

Original Article



miR-638 Serves as a Biomarker of 5-Fluorouracil Sensitivity to Neoadjuvant Chemotherapy in Breast Cancer

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ABSTRACT

Purpose: Neoadjuvant chemotherapy (NAC) is widely used to treat breast cancer (BC). The prediction and evaluation of chemotherapy responses remains a significant challenge.

Methods: MicroRNAs (miRNAs) play a crucial role in cancer drug resistance. We used a miRNA microarray and identified that miR-638 is downregulated in chemoresistant cases. However, the exact role of miR-638 and the underlying mechanisms of chemoresistance remain unclear. Using real-time quantitative reverse transcription polymerase chain reaction, we found significant downregulation of miR-638 in chemoresistant patients compared with chemosensitive patients. To explore the function of miR-638, we overexpressed and inhibited miR-638 expression in MDA-MB-231 and MCF-7 cells by transfecting them with miR-638 mimics and miR-638 inhibitor, respectively. Cell proliferation and apoptosis were measured using MTS and flow cytometry, respectively. A minimal patient-derived xenograft (MiniPDX™) model was established to evaluate the chemosensitivity to different drugs.

Results: The results showed that cell proliferation decreased and cell apoptosis increased in cells transfected with the miR-638 mimic, and cell proliferation and apoptosis were reversed with transfection of miR-638 inhibitor compared with the control group. Among patients who received 5-fluorouracil (5-FU), miR-638 expression levels were lower in the chemoresistant group than in the chemosensitive group. The MiniPDX™ model showed that MDA-MB-231 cells overexpressing miR-638 were more susceptible to 5-FU treatment *in vivo*.

Conclusion: We provided evidence of acquired resistance to 5-FU caused by miR-638 deficiency. Alterations in miR-638 may be used with 5-FU chemotherapy during NAC for BC.

Keywords: Breast Neoplasms; Fluorouracil; MicroRNA-638; Neoadjuvant Therapy

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Conflict of Interest

The authors declare that they have no competing interests.

Author Contributions

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INTRODUCTION

Neoadjuvant chemotherapy (NAC) is an important treatment strategy for breast cancer (BC). NAC is used to decrease tumor volume and lymph node metastasis [1] and increase the probability of successful breast-conserving surgery. Importantly, NAC predicts drug sensitivity and response to chemotherapy. Currently, NAC regimens are mainly based on traditional molecular subtypes [2], including the status of the estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki67 proliferation index determined using immunohistochemistry (IHC). However, BC is a highly heterogeneous tumor and the response to NAC may differ between BCs of the same subtype. Thus, we urgently need biomarkers that predict chemotherapeutic responses and guide chemotherapeutic strategies.

MicroRNAs (miRNAs) are small non-coding RNA molecules that are frequently dysregulated in different cancers [3]. Recent studies have revealed that specific miRNAs are associated with BC and can be used for diagnosis, prognosis, and as therapeutic strategies [4]. Our previous study identified a signature of five miRNAs that predicts chemotherapy response using miRNA microarrays. To explore the biological function of dysregulated miRNAs in BC chemoresistance, miR-638 was selected for further study because it was the most differentially expressed miRNA between the chemoresistant and chemosensitive groups according to quantitative polymerase chain reaction (qPCR) validation.

miR-638 participates in the occurrence and development of multiple tumors. For example, miR-638 promotes cell invasion and epithelial-mesenchymal transition in hepatocellular carcinoma [5]. In gastric cardia adenocarcinoma, miR-638 regulates cell proliferation, apoptosis, migration, and invasion by targeting *MACC1* [6]. Additionally, in BC, downregulation of miR-638 promotes cancer progression and results in poor prognosis [7]. miR-638 is also overexpressed in *BRCA1*-deficient triple-negative breast cancer (TNBC) [8]. However, the role and function of miR-638 in NAC in BC remains unclear.

In this study, we further verified the differential expression of miR-638 between the chemosensitive and chemoresistant groups and explored the role of miR-638 in cell proliferation and apoptosis *in vitro*. Moreover, we used the *in vivo* MiniPDX™ model to determine the chemosensitivity of miR-638 overexpression to various drugs. We aimed to evaluate the role of miR-638 as a potential biomarker for predicting response to NAC in BC.

METHODS**Clinical specimens**

BC specimens (n = 119) were obtained from Shandong University Qilu Hospital, Jinan, China between January 2009 and December 2013. All patients received NAC in varying cycles after diagnosis as confirmed using biopsy. Tumor resection was performed after NAC. According to the 2013 St. Gallen Consensus Recommendations [9], BC is divided into four subtypes based on immunohistochemical markers: Lumina A (ER+, PR+, Her-2-, and Ki67 ≤ 14%); Lumina B (ER+, PR+/-, Her-2-, Ki67 > 14% or ER+, PR+/-, and Her-2+); HER-2 positive (ER-, PR-, and Her-2+); and triple-negative type (ER-, PR-, and Her-2-). A cut-off value of 14% was used for the Ki67 index. Clinicopathological characteristics were obtained from patient files and pathology reports (Table 1). Informed consent was obtained from all patients. The Ethics Committee of

Table 1. Clinical characteristics of patients according to miR-638 expression level

Characteristics	Low expression (n = 60)	High expression (n = 59)	p-value
Age (yr)			0.6849
≤ 40	11	14	
41–60	36	35	
≥ 60	13	10	
Tumor size			0.4789
≤ 2	6	10	
2.1–5	48	42	
> 5	6	7	
Lymph node metastasis			0.2918
Absent	12	17	
Present	48	42	
Histological grade			0.3471
I	1	1	
II	44	36	
III	15	22	
Chemotherapy			1.0000
With fluorouracil	22	21	
Without fluorouracil	38	37	
Chemotherapy assessment			0.0028*
Resistance	44	27	
Sensitive	16	32	
ER			0.3193
Negative	15	20	
Positive	45	39	
PR			0.3546
Negative	22	27	
Positive	38	32	
HER2			0.1132
Negative	46	37	
Positive	14	22	
p53			1.0000
Negative	21	20	
Positive	39	39	
Ki-67			0.0056*
≤ 14%	8	22	
> 14%	51	38	
Molecular subtype			0.4712
HER2 overexpression	11	14	
Basal-like	4	6	
Luminal A	23	15	
Luminal B	22	24	

ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2.

*Significant difference.

Shandong University Qilu Hospital approved this study (KYLL-2019-2-010), and procedures involving human subjects were performed according to the Declaration of Helsinki.

NAC regimens and Miller and Payne response assessment

The NAC regimens used in this study were mainly of six types: adriamycin and cyclophosphamide (AC); adriamycin and docetaxel (AT); adriamycin, cyclophosphamide, and docetaxel (TAC); cyclophosphamide, docetaxel, and 5-fluorouracil (CTF); cyclophosphamide, epirubicin, and 5-fluorouracil (CEF); and cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) chemotherapy. Of these regimens, CTF, CEF, and CMF were included in the chemotherapy with 5-fluorouracil (5-FU) group, and the rest, including AC, AT, TAC, and other regimens, were integrated into the chemotherapy without 5-FU group. The detailed regimens of patients in this study are shown in **Supplementary Table 1**.

Miller and Payne (MP) grades were evaluated based on reduced tumor cellularity in the resected samples by comparing them with pre-treatment needle biopsy samples. Patients were divided into a chemoresistant group (MP grades 1–2; < 30% reduction in tumor cells) and a chemosensitive group (MP grades 3–5; 30%–100% reduction in tumor cells) [10]. Among the 119 patients, 49 (41.18%) were chemosensitive and 70 (58.82%) were chemoresistant.

Real-time qPCR

Tissue formalin-fixed paraffin-embedded (FFPE) needle biopsy samples of BC obtained before chemotherapy were used to evaluate miRNA expression. Total miRNA from FFPE sections was scraped with a 25-G needle under a microscope and extracted using a miRNeasy kit according to the manufacturer's instructions (Bioteke, Beijing, China). We reverse-transcribed 100 ng of miRNA and performed quantitative reverse transcription polymerase chain reaction (qRT-PCR) using a 7900HT system (Applied Biosystems, Foster City, CA, USA) and an All-In-One™ qRT-PCR detection kit (Genecopeia, Rockville, MD, USA) according to the manufacturer's instructions. Primers for miR-638 and the endogenous control U6 were purchased from Genecopeia. The relative expression levels of selected miRNAs were calculated using the traditional formula: $2^{-\Delta\text{Ct}} [\Delta\text{Ct} = (\text{Ct}_{\text{miRNA}}) - (\text{Ct}_{\text{U6}})]$. All reactions were performed in duplicates.

Cell proliferation and apoptosis assays

The human BC cell lines MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection and Tianjin Blood Institute (Tianjin, China). All cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum. One month prior to the experiments, both cell lines were authenticated using STR profile analysis and were confirmed to be Mycoplasma-negative. Cells were transfected with miR-638 mimics, miR-638 inhibitor, or negative control. After transfection, cell viability and proliferation were measured using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA) [11]. After incubation with 5-FU at a concentration of 10 μM for 24 h, the proliferation and apoptosis of cells transfected with miR-638 mimics, miR-638 inhibitor, or negative control were also measured. Apoptosis was measured using an Annexin V-FITC and PI staining flow cytometry kit (KeyGEN Biotech, Nanjing, China) and a flow cytometer (FACSCantoII, BD Science, Franklin, NJ, USA), according to the manufacturer's instructions [12].

Minimal patient-derived xenograft (MiniPDX™) model

The miR-638 mimic LV3 overexpression lentiviral vector (LV-miR-638) was purchased from GenePharma (Shanghai GenePharma Co., Ltd., Shanghai, China). MDA-MB-231 cells (a TNBC cell line with a more malignant phenotype [13]) were transfected with LV-miR-638 for 48 h, enriched, and filled into a MiniPDX™ efficacy evaluation system device (Shanghai LIDE Biotech Co., Ltd, Shanghai, China). The devices were subcutaneously injected into Nu/Nu-nude mice. Five different drugs, including epirubicin (Epi), docetaxel (Doc), olapalib (Ola), cisplatin (Cis), and 5-FU, were separately administered according to the clinical regimens (see **Supplementary Table 2**) every day. No drugs were administered to the control group. There were two mice in each group and three replicates for each mouse. Changes in the body weight of the mice after drug administration were measured daily. After 6 days, the vehicle devices were removed. Tumor cell viability was evaluated by measuring the ATPase activity. The proliferation rate ($T/C\% = \text{treatment group proliferation rate}/\text{control group proliferation rate}$) was calculated using the following equation:

$$\frac{\text{Mean RLU of the Treatment Group on Day 7} - \text{Mean RLU on Day 0}}{\text{Mean RLU of the Vehicle Group on Day 7} - \text{Mean RLU on Day 0}}$$

Based on this equation, the lower the T/C%, the greater the sensitivity of the tumor to the drug. Changes in the body weight of the mice after drug administration were measured to predict the side effects of the drugs. Relative change in body weight = $(BW_i - BW_0)/BW_0 \times 100\%$, where BW_i is the average body weight after drug administration and BW_0 is the average body weight at the first administration.

Statistical analysis

The microarray data were deposited in ArrayExpress (GSE73736). Differentially expressed genes in TNBC and normal cells were analyzed using the TCGA-BRCA public database. The target genes of hsa-miR-638 were predicted using TargetScan and miRanda algorithms. The intersection of these two parts was identified as a target of miR-638. Kyoto Encyclopedia of Genes (KEGG) pathway enrichment analysis was performed using the web-based tool StarBase (<http://starbase.sysu.edu.cn/>). The potential functional network of the selected miRNAs and their targets was constructed using the Cytoscape software (<http://www.cytoscape.org/>). Analyses were performed using the GraphPad Prism 5 statistical software (GraphPad Software Inc., La Jolla, CA, USA). Differences between the two groups were compared using the Student's *t* test. Fisher's exact test was used to analyze the association between miR-638 and the clinicopathological parameters. *P* values were considered statistically significant at $p < 0.05$.

RESULTS

miR-638 expression in BC samples receiving NAC

Our previous study on BC [14] found that miR-638 was downregulated in 10 cases of chemoresistance compared with 10 cases of chemosensitivity, as detected using microarray. It was further confirmed in the training, internal testing, and validation sets. In the present study, the expression of miR-638 in 119 BC cases treated with NAC was detected using qRT-PCR. miR-638 expression was divided into high or low expression levels, with the median as the cut-off point [15, 16]. The association between miR-638 expression and various clinicopathological parameters was analyzed (Table 1). miR-638 expression was significantly associated with chemoresistance ($p = 0.0028$). Moreover, a lower expression level of miR-638 was associated with a higher Ki67 proliferation index ($p = 0.0056$), suggesting a poor prognosis. There were no significant associations between miR-638 expression and other parameters, including age, tumor size, lymph node metastasis, histological grade, ER, PR, Her-2, p53, or molecular subtypes ($p > 0.05$) (Table 1).

Downregulated miR-638 was found in 5-FU chemoresistant cases

Differential expression of miR-638 between the chemoresistant and chemosensitive groups was further analyzed. The results showed that, compared to the chemosensitive group, miR-638 was significantly downregulated in the chemoresistant group ($p < 0.001$; Figure 1A), which was consistent with the results of our previous study. Furthermore, 43 of the 119 patients received 5-FU, including 30 with chemoresistance and 13 with chemosensitivity, and 22 of them had low expression levels of miR-638 and 21 had high expression levels of miR-638. In the miR-638 low expression group, 18 patients were chemoresistant and 4 were chemosensitive, whereas in the miR-638 high expression group, 12 patients were chemoresistant and 9 were chemosensitive. For these 43 cases, the relative expression levels of miR-638 were also compared between the chemoresistant and chemosensitive groups. The results showed higher miR-638 expression levels in the 5-FU chemosensitive group than in the chemoresistant group ($p = 0.0246$; Figure 1B), suggesting that high miR-638 expression levels might predict 5-FU sensitivity.

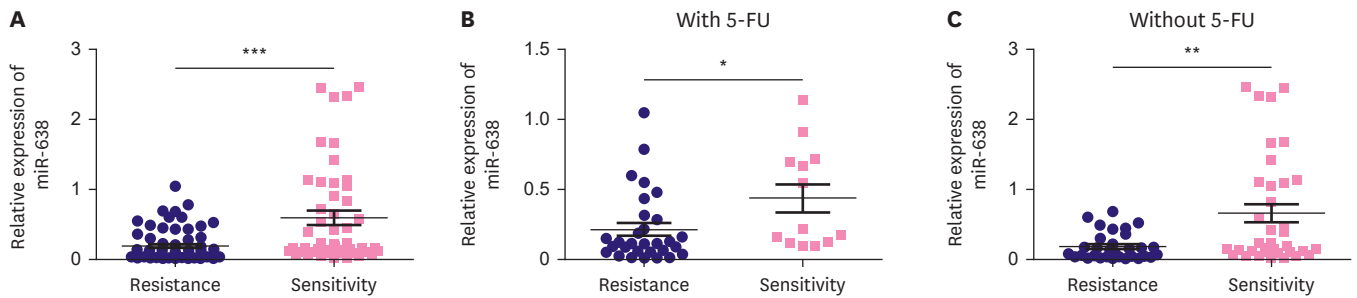


Figure 1. miR-638 expression level in breast cancer. Of a total of 119 cases, miR-638 was significantly downregulated in the chemoresistant group compared with the chemosensitive group (A; $p < 0.001$). Of the 43 patients who received 5-FU, compared with the chemosensitive group, miR-638 was significantly downregulated in the chemoresistant group (B; $p = 0.0246$). Of the 76 patients who received treatment without 5-FU, compared with the chemosensitive group, miR-638 was significantly downregulated in the chemoresistant group (C; $p = 0.0016$). 5-FU = 5-fluorouracil.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In the other group of 76 patients who did not receive 5-FU, there were 40 cases of chemoresistance (including 30 with low expression of miR-638 and 10 with high expression of miR-638) and 36 cases of chemosensitivity (including 13 with low expression of miR-638 and 23 with high expression of miR-638). In the 76 patients who received chemotherapy without 5-FU, the relative expression levels of miR-638 were also compared between the chemoresistant and chemosensitive groups. The results showed a lower miR-638 expression level in the chemoresistant group than in the chemosensitive group ($p = 0.0016$; **Figure 1C**), which also partly suggested that the low expression of miR-638 might be related to a more diverse chemotherapy response.

miR-638 inhibits cell proliferation and promotes apoptosis *in vitro*

The expression of miR-638 in MCF-7 and MDA-MB-231 cells was detected using qPCR. The results showed that miR-638 expression was lower in MDA-MB-231 cells than that in MCF-7 cells (**Supplementary Figure 1**). To explore the biological function of miR-638 in BC, we upregulated miR-638 expression in both MCF-7 and MDA-MB-231 cells by transfection with miR-638 mimics and downregulated it by transfection with an miR-638 inhibitor. After 24, 48, and 72 h of transfection, MTS assay was used to confirm the proliferation of MDA-MB-231 and MCF-7 cells. Compared to the negative control group, the viability and proliferation of both MDA-MB-231 and MCF-7 cells were significantly decreased in the miR-638 transfection group (**Figure 2A, D**; all $p < 0.05$). In contrast, cell viability and proliferation significantly increased in the miR-638 inhibitor transfection group compared with those in the inhibitor control group (**Figure 2B, E**; all $p < 0.05$). Furthermore, flow cytometry demonstrated enhanced apoptosis in miR-638 transfected-MCF-7 and MDA-MB-231 cells and reduced apoptosis in miR-638 inhibitor-transfected cells (**Figure 2C, F**) compared with the negative control and inhibitor control groups. These results suggest that miR-638 overexpression inhibits proliferation and promotes apoptosis of BC cells.

Overexpression of miR-638 is associated with sensitivity to 5-FU *in vivo* and *in vitro*

To identify the drug towards which a high sensitivity is associated with the upregulated expression of miR-638, we used a MiniPDX™ model *in vivo*—a good prediction tool to assess the sensitivity and resistance levels of chemotherapy drugs [17]. The workflow of this experiment is shown in **Supplementary Figure 2**. We assessed the sensitivity of MiniPDX™ to chemotherapeutics, including Epi, Doc, Cis, and 5-FU, as well as the new target drug, Ola. Standard regimens of these five drugs were administered (**Supplementary Table 2**). The lower

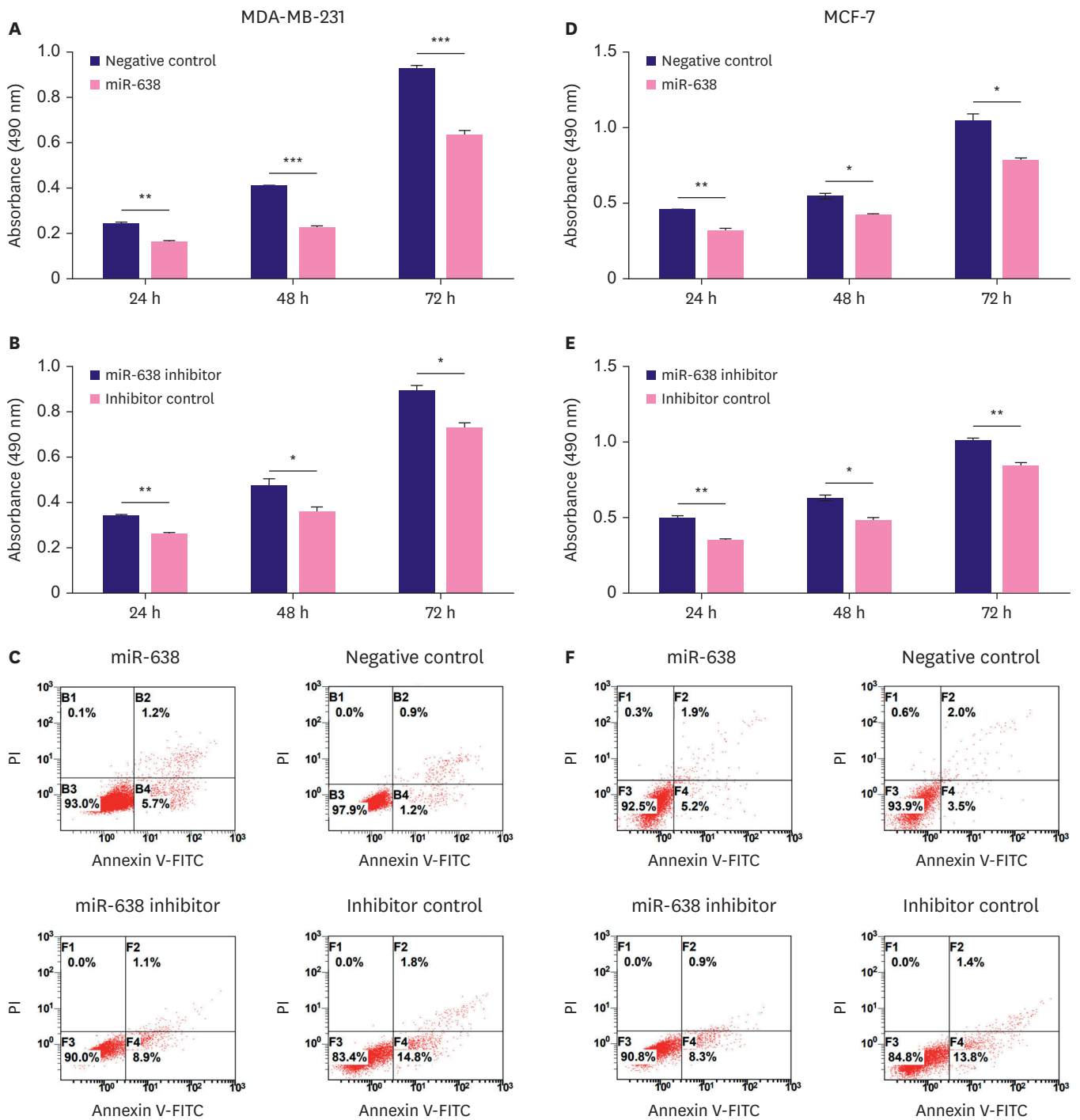


Figure 2. *In vitro* function of miR-638. Compared with the negative control group, cell proliferation significantly decreased in MDA-MB-231 cells after transfection with miR-638 for 24, 48, and 72 hours (A; $p = 0.0031$, $p = 0.0002$, and $p = 0.0007$, respectively). Increased MDA-MB-231 cell proliferation was observed at 24, 48, and 72 hours in the miR-638 inhibitor transfection group (B; $p = 0.0031$, $p = 0.0455$, and $p = 0.0107$, respectively). The proliferation ability of MCF-7/ADM cells was suppressed in the miR-638 transfection group (D; $p = 0.0039$, $p = 0.0183$, and $p = 0.0124$, respectively) and increased in the miR-638 inhibitor transfection group (E; $p = 0.0025$, $p = 0.0271$, and $p = 0.0064$, respectively) compared with the negative control and inhibitor control groups. Flow cytometry analysis showed that enhanced apoptosis activity was observed in the MDA-MB-231 and MCF-7 cells transfected with miR-638 (7.1% and 5.5%, respectively) compared with the negative control or inhibitor control groups (6.9% and 2.1%, respectively), and reduced apoptosis in both cell types treated with miR-638 inhibitor (10.0% and 16.6% [control]; and 9.2% and 15.2% [treatment group], respectively. C, F) was observed. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

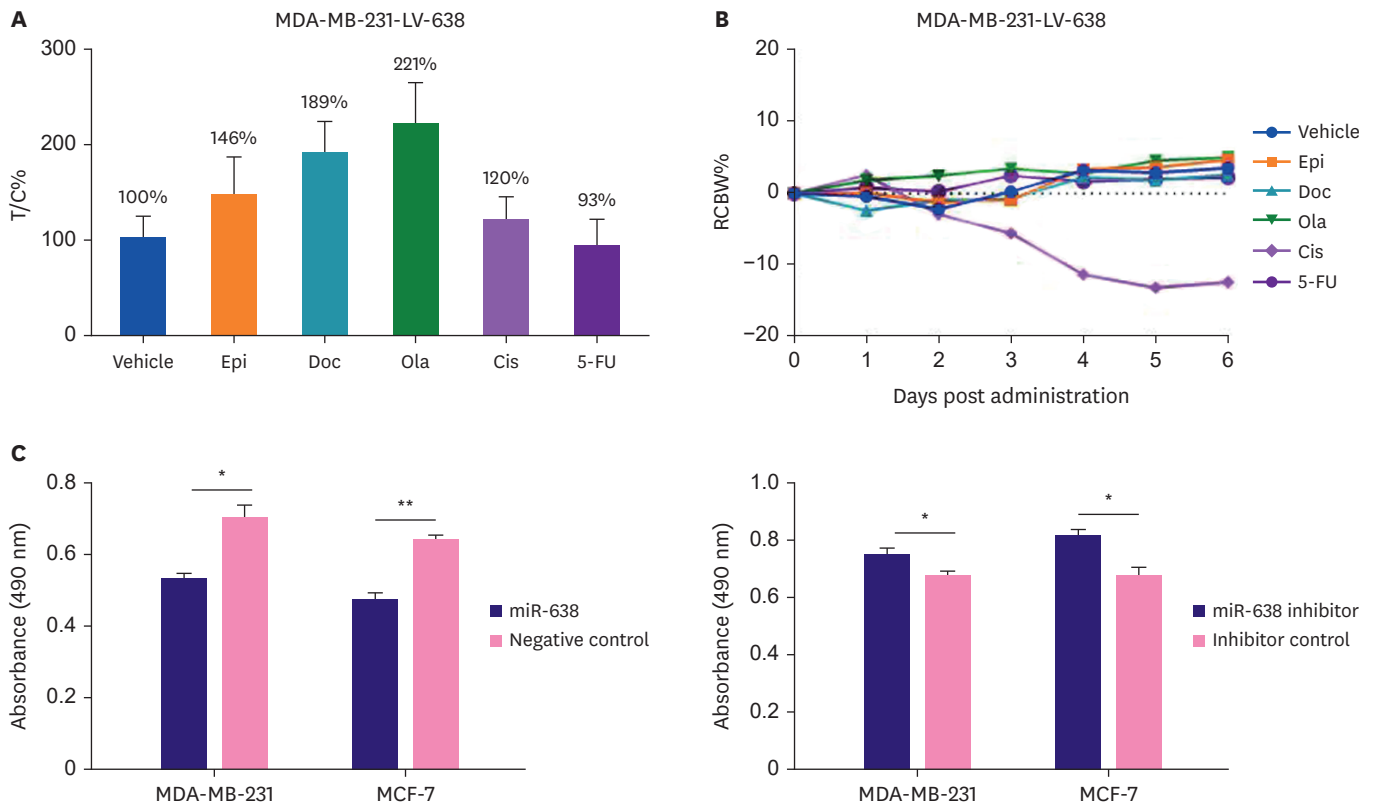


Figure 3. MiniPDX™ model. The T/C% value of Epi, Doc, Ola, Cis and 5-FU group was 146%, 189%, 221%, 120%, and 93% (A). The T/C value of Epi, Doc, Ola and Cis group was higher than that of the control group and that of 5-FU was lower than the control group. A low proliferation rate of tumor cells was observed in the 5-FU group. There was no statistically significant decrease in the body weight of mice in the five groups (B). RCBW% of mice in Epi (range, 1.32–4.87), Doc (range, 2.76–6.83), Ola (range, 1.58–4.80), Cis (range, 13.64–2.02), and 5-FU (range, 2.46–5.34) group is shown. There was a decrease in body weight on 4–6 day in Cis group, but it was statistically insignificant (RCBW%: $\leq 15\%$). With 5-FU (10 μM) *in vitro*, MTS showed that cell viability and proliferation of both MDA-MB-231 and MCF-7 cells significantly decreased in the miR-638 transfection group ($p = 0.0188$ and $p = 0.0033$) and increased in the miR-638 inhibitor transfection group ($p = 0.0199$ and $p = 0.0320$; C) compared to the control group.

T/C% = treatment group proliferation rate/control group proliferation rate; Epi = epirubicin; Doc = docetaxel; Ola = olaparib; Cis = cisplatin; 5-FU = 5-fluorouracil; RCBW% = rate of change in body weight.

the T/C%, the higher the inhibition rate of the tumor cells by the drug. The MiniPDX™ model results showed that the T/C% values of the Epi, Doc, Ola, Cis, and 5-FU groups were 146, 189, 221, 120, and 93%, respectively (Figure 3A; see Supplementary Table 3). The T/C values of the Epi, Doc, Ola, and Cis groups were higher than those of the control group, and the T/C value of 5-FU group was lower than that of the control group. The highest inhibition rate was observed in the 5-FU group, indicating that cells overexpressing miR-638 were sensitive to 5-FU, and these cells were resistant to Epi, Doc, Cis, and Ola. The body weight of each mouse is shown in Supplementary Table 4. Rate of change in body weight (RCBW%) of mice in Epi (range: 1.32–4.87), Doc (range: 2.76–6.83), Ola (range: 1.58–4.80), Cis (range: 13.64–2.02) and 5-FU (range: 2.46–5.34) group is shown in Figure 3B. Although there was a decrease in body weight on 4–6 day in the Cis group, RCBW% was no more than 15%, and the difference was not statistically significant.

To further verify the role of miR-638 in 5-FU sensitivity *in vitro*, after 24 h of 5-FU treatment at a concentration of 10 μM , the MTS assay was used to confirm the proliferation of MDA-MB-231 and MCF-7 cells transfected with miR-638 or miR-638 inhibitor. The results showed that with 5-FU the viability and proliferation of both MDA-MB-231 and MCF-7 cells significantly decreased in the miR-638 transfection group (Figure 3C, left; $p=0.0188$; 0.0033)

and increased in the miR-638 inhibitor transfection group (Figure 3C right; $p=0.0199$; 0.0320) compared with the negative control group, proving that miR-638 overexpression was associated with enhanced chemotherapy sensitivity to 5-FU *in vitro*.

Identification of targets and pathways of drug resistance regulated by miR-638

To further explore the potential role of miR-638 in drug resistance in TNBC, putative targets of miR-638 were predicted using the TargetScan and miRanda algorithms. Differentially expressed genes in TNBC and normal cells were analyzed using the TCGA-BRCA public database. Intersecting genes from these databases were collected. KEGG pathway enrichment analysis showed that the targets of miRNAs were mainly involved in the calcium signaling and PI3K-Akt signaling pathways (Figure 4A, B), as well as in other significant pathways, such as transcriptional misregulation in cancer, EGFR tyrosine kinase inhibitor resistance, small cell lung cancer, circadian entrainment, glycerophospholipid metabolism, HIF-1 signaling pathway, maturity-onset diabetes of the young, and type II diabetes mellitus. Twenty-three genes targeted by miR-638 were found to be involved in these pathways. Potential target genes of miR-638, such as CACNA1A, VEGFA, CACNA1I, FGF23, CAMK2A,

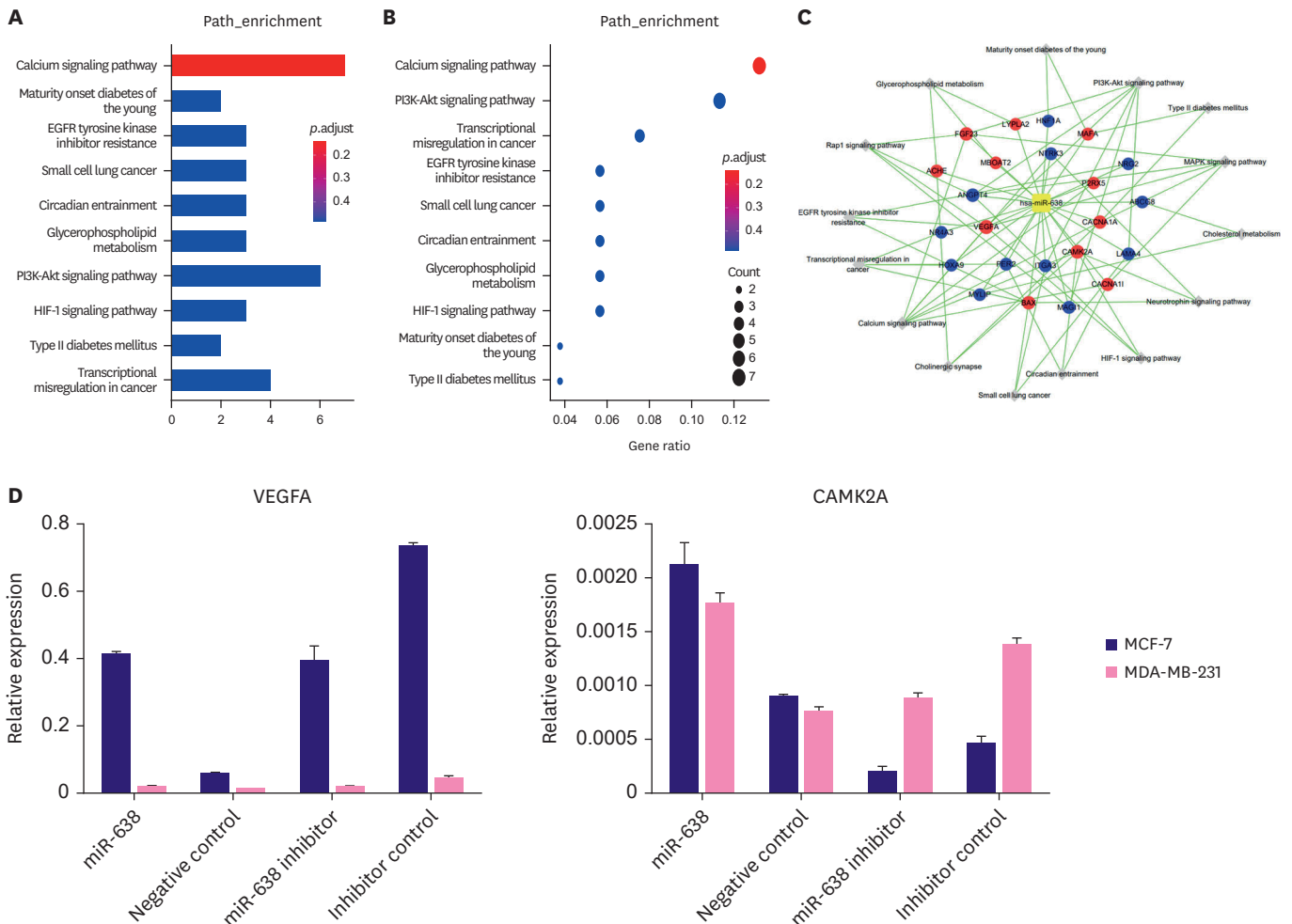


Figure 4. Genes and pathways regulated by miR-638. The signaling pathway regulated by miR-638 ($p < 0.05$; A). Signaling pathways affected by the genes regulated by miR-638 (B). The network of targeted genes and related signaling pathways of miR-638 (C). The expression of VEGFA and CAMK2A. Compared with negative control or inhibitor control group, the expression of VEGFA (D; left) and CAMK2A (D; right) in both MCF-7 and MDA-MB-231 cells was upregulated in miR-638 transfection group and downregulated in miR-638 inhibitor group.

NTRK3, and P2RX5 are mainly involved in the calcium signaling pathway. Additionally, VEGFA, LAMA4, ANGPT4, FGF23, MAGI1, and ITGA3 are involved in the PI3K-Akt signalling pathway. The network of miR-638 and 23 target genes is shown in **Figure 4C** ($p < 0.05$).

As the resistance mechanism of miR-638 is mediated, we preliminarily selected five potential target genes, including four upregulated genes (CACNA1A, VEGFA, FGF23, CAMK2A) and one downregulated gene (NTRK3) mainly involved in the calcium signaling pathway for further validation. After transfection with miR-638 or miR-638 inhibitor for 48 h, the relative expression of these five genes in MCF-7 and MDA-MB-231 cells compared with negative or inhibitor control was quantified using qPCR. The results showed that the expression of VEGFA and CAMK2A (**Figure 4D**) in both cell lines was upregulated in the miR-638 transfection group and downregulated in the miR-638 inhibitor group, consistent with the results predicted by the software. However, the expression of CACNA1A, FGF23, and NTRK3 (**Supplementary Figure 3**) in both cell lines did not show a similar result with the software. These results suggest that VEGFA and CAMK2A are potential targets of miR-638 in BC.

DISCUSSION

NAC has been used in the treatment and management of patients with BC. Larger tumor size (> 5 cm), HER-2 overexpression, TNBC with tumor size > 3 cm, and lymph node metastasis are indications for NAC [18]. However, NAC has some risks. If NAC is not efficacious, it may add to the physical and economic burdens on patients. Thus, it is important to identify biomarkers that can predict the response to chemotherapy and guide treatment.

miR-638 was downregulated in chemoresistant cases compared to chemosensitive cases as confirmed using miRNA microarray analysis and qPCR. Decreased expression of miR-638 has also been reported in non-small cell lung cancer [19] and colorectal carcinoma [20], suggesting a tumor-suppressive role of miR-638 [21]. The function of miR-638 is related to cell proliferation and invasion. Our results showed that downregulation of miR-638 was associated with a higher Ki67 index, indicating a potential effect on cell proliferation. After transfection with miR-638 mimics *in vitro*, the proliferation of MCF-7 and MDA-MB-231 cells decreased and cell apoptosis increased compared with the negative control group. Additionally, cell proliferation and apoptosis were reversed by transfection with the miR-638 inhibitor compared with the inhibitor control group. These results suggest an important role of miR-638 in cell proliferation and apoptosis.

Furthermore, we evaluated different drugs to identify the drug towards which there was highest sensitivity in cases with downregulated miR-638 expression. Epi, Doc, Cis, and 5-FU are commonly used as NAC in patients with BC. Ola, a new drug for BC with *BRCA* mutations, was also evaluated. Drug sensitivity was evaluated using the MiniPDX™ model *in vivo*. MDA-MB-231 cells transfected with LV-miR-638 were subcutaneously injected into Nu/Nu-nude mice. The viability of MDA-MB-231 cells was measured after administration of the five drugs. The results showed the lowest T/C% value in the 5-FU administration group, suggesting that miR-638 overexpression enhanced sensitivity to 5-FU. Additionally, among the patients who received 5-FU, miR-638 expression levels were lower in the chemoresistant group than in the chemosensitive group. These results indicated that miR-638 may have a regulatory effect on 5-FU chemosensitivity. Furthermore, with 5-FU at a concentration of 10 μ M, MTS assay results showed that the proliferation of both MDA-MB-231 and MCF-7 cells

significantly decreased in the miR-638 transfection group and increased in the miR-638 inhibitor transfection group compared with the negative control group. These conclusions proved that miR-638 overexpression was associated with enhanced chemotherapy sensitivity to 5-FU *in vitro* and *in vivo*.

Tan et al. found that miR-638 overexpression increased the sensitivity to ultraviolet light and cisplatin, and miR-638 affected DNA repair by downregulating BRCA1 expression in TNBC [22]. Zhao et al. [23] reported that miR-638 overexpression is associated with increased sensitivity to Doc in MCF-7 cells; however, our preliminary study showed conflicting results. Our study indicates the feasibility of using miR-638 as a predictive biomarker of 5-FU sensitivity in BC. Olaparib may not show anti-cancer effects *in vivo*. Olaparib may be effective for BRCA1 mutation, but not for expression downregulation in TNBC. miR-638 overexpression also showed association with resistance to Doc. This discrepancy may be due to differences in the function of miR-638 in different cancer cells, leading to differences in drug response. Additionally, *in vitro* cell experiments for drug sensitivity produced results that were different from the results of *in vivo* experiments. The results may also be affected by other factors, such as metabolism and the tumor microenvironment. However, in the cases without 5-FU, the expression of miR-638 was also downregulated in the chemoresistant group compared with the chemosensitive group, partly suggesting that miR-638 might be related to other drug sensitivities. Other chemotherapy drugs or combined drug regimens were not verified in the MiniPDX™ experiment. Further research is needed on the association of miR-638 expression and drug sensitivity.

Potential target genes of miR-638, such as CACNA1A, VEGFA, CACNA1I, FGF23, CAMK2A, NTRK3, and P2RX5 are mainly involved in the calcium signaling pathway. Additionally, VEGFA, LAMA4, ANGPT4, FGF23, MAGI1, and ITGA3 are involved in the PI3K-Akt signaling pathway. The calcium signaling pathway regulates cancer cell sensitivity to chemotherapeutic drugs by affecting intracellular calcium influx [24]. The PI3K-Akt signaling pathway modulates chemoresistance in many human cancers, including ovarian [25], small-cell lung [26], and gastric cancers [27] and is a pivotal pathway that regulates multidrug resistance in cancers [28]. Of these genes, we detected five for further validation using qPCR. The results showed that the expression of VEGFA and CAMK2A in both MDA-MB-231 and MCF-7 cells was upregulated in the miR-638 transfection group and downregulated in the miR-638 inhibitor group, suggesting that VEGFA and CAMK2A may be potential targets of miR-638 in BC. However, the expression of CACNA1A, FGF23, and NTRK3 in both cell types was disordered and inconsistent with the results predicted by the software. The exact molecular mechanism of the targeted genes and signaling pathways regulated by miR-638 require further study.

Overall, our findings uncovered a novel role for miR-638 in the regulation of 5-FU chemosensitivity in BC patients. miR-638 may serve as a potential biomarker for NAC response in patients with BC.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

The regimes of patients in the study

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Supplementary Table 2

The dosage regimen of this study

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Supplementary Table 3

The cell viability in different groups

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Supplementary Table 4

Body weight changes in Nu/Nu mice treated with different drugs

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Supplementary Figure 1

qRT-PCR showed the expression of miR-638 in MDA-MB-231 cells is relatively lower than MCF-7 cells.

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Supplementary Figure 2

The flowchart of the miniPDX™ model.

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Supplementary Figure 3

The expression of CACNA1A, FGF23, and NTRK3. The expression of CACNA1A (A) was downregulated in both miR-638 and miR-638 inhibitor transfection group compared with negative control or inhibitor control group. The expression of FGF23 was high in miR-638 and miR-638 inhibitor transfection group in MDA-MB-231 cells but low in MCF-7 cells (B). The expression of NTRK3 (C) in both MCF-7 and MDA-MB-231 cells was upregulated in miR-638 transfection group and downregulated in miR-638 inhibitor group. The expression of these 3 genes were disordered and not consistent with the predicted result.

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