in various signaling pathways, mutations in specific genes, and epigenetic dysregulations, including downregulation of microRNAs [10]. Previous studies have proved that microRNA are deregulated in BC cells [11]. MicroRNAs could be either oncogenic miRNA (oncomiR) or tumor suppressor miRNA (tsmiR). Tumor suppressor miRs are downregulated in various cancers like BC. Therefore, microRNA replacement therapy could effectively increase the efficacy of chemotherapeatics used in BC treatment [12]. MiR-138-5p is a known strong tsmiR whose anti-cancerous effects on various cancers, including BC, have been illustrated in numerous types of researches [13–15]. A study on pancreatic cancer showed that miR-138-5p restoration, by targeting vimentin, could increase the chemosensitivity of cancerous cells to 5FU (5fluorouracil) [16]. Moreover, recent studies demonstrated that miR-138-5p improves the sensitivity of cervical cancer cells to Cisplatin and Glioblastoma (GBM) cells to temozolomide (TMZ) by targeting H2AX and Survivin, respectively [17, 18]. We showed, in our previous research, that miR-138-5p has several anti-tumorigenic effects on BC cells by targeting PD-L1 (programmed cell death ligand 1) and suppressing cell proliferation and migration and the induction of apoptosis [15]. Recent investigations have proved the synergistic effects of miR-424-5p and miR-383-5p on enhancing the chemosensitivity of BC cells to PTX [19, 20]. However, there is no report on the impacts of miR-138-5p restoration on the chemosensitivity of BC cells to PTX so far. In this study, we hypothesized that miR-138-5p might increase the toxic effects of PTX in the MDA-MB-231 breast cancer cell line. Our data represented that miR-138-5p could enhance the cytotoxic effects of lower doses of PTX on BC cell migration, apoptosis, cell proliferation, colony formation, and cell cycle. The mentioned findings suggest that miR-138-5p restoration and paclitaxel therapy combined might be a novel therapeutic approach that improves the efficacy of chemotherapy in BC treatment.

# 2. Materials and Methods

**2.1. Cell line culture:** Human Breast Cancer cell line MDA-MB-231 was purchased from Pasteur Institute's Cell Bank of Iran (Iran) and cultured in RPMI-1640 culture medium (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Sigma-Aldrich, Merck, Germany) and cultivated in the incubator with the temperature of 37 °C and 5% CO2.

**2.2. miR transfection and Paclitaxel (PTX) treatment:** Negative control mimics (5'-GAUGGCAUUCGAUCAGUUCUA-3') and miR-138-5p mimics (5'-AGCUGGUGUUGUGAAUCAGGCCG-3') obtained from ShineGene (china) were transfected in a concentration of 40 pmol into a $1\times10^6$ numbers of MDA-MB-231 cells, using Gene Pulser electroporation system (Bio-Rad) separately. Then, $3\times10^5$ numbers of transfected cells were transferred into each well of 6-well plates and cultivated for 48 h. In the following, MACSQuant Analyzer 10 flow-cytometry (MiltenyiBiotec, Germany) and qRT-PCR were utilized to evaluate transfection efficacy. Six cellular groups were compared in the MTT assay: the N-control group, which was transfected with NC-miR mimics, the miR-138-5p group, transfected with miR-138-5p mimics, the PTX (IC25) group, treated with $IC_{25}$ (quarter maximal inhibitory concentration) of PTX, the

PTX (IC50) group, treated with $IC_{50}$ (half maximal inhibitory concentration) of PTX, the miR-138-5p+PTX (IC25) group, transfected with miR-138-5p mimics and treated with $IC_{25}$ of PTX, and the miR-138-5p+PTX (IC50) group, which was transfected with miR-138-5p mimics and treated with $IC_{50}$ of PTX. Finally, four groups of cells were selected and participated in following tests.

**2.3. Cell viability assay:** to identify the inhibitory doses of PTX on the viability of MDA-MB-231 cells after transfection with miR-138-5p and treatment with PTX in a combined or separate manner, an MTT assay was applied. Briefly, transfected cells were seeded into the 96-well plates at a total of $1\times10^4$ cells per well and, after 24 h incubation, treated with different doses of PTX (0-12.5 µg/ml). The cells were incubated at 37 °C for 24 h followed by washing with PBS and incubating with 50µl of 2 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 h at 37 °C. In the next stage, the medium of each well was replaced with 150µl of DMSO (dimethyl sulfoxide) and incubated for further 30 min at 37 °C. Finally, the optical absorbance of each well was determined at the 570 nm wavelength by the Eliza reader (Tecanmicroplate, Switzerland). All experiments were conducted three times in a triplicate manner.

**2.4. RNA extraction and qRT-PCR:** cellular RNAs were extracted from breast cancer cells by TRIzol reagent (GeneAll, Korea), and their purity and concentration were revealed by NanoDrop spectrophotometer (TermoFisher). Then cDNAs were synthesized from mRNAs using BioFACT Kit (BR631-096, Korea). Finally, qRT-PCR was carried out by a BioFACT 2×SYBR green kit in an ABI Biosystem instrument (USA) to evaluate the transcript levels of Caspase 3, Caspase 8, Caspase 9, BAX, Bcl-2, P21, CCND1, and CDK4. The fold changes of gene expression were analyzed using the $2^{-\Delta\Delta Ct}$ formula and β-actin was used as an internal control. Primer sequences were listed in Table 1.

Table 1 Primer Sequences

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| **β-actin** | 5'-AGAGCTACGAGCTGCCTGAC-3' | 5'-AGCACTGTCTTGGCGTACAG-3' |
| **P21** | 5'-CCTCAAAGGCCCGCTCT-3' | 5'- ACCTCTCATTCAACCGCCTA-3' |
| **CCND1** | 5'- TGGATGCTGGAGGTCTGCGA-3' | 5'-GTCTCCTTCATCTTAGAGGCCACG-3' |
| **CDK4** | 5'-CTACCTCTCGATAT GAGCCAGT-3' | 5'-CATCTGGTAGCTGTAGATTCTG-3' |
| **Bcl-2** | 5'-CTGTGGATGACTGAGTACCTG-3' | 5'- GAGACAGCCAGGAGAAATCA-3' |
| **BAX** | 5'-TTTGCTTCAGGGTTTCATCCA-3' | 5'-TCTGCAGCTCCATGTTACTGTC-3' |
| **Caspase-3** | 5'-CAAACCTCAGGGAAACATTCAG-3' | 5'-CACACAAACAAAACTGCTCC-3' |
| **Caspase-8** | 5'-GACCACGACCTTTGAAGAGCTTC-3' | 5'-CAGCCTCATCCGGGATATATC-3' |
| **Caspase-9** | 5'-CTGTCTACGGCACAGATGGAT-3' | 5'-GGGACTCGTCTTCAGGGGAA-3' |

**2.5. Wound healing assay:** MDA-MB-231 cells were separately transfected with the NC-miR and miR-138-5p mimics and cultured at the initial number of 2×10  cells in each well of 24-well plates. The cells, after 24 h incubation at 37 °C, were treated with PTX and incubated for a further 24 h. The wound area was scratched using a sterile 100µl pipette tip on a cellular monolayer, and removing of dead cells was done by the medium exchange. In the following, to evaluate the migratory potential of cells, the inverted microscope (Optika, XDS-3, Italy) was applied to measure the width of the wound at 0, 24, and 48 h after scratching.

**2.6. Apoptosis assay using AnnexinV/PI:** the rate of apoptosis induced by miR-138-5p, PTX, or their combination was determined using Annexin V/PI kit (BD Biosciences). Briefly, a number of $3\times10^5$ transfected cells were seeded into each well of the 6-well plate and incubated for 24 h at 37 °C, followed by treatment with PTX in a separate or combined manner. Then, the cells were harvested after further 24 h incubation, and other steps, including washing with PBS and staining with Annexin V/PI, were done. The apoptotic cells were distinguished by the MACSQuant 10 flow-cytometry, and obtained data were analyzed by FlowJo software 7.6 (TreeStar Inc, USA).

**2.7. Apoptosis test by DAPI staining:** in proof of induced apoptosis, chromatin fragmentation was determined using DAPI staining in treated cells. In summary, transfected cells were calculated and cultivated at the initial concentration of $15\times10^3$ cells per well in 96-well plates. The cells were treated with PTX after overnight incubation and cultured at 37 °C for a further 24 h. After that, the cells were washed three times with PBS, and fixing with 4% paraformaldehyde was performed for one h. Then, penetrating of cell membranes was accomplished using Triton-X-100 (0.1%) for 10 min, followed by washing with PBS and staining with 60µl of 0.1% DAPI for 15 min in a dark place. Eventually, chromatin fragmentation, as a sign of apoptosis, was determined by the cytation 5 cell imaging system (Biotek, USA).

**2.8. Colony formation assay:** To explore the inhibitory effect of miR-138-5p and PTX on colony-forming ability, transfected BC cells were seeded into the 6-well plates at a density of $5 \times 10^3$ cells per well and treated with PTX in a combined or separate manner after 24 h incubation. After incubation for 12 days, the cells were washed with PBS and fixed with paraformaldehyde (5%). In the end, the colonies formed via cell division were stained using 0.5% crystal which was washed with PBS after 30 min. Finally, the numbers of colonies were detected by imaging with OPTICA inverted microscope.

**2.9. Cell cycle:** BC cells were transfected with the miR-138-5p or NC-miR mimics, and seeded at the initial numbers of $3\times10^5$ cells/well in the 6-well plates. After 24 h incubation, the cells were treated with PTX in a combined or separate manner and kept in an incubator for 24 h at 37 °C. Thereafter, cells were detached, washed and fixed with PBS, and chilled at 70% ethanol for 24 h. Next, the cells were treated with RNase A (Bioneer, Korea) and incubated with PI at 25°C in a dark place for 30 min. The cellular populations in different cell cycle phases were determined using MACSQuant flow-cytometry, and FlowJo software was applied to analyze the data.

**2.10. Western blotting:** The extraction of cellular proteins was carried out by RIPA lysis buffer (Santa Cruz Biotechnology) based on manufacturer protocol. The SDS-PAGE (12%) was accomplished to separate 25µg protein per lane, which was transferred to a polyvinylidene fluoride membrane by the semi-dry blotting method. A blocking buffer containing 0.5% Tween-20 in PBS was employed to block the membrane for 2 h under the shaking condition at 25°C. In the following, specific primary monoclonal antibodies were added to the membrane to interact with the specific proteins: β-actin (sc-47778,1:300), MMP2 (sc-10736,1:200), vimentin (Invitrogen 14-9897-82,1:1000), E-cadherin (sc-21791,1:200), AKT (E-AB-30471,1:1000), p-AKT (sc-271966,1:100), PI3K (sc-67306,1:200), p-PI3K (ab182651,1:500). After 24 h incubation at 4°C, the membrane was rinsed with PBS and further incubated for 75 min with the secondary antibody (mouse anti-rabbit IgG -HRP: sc-2357, 1:1000) under the shaking condition at 25°C. The electro-Chemo-Luminescence system (Roche, Germany) was employed to visualize the bands imaged by the western blotting imaging system (Sabz Biomedicals, Iran). β-actin was used as a housekeeping protein to normalize the bands. The sharpness of western blot bands was analyzed and quantified using ImageJ software.

**2.11. Statistical analysis:** GraphPad Prism version 6.0 (GraphPad, San Diego, CA, USA) was applied for analyzing all data. To determine the statistical significance between two groups, Student's paired t-test was used, and the statistical significance among several groups was calculated and analyzed by the Kruskal-wallis test of one-way ANOVA or multiple comparisons test of Two-way ANOVA. Mean ± S.E.M was used to express all data. All experiments were performed in a triplicate and repeated three times separately.

# 3. Results

### 3-1. $IC_{25}$ and $IC_{50}$ determination of PTX in MDA-MB-231 cells

As we reported in the previous study, the MDA-MB-231 cell line was selected as the main cell line to restore miR-138-5p between four different breast cancer cell lines (MDA-MB-468, MDA-MB-231, SKBR-3, and MCF-7) based on the least level of miR-138-5p transcript and the most level of PD-L1 expression. In the present study, we continued our experiments using the MDA-MB-231 cells as the main cell line to explore the effects of miR-138-5p restoration and paclitaxel therapy combined. A 40 pmol concentration of miR-138-5p mimics was introduced as an optimal transfection dose in MDA-MB-231 cells in our previous study [15]. Here, cells were incubated with various concentrations of PTX (0 to 12.5µg/ml) for 24 h, and the cell viability was determined using an MTT assay to indicate the inhibitory doses of PTX. According to our results, 5 µg/ml and 10 µg/ml of PTX decreased the viability of BC cells to 75% and 50%, respectively. Therefore, 5µg/ml and 10µg/ml of PTX were selected as $IC_{25}$ and $IC_{50}$, respectively (**Fig 1:A**).

### 3-2. miR-138-5p strengthened the toxicity of PTX in lower doses

MTT assay revealed that the survival rates of BC cells have decreased by 25%, 50%, 40%, 51% and 70% in the PTX (IC25), PTX (IC50), miR-138-5p, miR-138-5p+PTX (IC25) and miR-138-5p+PTX (IC50) groups compared to the N-control group respectively (**Fig 1:B**). Besides, the miR-138-5p+PTX (IC25) group showed a significantly less cell survival rate rather than the PTX (IC25) group or the miR-138-5p group alone, which means PTX (IC25) combined with miR-138-5p approximately has toxicity as the same of PTX (IC50). Treatment with 10μg/ml of PTX ($IC_{50}$), separately or in combination with miR-138-5p, was highly toxic and led to more than 50% cell death. So, two groups treated with the mentioned dose were removed from the next tests.

### 3-3. MiR-138-5p reinforced the suppressive effect of PTX on BC cell migration

Using wound healing assay, the migratory potential of MDA-MB-231 cells after transfection with miR-138-5p and, or treatment with PTX (IC25) was explored for 0, 24 and 48 h after scratching via inverted microscope. As mentioned in our previous study, the migration to the wound area was sustained in miR-138-5p-overexpressing cells by downregulation of MMP9, MMP2, vimentin, and upregulation of E-cadherin. Here, results indicated that cell migration was repressed in all groups compared to N-control one. As well, the potential of migration was suppressed more significantly in cells transfected with miR-138-5p and treated with PTX (miR-138-5p+PTX (IC25) group), compared to a separate manner (**Fig 2: A**). Furthermore, the western blotting assay represented a more significantly decreased protein level of MMP2 versus an increased level of E-cadherin protein in the miR-138-5p+PTX (IC25) group against the miR-138-5p group ($p<0.01$, $p<0.0001$) or the PTX (IC25) group alone ($p<0.0001$, $p<0.001$) **(Fig 2: B, C)**. The colored full-length western blots were listed in supplementary figures S1, S2, S3, and S4.

### 3-4. miR-138-5p enhanced the inhibitory potential of PTX on colony formation and cell proliferation in MDA-MB-231 cells

To investigate the effects of miR-138-5p and PTX in combination or a separate manner on cloning and cell expansion, the colony formation and western blotting assays were employed. In our previous study, we showed the inhibitory effects of miR-138-5p on colony formation and proliferation. Here, obtained data displayed that PTX could decrease colony forming and cell proliferating by downregulation of the PI3K/AKT pathway. Moreover, colony formation and the PI3K/AKT pathway were suppressed more significantly in the miR-138-5p+PTX (IC25) group versus other groups ($p<0.0001$) (**Fig 3**). The colored full-length western blots were listed in supplementary figures S1, S5, S6, S7, and S8.

### 3-5. miR-138-5p restoration and PTX therapy combined had a synergistic effect on the induction of apoptosis in MDA-MB-231 cells

Data obtained from flow-cytometry and DAPI staining illustrated induced apoptosis in all MDA-MB-231 treated groups compared to the N-control one. Also, significant increased early apoptosis (25.5% apoptotic cells) and increased total apoptosis (36.6% apoptotic cells) were observed in the miR-138-5p+PTX (IC25) group versus other groups ($p<0.0001$) **(Figure 4)**. Furthermore, elevated remarkable chromatin fragmentation was detected in the miR-138-5p+PTX (IC25) group compared to other ones

**(Figure 5: A)**. Also, qRT-PCR was accomplished to evaluate the transcript levels of several biomarkers involved in apoptosis, including BAX/Bcl-2 ratio, Caspase 3, Caspase 8, and Caspase 9 **(Figure 5: B)**. Our data showed a remarkable increase in BAX/Bcl-2 ratio and elevated transcript levels of Caspase 3 and Caspase 9 in the miR-138-5p+PTX (IC25) group against others ($p$<0.0001).

### 3-6. PTX therapy combined with miR-138-5p restoration improved arresting of BC cells in sub-$G_1$ and $G_1$ phases

As mentioned earlier, PTX arrests the cell cycle in the $G_2$/M phase. Here, using cell cycle assay, we detected 79.9% of PTX (IC25) cells arrested in the $G_2$/M phase. Also, 22.9% and 37.7% of cells belonging to the miR-138-5p group were arrested in the sub-$G_1$ and $G_1$ phases, respectively. Interestingly, in the miR-138-5p+PTX (IC25) group, the percentage of arrested cells in the $G_2$/M phase significantly decreased into 53.2% while 19.9% and 14.6% of them were arrested in the sub-$G_1$ and $G_1$ phases, respectively (**Figure 6: A, B**). In order to investigate the mechanisms of cell-cycle arresting, the transcript levels of several factors involved in cell-cycle, including Cyclin-dependent kinase 4 (CDK4), cyclin D1 (CCND1), and p21 (cyclin-dependent kinase inhibitor 1), were evaluated by qRT-PCR. According to data obtained from qRT-PCR, there was no significant difference between the PTX (IC25) group and the N-control group in transcript levels of named factors. However, considerable downregulation in CDK4 and CCND1 and remarkable upregulation of p21 were observed in both the miR-138-5p and miR-138-5p+PTX (IC25) groups (**Figure 6: C**).

## 4. Discussion

Breast Cancer (BC) is the cause of 25% of all death cases in women worldwide. One of the conventional chemotherapeutic agents is paclitaxel (PTX) [21] which has been utilized to treat BC since 1994 [22]. Paclitaxel is used in adjuvant settings also. Recently, Ke-Da Yu, et al., have suggested a paclitaxel-plus-carboplatin regimen as a practical alternative adjuvant chemotherapy choice for patients with operable triple negative breast cancer [23]. Resistance to chemotherapeutic drugs is considered as the main obstacle in the BC chemotherapy and the insensitivity of BC cells to PTX limits the anti-tumor effects of this agent [26]. On the other hand, PTX have several side effects on patients receiving higher doses of PTX [27]. Dulcie Lai, et al., have suggested the possible mechanism of paclitaxel resistance; TAZ (transcriptional co-activator with PDZ-binding motif) or WWTR1 is a transcriptional coactivator which acts as a downstream regulatory target in the Hippo signaling pathway and forms a complex with TREAD which translocate to the nucleus and binds to the promoter of *Cyr61* or *CTGF* genes and promotes the expression of mentioned genes which subsequently Cyr61 or CTGF protein is secreted out of the cells and activates integrin heterodimers on the cell membrane leading to cell survival and resistance to paclitaxel-induced apoptosis [28]. MicroRNAs are involved in various cellular processes which their deregulation might influence on cellular behaviors leading to multiple disorders and cancers including BC [29]. miR-138-5p as a tumor suppressor miRNA has different roles in biological processes indicated in our previous study. This miRNA is downregulated in the BC cells and its restoration has multiple anti-tumorigenic

effects on BC cells via targeting PD-L1, and suppressing cell proliferation, cell migration and inducing programmed cell death [15]. Several studies demonstrated that miR-138-5p enhances the chemosensitivity of cervical cancer cells to Cisplatin and Glioblastoma (GBM) cells to temozolomide (TMZ), respectively [17, 18]. Besides, restoration of miR-138-5p could improve the chemosensitivity of cancer cells to 5fluorouracil (5FU), through targeting vimentin [16]. In addition, a recent study showed that miR-138-5p restoration could sensitize PTX-resistant epithelial ovarian cancer cells via targeting cyclin-dependent kinase 6 (CDK6) [30]. Another study showed that LncRNA UCA1 through inhibition of miR-138-5p contributes to oxaliplatin (OXA) resistance and activation of the AKT/mTOR signaling pathway in hepatocellular carcinoma cells [31]. On the other hand, recent investigations have proved the synergistic effects of miR-424-5p and miR-383-5p reinforcing the chemosensitivity of BC cells to PTX [19, 20].

So far, the impression of miR-138-5p restoration has not been examined on the chemosensitivity of BC cells to the PTX. The present investigation, indicates on the synergistic effects of overexpressed miR-138-5p on elevating the chemosensitivity of MDA-MB-231 breast cancer cells to PTX. Several investigations have emphasized on suppressive effects of PTX on cell migration in cancerous smooth muscle cells and BC cells [32–35]. We showed the inhibitory effect of miR-138-5p on MDA-MB-231 cells migration, in our previous study. Here, miR-138-5p restoration and PTX therapy combined illustrated their synergistic effect on inhibiting cell migration by modulating the protein levels of MMP2, vimentin, and E-cadherin. A former research has demonstrated that 3'-UTR of vimentin mRNA is a direct target of miR-138-5p [36]. Therefore, it may be concluded that miR-138-5p, through suppressing vimentin and modulating MMP2 and E-cadherin, would intensify the anti-migratory effect of PTX on BC cells. Furthermore, we proved that miR-138-5p in combination with PTX could deduce the potential of cell proliferation and colony formation in MDA-MB-231 cells rather than PTX (IC25) treatment alone. We evaluated the PI3K/AKT pathway in the N-control, miR-138-5p-transfected, PTX (IC25)-treated, and miR-138-5p + PTX (IC25) groups to investigate the molecular mechanisms underlying the anti-proliferative effects of miR-138-5p and PTX on the BC cells. Our findings obtained from MDA-MB-231 cells showed significantly less levels of PI3K, p-PI3K, and AKT proteins in the miR-138-5p + PTX (IC25) group rather than the miR-138-5p group or the PTX (IC25) one. Also, the decreased number of colonies in the miR-138-5p + PTX (IC25) group versus the PTX (IC25) group might reflect the booster effect of miR-138-5p restoration on the anti-mitotic impression of PTX in MDA-MB-231 cells. Likewise, our study indicated that miR-138-5p and PTX therapy combined could significantly elevate the percentage of the sub-$G_1$ and $G_1$ arrested cells compared to the PTX therapy alone. It has been proved that CCND1 overexpression correlates with tumor progression [37] and the CCND1-CDK4 complex promotes the transition from $G_1$ to S phase by sustaining the retinoblastoma protein (pRb) [38]. p21, a cyclin-dependent kinase inhibitor, inhibits all cyclin/CDK complexes and functions as a regulator of cell cycle progression in the $G_1$ and S phases [39–41]. According to the data obtained from MDA-MB-231 cells, CDK4 and CCND1 had less transcript levels in the miR-138-5p + PTX (IC25) group rather than the PTX (IC25) cells. Moreover, significantly up-regulation of p21 was observed in the miR-138-5p + PTX (IC25) group versus the PTX (IC25) group or miR-138-5p-transfected group. Liu et al., introduced CCND1 mRNA as a direct target of miR-138-5p in nasopharyngeal carcinoma cells [42]. miR-138-5p could block the $G_1$/S transition by suppressing the cell-cycle regulators in prostate cancer

cells [43]. Overall, targeting of CCND1 mRNA by miR-138-5p restoration, downregulation of CDK4, and upregulation of p21 may explain the significantly higher percentage of sub-G1 and G1 arrested cells in the miR-138-5p + PTX (IC25) group, and it is another sign of apoptosis too. Therefore, the combination of miR-138-5p with PTX may improve the efficiency of PTX as a chemotherapeutic drug in BC treatment.

# 5. Conclusion

Overall, miR-138-5p restoration in combination with PTX therapy could improve the inhibition of cell migration, apoptosis induction, and the cell cycle arrest. Also, this combination decreases the potency of proliferation and colony-formation in BC cells synergistically. In conclusion, based on our information obtained from the current project, the combined therapy might be a new therapeutic approach to decrease the efficacious consuming PTX dose in chemotherapy and enhance the efficacy of BC treatment. However, further experiments are essential to detect precisely the unknown cellular pathways underlying these synergistic effects or signaling pathways involved in the reversion of paclitaxel resistance by miR-138-5p restoration.

# Abbreviations

| Abbreviation | Full length word |
|---|---|
| BC | Breast cancer |
| PTX | Paclitaxel (Taxol) |
| FDA | Food and drug administration |
| 5-FU | 5-fluorouracil |
| GBM | Glioblastoma |
| TMZ | Temozolomide |
| PD-L1 | programmed cell death ligand1 |
| NC-miR | Negative control miRNA |
| $IC_{50}$ | half maximal inhibitory concentration |
| $IC_{25}$ | quarter maximal inhibitory concentration |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide |
| DMSO | Dimethyl sulfoxide |
| MMP2 | matrix metalloproteinase-2 |
| E-cadherin | epithelial cadherin |
| AKT | Protein kinase B (PKB) |
| p-AKT | phosphorylated Protein kinase B (PKB) |
| p-PI3K | phosphorylated Phosphoinositide 3-kinases |
| PI3K | Phosphoinositide 3-kinases |
| BCL-2 | B-cell lymphoma-2 |
| BAX | Bcl-2-associated X protein |
| Caspase 3 | Cysteine-Aspartic Acid Protease 3 |
| Caspase 8 | Cysteine-Aspartic Acid Protease |
| Caspase 9 | Cysteine-Aspartic Acid Protease 9 |
| P21 | Cyclin-dependent kinase inhibitor 1 |
| CDK4 | Cyclin-dependent kinase 4 |
| CCND1 | cyclin D1 |
| CDK6 | cyclin-dependent kinase 6 |
| OXA | oxaliplatin |

| | |
|---|---|
| pRb | retinoblastoma protein |

# Declarations


### Acknowledgements

We thanks Tabriz Immunology Research Center (IRC), Iran for providing facilities to carry out this research.

**Data Availability Statement:** No Data associated in the manuscript.

**Funding:** *The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.*


**Competing interests:** *The authors have no relevant financial or non-financial interests to disclose.*

**Authors contributions:** *Conceptualization and Study design: BB and RS. Experiments: MR, ERA and PL. Data analysis and interpretation: MR, RS. Writing original draft preparation: MR. Writing review and editing: MR, RS, BB, MAHF and SMBK. Funding acquisition: RS and SMBK. Supervision: RS and BB.*

**Ethics approval:** *This article does not contain any studies with human participants or animals performed by any of the authors.*

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# Figures

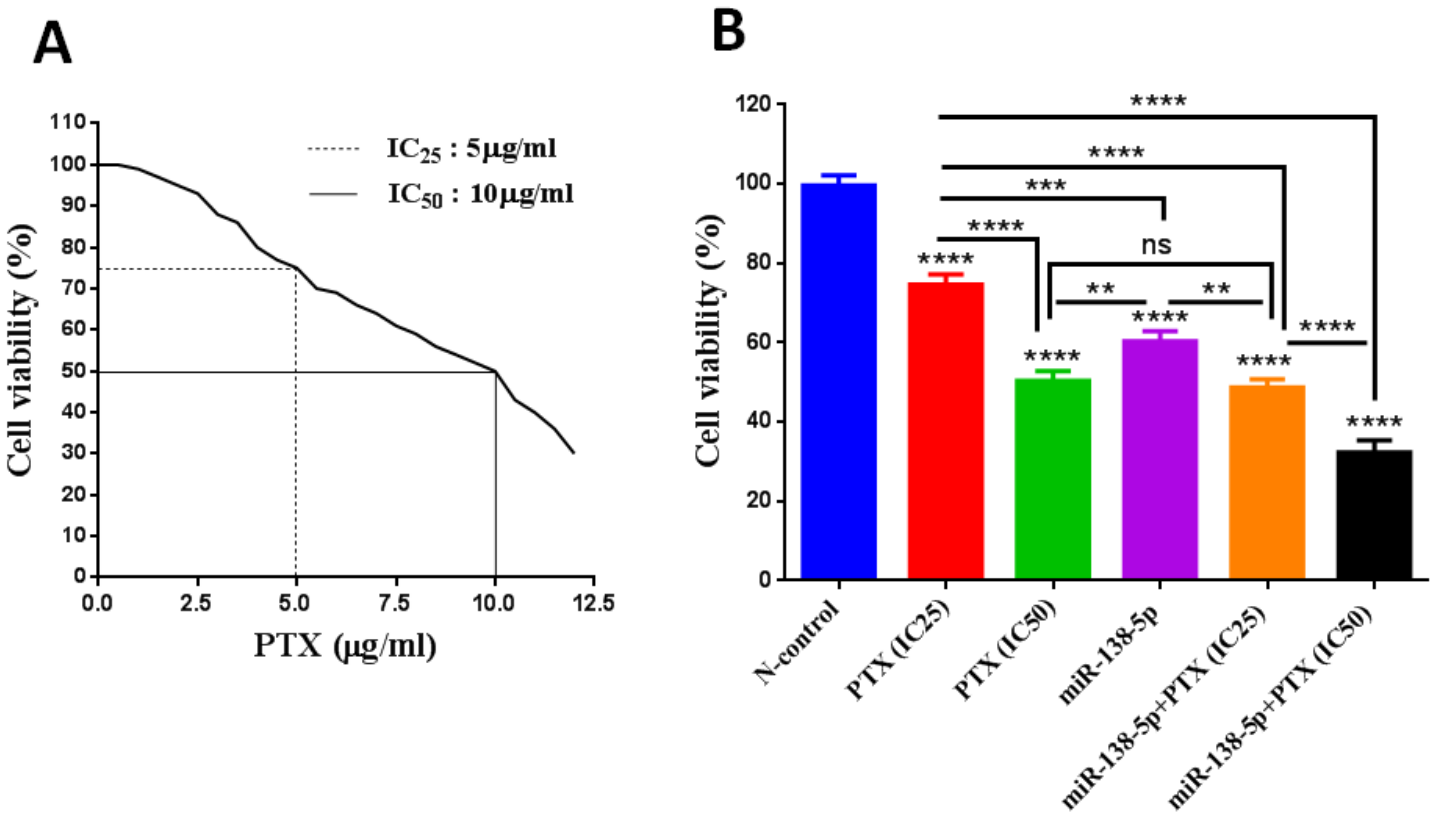
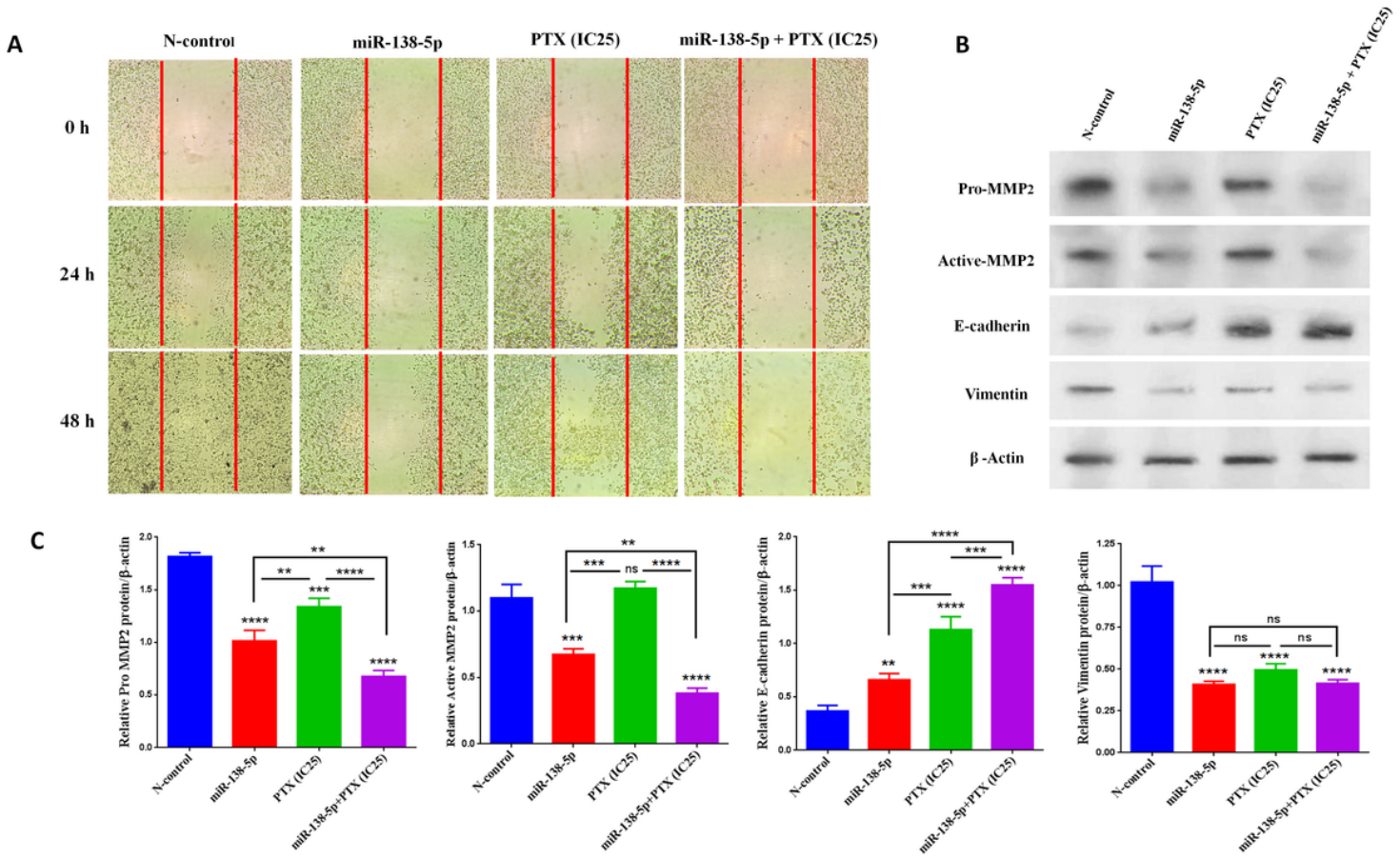


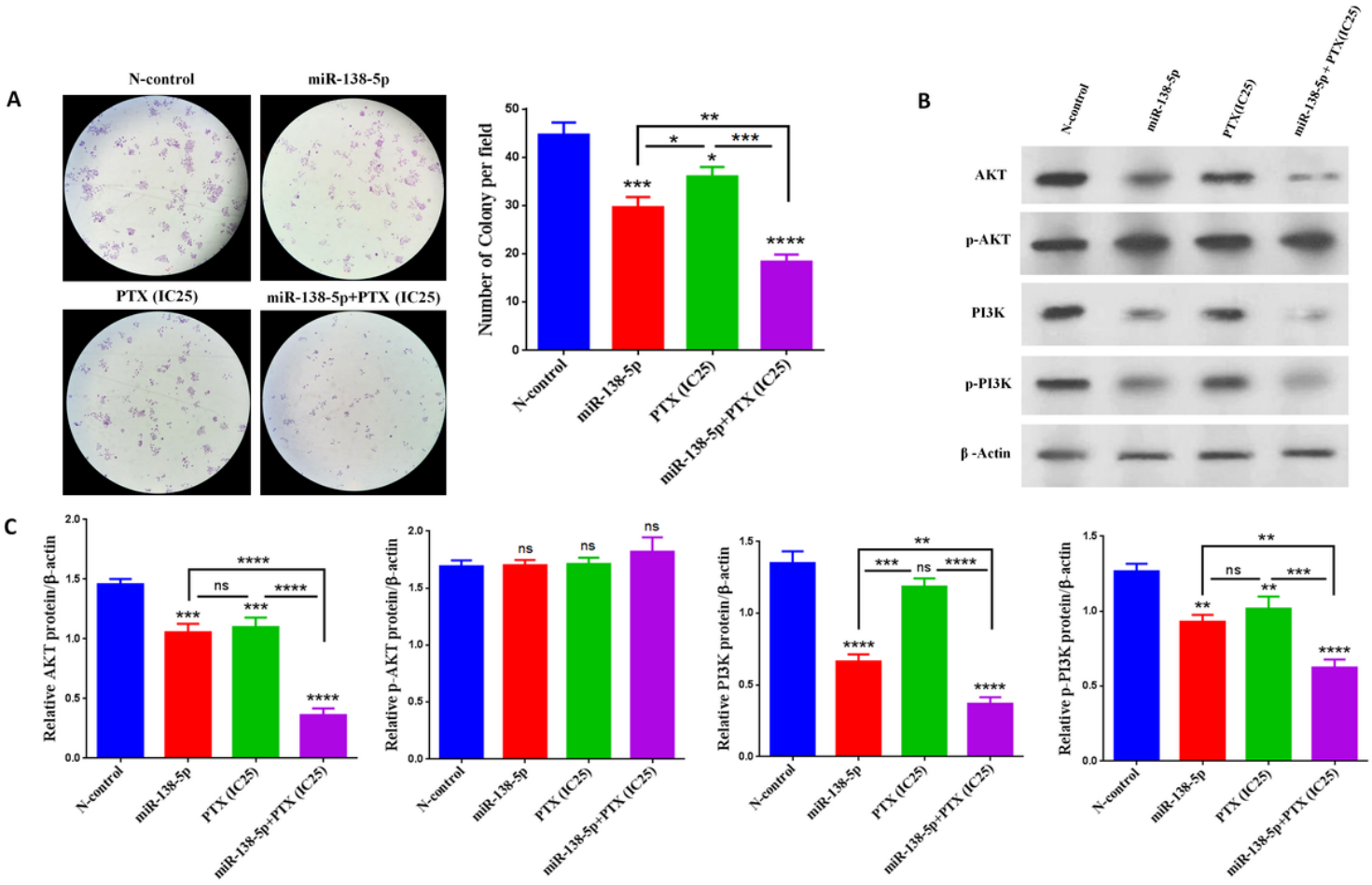

**Figure 1**

Identification of the inhibitory doses of PTX. A. BC cells were treated with 0 to 12.5μg/ml of PTX and MTT assay which applied 24 h after incubation determined 5μg/ml and 10μg/ml of PTX as $IC_{25}$ and $IC_{50}$ respectively in MDA-MB-231 cells. B. The cell viability was investigated among six cellular groups including the N-control, PTX (IC25), PTX (IC50), miR-138-5p, miR-138-5p+PTX (IC25) and the miR-138-5p+PTX (IC50) which detected the increased toxicity of PTX (IC25) in combined with miR-138-5p up to the toxicity of PTX (IC50). (Multiple comparison Two-way ANOVA, ns means no significant, ****$p$<0.0001, ***$p$<0.001 **$p$<0.01).



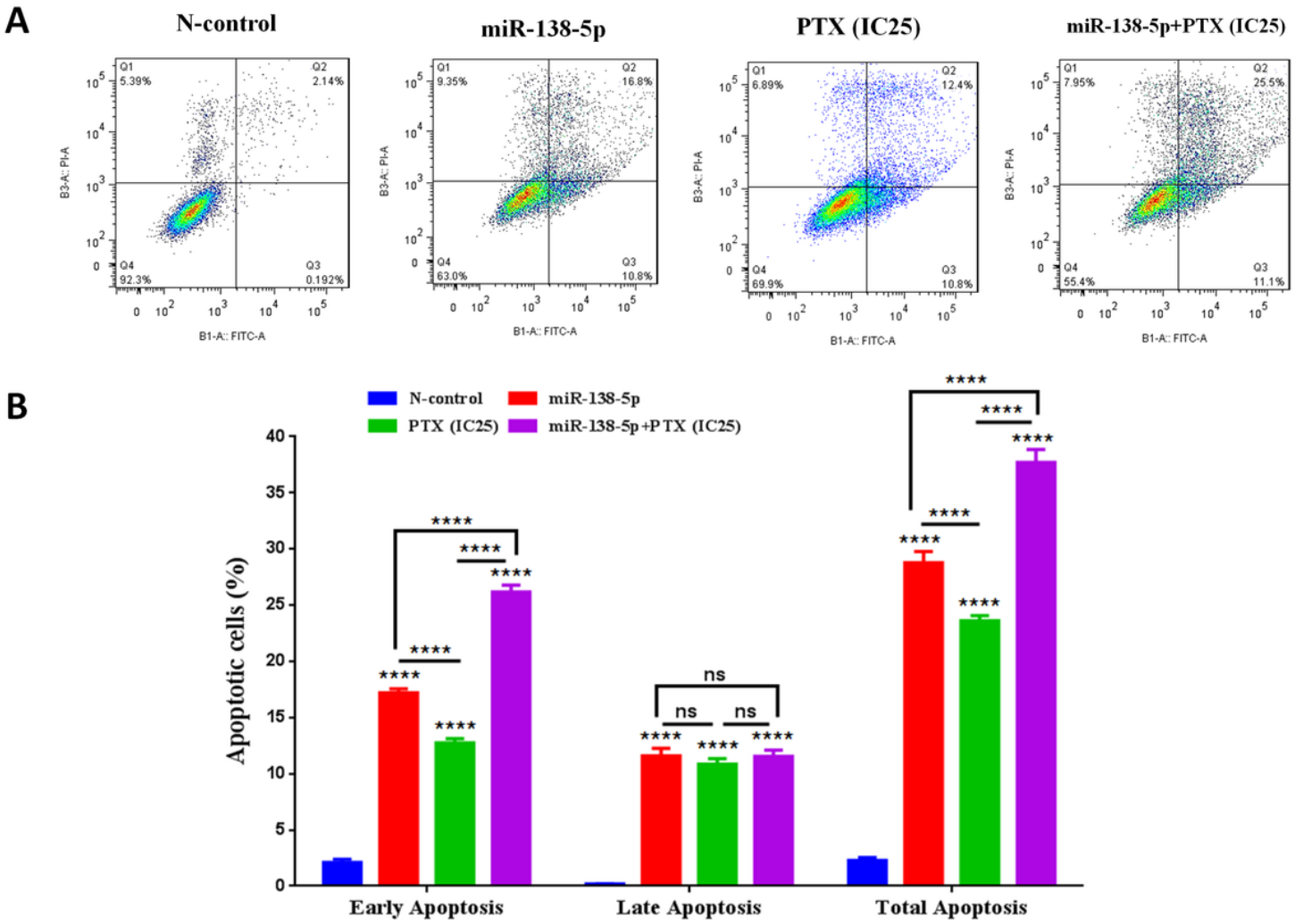

## Figure 2

MiR-138-5p reinforced the suppressive effects of PTX on the migration of MDA-MB-231 cells. A: Cell migration was repressed in all groups compared to the N-control group and also the potency of migration was suppressed more significantly in the miR-138-5p+PTX (IC25) group in comparison to treatment with PTX (IC25) or miR-138-5p separately. B, C. The western blot indicated more significantly decreased level of MMP2 protein but increased level of E-cadherin protein in the miR-138-5p+PTX (IC25) group versus the miR-138-5p group or PTX (IC25) one. (Multiple comparison Two-way ANOVA, ns means no significant, $^{****}p<0.0001$, $^{***}p<0.001$, $^{**}p<0.01$). MMP2: matrix metalloproteinase-2, E-cadherin: epithelial cadherin



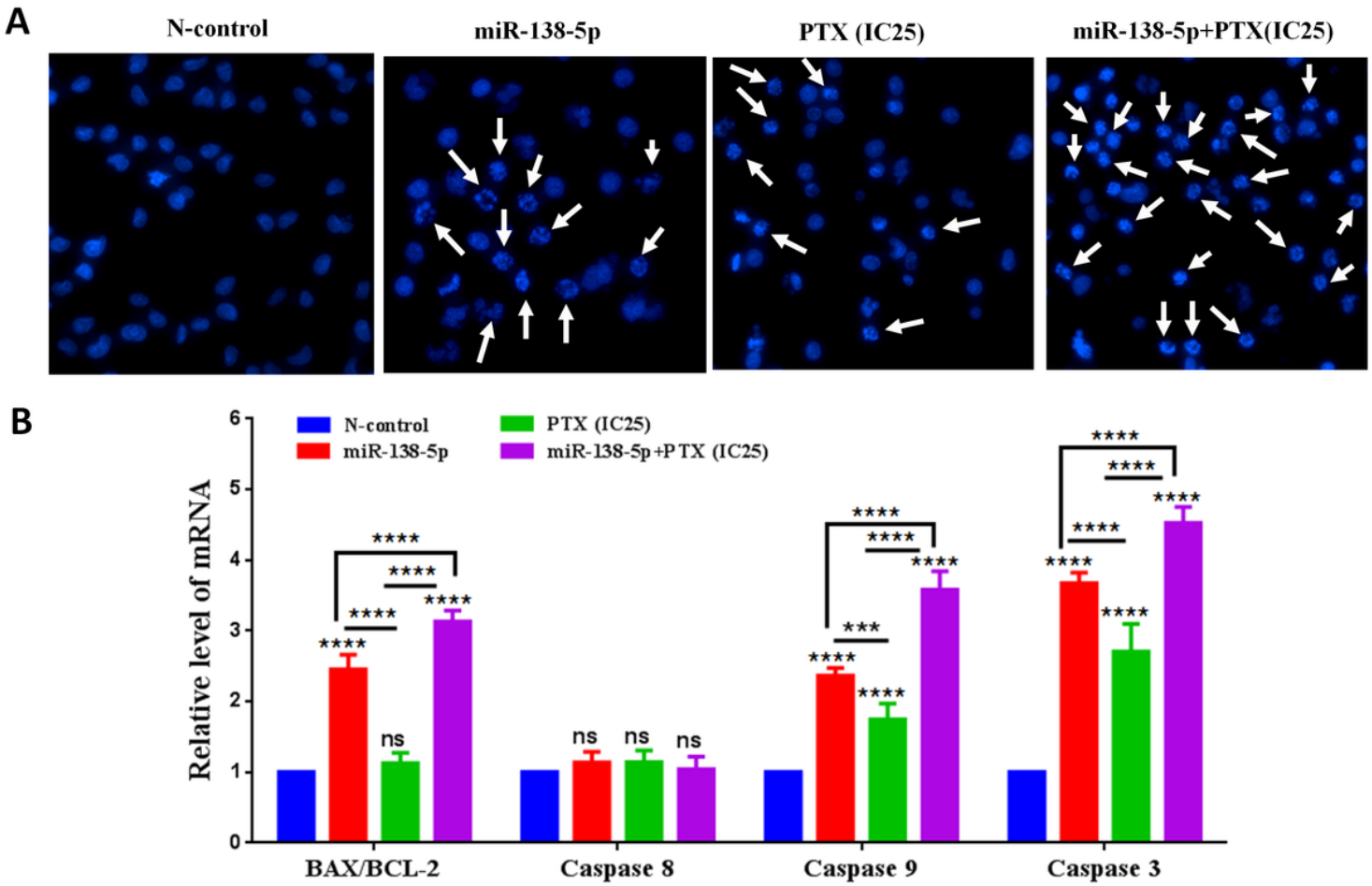

## Figure 3

miR-138-5p enhanced the inhibitory potential of PTX on colony formation and cell proliferation in the BC cells. A. Number of colonies in the miR-138-5p+PTX (IC25) group were significantly less than the PTX (IC25) group or the miR-138-5p one. B, C. Western blotting represented remarkable decreased protein levels of AKT, PI3K and p-PI3K in the miR-138-5p+PTX (IC25) group versus the PTX (IC25) group or the miR-138-5p alone. (Multiple comparison Two-way ANOVA, ns means no significant, * $p$<0.05, ** $p$<0.01, *** $p$<0.001 and **** $p$<0.0001). AKT: Protein kinase B (PKB), p-AKT: phosphorylated Protein kinase B (PKB), PI3K: Phosphoinositide 3-kinases, p-PI3K: phosphorylated Phosphoinositide 3-kinases




## Figure 4

miR-138-5p restoration and PTX therapy combined had synergistic effect on the induction of apoptosis in MDA-MB-231 cells. A, B. Flow-cytometry illustrated induced apoptosis in all treated groups. Increased early apoptosis (25.5% apoptotic cells) and total apoptosis (36.6% apoptotic cells) were detected in the miR-138-5p+PTX (IC25) group rather than others. (Multiple comparison Two-way ANOVA, ns means no significant and **** $p < 0.0001$).




## Figure 5

miR-138-5p restoration and PTX therapy combined had synergistic effect on the chromatin fragmentation and the induction of apoptotic biomarkers in BC cells. A. DAPI staining revealed elevated remarkable chromatin fragmentation in the miR-138-5p+PTX (IC25) group compared to other groups. B. The transcript levels of apoptotic biomarkers, including BAX/Bcl-2 ratio, Caspase3, and Caspase 9 were significantly overexpressed in the miR-138-5p+PTX (IC25) group. (Multiple comparison Two-way ANOVA, ns means no significant and **** $p< 0.0001$). BCL-2: B-cell lymphoma-2, BAX: Bcl-2-associated X protein, Caspase 9: Cysteine-Aspartic Acid Protease 9, Caspase 8: Cysteine-Aspartic Acid Protease, Caspase 3: Cysteine-Aspartic Acid Protease 3

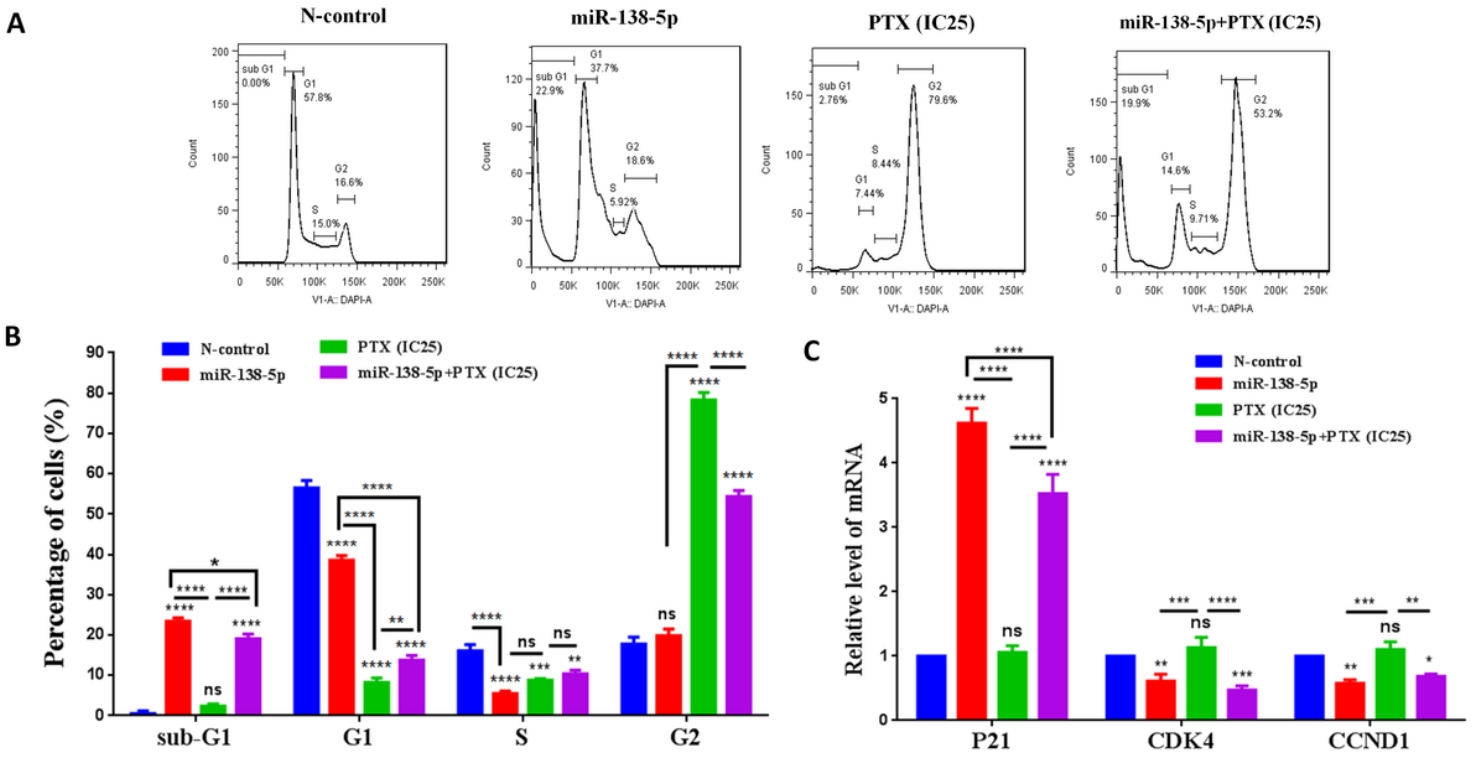



**Figure 6**

PTX therapy combined with miR-138-5p restoration improved arresting of BC cells in the sub-$G_1$ and $G_1$ phases. A, B. Cell-cycle assay detected 79.9% of PTX (IC25) cells arrested in the $G_2$/M phase. In addition, 22.9% and 37.7% of miR-138-5p cells were arrested in the sub-$G_1$ and $G_1$ phases respectively. However, the percentage of arrested cells in the $G_2$/M phase significantly decreased into 53.2% in the miR-138-5p+PTX (IC25) group and also 19.9% and 14.6% of mentioned cells were arrested in the sub-$G_1$ and $G_1$ phases respectively. C. The transcript levels of CCND1, CDK4, and p21 were evaluated by qRT-PCR which there was no significant difference in expression levels of named factors between the PTX (IC25) group and N-control one. But, significantly downregulation in CDK4 and CCND1 and significantly upregulation of p21 were observed in both miR-138-5p and miR-138-5p+PTX (IC25) groups. (Multiple comparison Two-way ANOVA, ns means no significant, * $p$<0.05, ** $p$<0.01, *** $p$<0.001 and **** $p$< 0.0001). P21: Cyclin-dependent kinase inhibitor 1, CCND1: cyclin D1, CDK4: Cyclin-dependent kinase

# Supplementary Files

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