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ORIGINAL RESEARCH

RETRACTED ARTICLE: The Multifunction Of miR-218-5p-Cx43 Axis In Breast Cancer

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Chen Xia[1,*]
Hong Jiang[2,*]
Fugui Ye[3,*]
Zhigang Zhuang[4]

[1]Department of Medical Oncology, Hepatobiliary and Pancreatic Unit, Hunan Cancer Hospital, the Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, Changsha 410013, People's Republic of China; [2]Medical Department, Hunan Provincial Maternal and Child Health Care Hospital, Changsha 410008, People's Republic of China; [3]Key Laboratory of Breast Cancer in Shanghai, Department of Breast Surgery, Fudan University Shanghai Cancer Center, Shanghai 200032, People's Republic of China; [4]Department of Breast Surgery, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai 200040, People's Republic of China

*These authors contributed equally to this work

Background: Gemcitabine is proven to be the first-line standard treatment of breast cancers. Yet, little is known involving gemcitabine resistance and remains largely to be elucidated.

Materials and methods: We evaluated the expression of Cx43 in gemcitabine-resistant cells and parental cells by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot analyses. Dual-luciferase reporter assay was applied to examine the epigenetic regulator of Cx43. The role of miR-218-5p/Cx43 axis on cell cytotoxicity, cell proliferation, colony formation, chemoresistance and migration was detected via mammalian expression vector and small short RNA (shRNA) transfection in vitro.

Results: In this study, we found that Cx43 expression levels were significantly lower in gemcitabine-resistant cells than in the parental cells. On deep investigation of the epigenetic regulation of Cx43, a few siRNA candidates targeting Cx43 were derived. Through dual-luciferase reporter assay, Cx43 was proved to be a direct target of miR-218-5p. Besides, qPCR, Western blot demonstrated the inverse correlation between miR-218-5p and Cx43 expression in breast cancer cells, thus forming the miR-218-5p-Cx43 axis. Notably, miR-218-5p-Cx43 axis was found to be involved in the process of gemcitabine chemoresistance, cell proliferation and migration in breast cancer cells.

Conclusion: Our findings suggested that miR-218-5p-Cx43 axis was versatile and indicated significant potency in breast cancer cells. More importantly, miR-218-5p-Cx43 axis might be valuable in translational medicine, with therapeutic and prognostic information.

Keywords: Cx43, breast cancer, miR-218-5p, gemcitabine, multifunction

Introduction

Breast cancer is the most common cancer in women worldwide.[1] Currently, the treatment strategies for breast cancer include surgery, cytotoxic chemotherapy, hormonal therapy, targeted drugs, or a combination of these methods. However, drug resistance is becoming a major clinical obstacle in treating breast cancer.[2] In heavily pretreated or treatment-resistant cases of breast cancer, there are limited treatment options. Although a plenty of studies have been conducted to uncover the underlying mechanisms of chemoresistance and many predictive biomarkers have been identified so far, much remains to be elucidated.[3–5]

Recently, gemcitabine, also known as 2′,2′-difluorodeoxycytidine (dFdC), is an analogue of deoxycytidine. It has been widely used in the treatment of several types of cancer, including pancreatic cancer, non-small cell lung cancer, bladder and metastatic breast cancer.[6,7] After entering the cell, dFdCTP, gemcitabine triphosphate metabolite, may be incorporated into DNA, leading to strand termination and cellular apoptosis. Other anticancer mechanisms of gemcitabine include

Correspondence: Chen Xia
Department of Medical Oncology, Hepatobiliary and Pancreatic Unit, Hunan Cancer Hospital, The Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, No.283 Tongzipo Road, Changsha 410013, People's Republic of China
Tel +86 731 8976 2051
Email summering53@126.com

Zhigang Zhuang
Department of Breast Surgery, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, 536 Chang-Le Road, Shanghai 200040, People's Republic of China
Tel +86 133 7198 5057
Email zhigangzhuangfmih@gmail.com

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ribonucleotide reductase (RNR) inhibition, RNA incorporation and thymidylate synthase inhibition.[8,9] Emerging evidence demonstrate that gemcitabine-based regimens have confirmed effect in the first-line treatment of breast cancer.[10] However, one of the main factors hindering gemcitabine application is chemoresistance.[11] Previous investigations indicate that connexins (Cxs) and gap junctional intercellular communications (GJICs) have a broad physiological and pathological function in cancer cell development, growth, differentiation and homeostasis.[12] Experimental studies suggest that targeting Cxs may be a novel technique, either to inhibit tumor cell growth directly or to sensitize to various therapeutics.[13] Cx43 is the best known Cxs, while little is known about its relationship with gemcitabine sensitivity in breast cancer.

miRNAs are noncoding RNA molecules of approximately 21 to 25 nucleotides that modulate gene expression by directly interacting with an mRNA target, thus leading to either degradation of the mRNA transcription or inhibition of the translation process. miRNAs play pivotal roles in tumor cell proliferation, migration, invasiveness and metastasis, involved in tumor progression, patient prognosis.[14–17] MiRNAs also regulate chemosensitivity of different drugs in multiple cancers. For an example, it is reported that microRNA-181b regulated gemcitabine resistance in pancreatic cancer.[18,19] The past decades' diligence witnesses the importance and utility of miRNAs in the field of cancer. However, the epigenetic regulation of miRNAs to chemosensitivity is complex and needs further investigation.

In this study, based on our previously published mRNA and miRNA microarray data, we aimed to investigate the roles of Cx43 and miRNAs in gemcitabine resistance in breast cancer cells. Meanwhile, the broad function of Cx43 and miRNAs will be explored. Here, we found that miR-218-5p-Cx43 axis not only acted as a regulator of gemcitabine resistance, but also functioned as a modulator of cell proliferation and migration in breast cancer cells, with tremendous potency to further research.

Materials And Methods

Cell Lines And Cell Culture

The MDA-MB-231 (MDA-231) and the HEK293T cell lines were obtained from the Shanghai Cell Bank Type Culture Collection Committee (CBTCCC, Shanghai, China). All cells used were passaged for less than 6 months. The MDA-231 gemcitabine-resistant subline (MDA-231-Gem) was gifted from the Key Laboratory of Breast Cancer in Shanghai, Fudan University Shanghai Cancer Center. All cells were cultured according to the manufactures' instruction, supplemented with 10% fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA), 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco-BRL, Carlsbad, CA, USA) at 37°C in a humidified atmosphere with 5% CO2.

Plasmids Construction And Stable Cells Establishment

The coding sequence of Cx43 was amplified by polymerase chain reaction (PCR) and then cloned into the adenoviral vector plasmid with the HA-Flag tag, using the Gateway Cloning system (Invitrogen Life Technologies, Carlsbad, CA, USA). The pre-miR-218 bp precursor sequences were constructed in lentiviral based pEZX constructs by GeneCopoeia, Inc. The plasmids containing target sequences and corresponding vector plasmids were transfected into HEK293T cells with packaging and envelope plasmids using polyethylenimine reagent. The medium containing virus particles was harvested and used to transfect the indicated cells with polybrene. Medium containing 5 μg/mL puromycin was used to select the stable cells.

Transient-Transfected Cell Construction

The miR-135b, miR-186-5p and miR-218-5p mimics duplexes were synthesized by Genepharma (Shanghai, China). HEK293T cells were transfected with miRNAs mimics using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the instruction. Transient-transfected cells were subjected to qPCR and Western blot to prove the function of miRNAs.

RNA Isolation, qRT-PCR

Total RNA was extracted from cells with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and the reverse transcription reaction was performed using the reverse transcription system (Promega Corporation, Shanghai, China). qPCR was performed using SYBR Premix Ex Taq (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China), and GAPDH was used as an internal control. The primer sequences used were as follows: Forward, 5′- GGTGGACTGTTTCCTCTCTCG-3′ and Reverse, 5′-GGAGCAGCCATTGAAATAAGC-3′ for Cx43; forward, 5′-GGATTTGGTCGTATTGGG-3′ and reverse, 5′-GGATTTGGTCGTATTGGG-3′ for GAPDH.

Western Blot

T-PER tissue extraction buffer (Thermo Fisher Scientific, Rockford, IL, USA) supplemented with protease and phosphatase inhibitor tablets (Roche Diagnostics, Indianapolis, IN, USA) was applied for protein extraction. A total of 20 μg protein was resolved by SDS-PAGE and transferred to polyvinylidene fluoride film. Antibodies used included anti-Cx43 antibody (1:1000, Abcan, America) and anti-GAPDH antibody (1:1000, Sigma-Aldrich). Goat anti-rabbit and goat anti-mouse (1:5000, Jackson ImmunoResearch Laboratories, Inc., West Grove, MA, USA) secondary antibodies were used. The signals were detected using chemiluminescent horseradish peroxidase substrate (Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

Dual-Luciferase Reporter Assay Targeting Cx43-3′UTR

The 3′-UTR of Cx43 was amplified from human genomic DNA and cloned into the region directly downstream of the Renilla gene stop codon in the psiCHECK2/Luciferase vector (Promega, Madison, WI) to generate psiCHECK2-CDA-3′-UTR constructs. The mutant 3′-UTR of Cx43 was amplified using psiCHECK2-CDA-3′-UTR as the template and cloned into the downstream of psiCHECK2/Luciferase vector. The miR-135b, miR-186-5p and miR-218-5p mimics duplexes were synthesized by Genepharm (Shanghai, China). HEK293T cells were co-transfected with a mixture of reporter constructs and miRNA duplexes using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After incubation for 48 hrs, firefly and renilla luciferase intensity were measured using the dual-luciferase reporter assay system (Promega, Madison, WI) from the cell lysates.

Cytotoxicity And Cell Proliferation Assays

Cells at the logarithmic growth phase were plated in 96-well plates. Following overnight adherence, complete medium was replaced with medium containing 16 different concentrations of gemcitabine ranging between 0.00001 and 900 μmol/L. Cell cytotoxicity was measured by the Cell Counting Kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) 6 days later. The IC50 value of gemcitabine was estimated from semilogarithmic dose–response curves generated using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Each experiment was performed in triplicate. Cell proliferation rate was also measured using the CCK-8 kit every 24 hrs for seven days and a proliferation curve was generated using GraphPad Prism.

Colony Formation Assay

The cells were seeded in 60-mm dish. Following 24 hrs, complete medium was replaced with medium containing different concentrations of gemcitabine (6, 9 and 12 nmol/L). In addition, cells were seeded without gemcitabine as standard controls. After 14 days, clones were fixed and stained with crystal violet. Stained clones with a diameter of >1 mm were counted and standardized. The cloning efficiency was calculated using the following formula: Cloning efficiency (%) = (clone number/total cell number)/(control clone number/control total cell number) × 100. Each independent experiment was performed in triplicate.

Immunofluorescence Assay

Briefly, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 15mins. The cells were washed with PBS, blocked with 5% BSA in PBS for 1hr and incubated with primary antibody overnight at 4°C. Secondary antibody was conjugated with Alexa fluorescence 568 (1:1000, Ivigrogen) in 37°C. DAPI was used to stain nuclei (1:5000, Sigma). Cells were visualized by confocal fluorescence microscopy.

Migration Assay

For transwell migration assay, cells were resuspended in serum-free media and added to the upper compartment of the chamber, and 600mL of complete medium was added to the lower chamber. Cells were incubated in a humidified environment with 95% air and 5% CO_2 at 37°C and allowed to migrate for 12hrs. After removal of the non-migrated cells, cells that had migrated through the filter were stained with crystal violet, photographed, and counted using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc.) and SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). ANOVA and the Student *t*-test were used to determine the statistical significance between experimental groups. All *p* values were two-sided, and $p<0.05$ was considered to indicate a statistically significant difference.

Results

Cx43 Sensitized Breast Cancer Cell To Gemcitabine

Given the multiple facets of Cxs in cancer biology and tight correlation with treatment sensitivity. Previously, we conducted the mRNA microarray (the accession number: GSE63140) between MDA-231 and MDA-231-Gem cells. By intersecting the Cxs with the mRNA microarray data, Cx43 was the only one with remarkably differential expression (Figure 1A). Therefore, we focused on Cx43 in our further investigation. We determined the expression of Cx43 in the indicated cells by both qPCR and Western blot assays, and the result showed that Cx43 expression level was significantly lower in gemcitabine-resistant MDA-231-Gem cells than in the parental cells (Figure 1B and C). Vice versa, reintroduction of Cx43 in MDA-231-Gem (MDA-231-Gem/Cx43) carried out by lentivirus transduction and confirmed by Western blot assay (Figure 1D) demonstrated that the IC50 value of MDA-231-Gem/Cx43 was 33.90 nM significantly lower than that of MDA-231-Gem/HF cells, which was 63.1 nM ($p<0.001$), whereas tremendously higher than that of MDA-231 cells (Figure 1E–G). Collectively, overexpression of Cx43 re-sensitized gemcitabine-resistant cells to gemcitabine in breast cancer cell model.

miR-218-5p Was A Post-Transcriptional Regulator Of Cx43 Expression By Directly Targeting Its 3′-UTR

Previous literature indicated that Cx43 was a novel therapeutic target; therefore, deep understanding of its regulation was urgent. Hence, in order to explore the epigenetic regulation of Cx43, four prediction algorithms were used to predict potential miRNAs that target the 3′-UTR sequence of Cx43: PITA, TargetScan, miRanda and miRWalk. Consequently, 8 candidate miRNAs were extracted by four algorithms (Figure 2A). Besides, our previously published miRNA microarray data (the accession number: GSE63140) were adopted to further inspect the potential miRNAs. By intersecting the 118

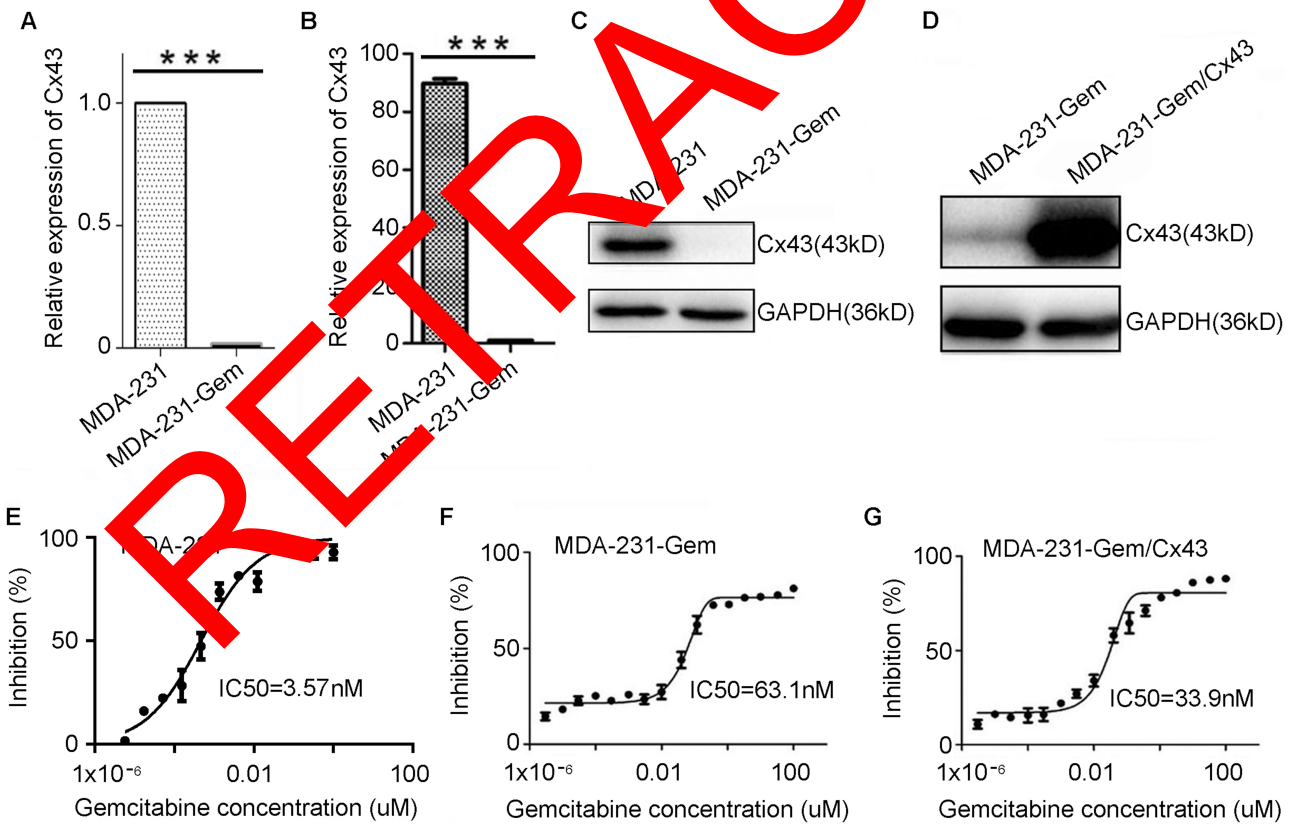

Figure 1 Cx43 up-regulation was associated with gemcitabine sensitivity in breast cancer cells. (**A**) Relative transcriptional expression level of Cx43 in mRNA microarray. (**B**) Relative transcriptional expression level of Cx43 in cell model. (**C** and **D**) The protein expression level of Cx43 in gemcitabine resistance cells and established stable cells, respectively. (**E-G**) Response of parental cells, gemcitabine resistance cells and established stable cells to different doses of gemcitabine. The IC50 for each cell line was presented. The assays were performed in triplicate. ***$p<0.001$.

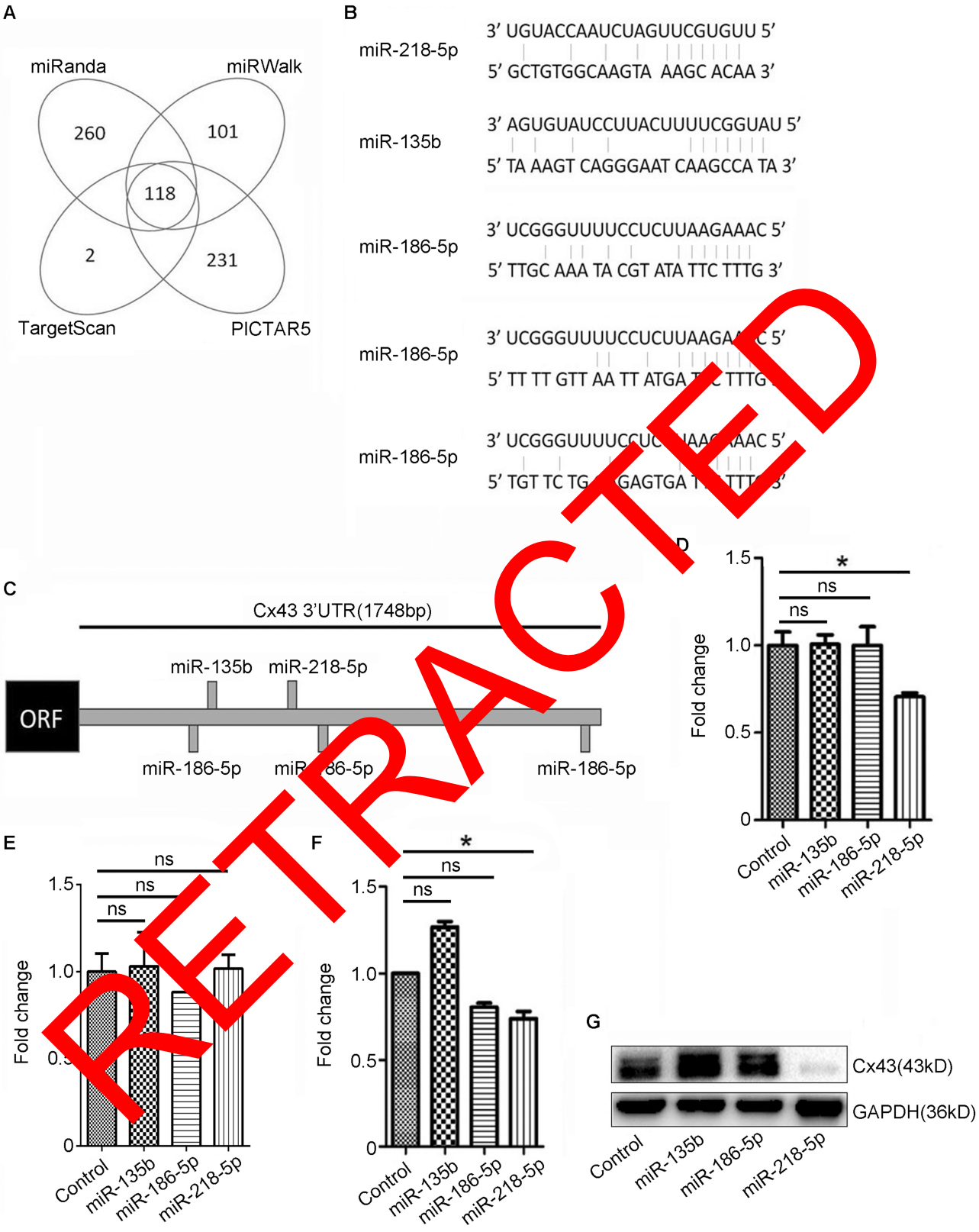

Figure 2 Cx43 was a direct target of miR-218-5p. (**A**) Candidates miRNAs targeting Cx43 were predicted by using miRanda, miRWalk, TargetScan and PICTAR5. The numbers in overlapping circles were simultaneously predicted by different algorithms. (**B**) Cx43 3′UTR and corresponding binding site fragments were introduced into the luciferase 3′UTR reporter constructs. (**C**) Schematic model of the miR-135b, miR-186-5p and miR-218-5p seed matches in Cx43 3′UTR. (**D** and **E**) Relative luciferase activities of luciferase reporters with wild-type and mutant Cx43 3′UTR were assessed in HEK293T cells. (**F**) Cx43 mRNA levels were determined by qPCR in miRNAs stable cell lines. (**G**) Cx43 protein levels were determined by Western blot in miRNAs stable cell lines. *p<0.05.

candidate miRNAs with more than threefold elevated miRNAs of the microarray, miR-135b, miR-186-5p and miR-218-5p were shown to be the most potential Cx43-associated miRNAs. The possible binding sites of the Cx43 3′-UTR of these three miRNAs were presented by using TargetScan (Figure 2B and C). Based on the above analyses, dual-luciferase reporter assay was conducted to examine the epigenetic regulators of Cx43, and the results confirmed that the relative luciferase activity of miR-218-5p, other than miR-135b and miR-186-5p, was remarkably reduced in full-length wild-type 3′TUR of Cx43 (Figure 2D). However, the reduction disappeared as shown in mutant 3′TUR of Cx43 (Figure 2E). That is, miR-218-5p was the directly epigenetic regulator of Cx43.

In addition, qPCR assay and Western blot analyses of miRNA-transiently transfected cells were used to confirm the effect of miRNAs on Cx43 expression. The qPCR result indicated that mRNA level of Cx43 was reduced significantly in miR-218-5p group ($p<0.05$) and marginal significance in miR-186-5p group ($p=0.053$), but increased in miR-135b group (Figure 2F). Western blot assay showed a consistent trend with the qPCR result (Figure 2G).

Taken together, we could draw the conclusion that miR-218-5p was the most efficient miRNA that could down-regulate Cx43 expression by directly targeting its 3′-UTR in breast cancer cells.

miR-218-5p-Cx43 Axis Modulated Chemosensitivity Of Gemcitabine In Breast Cancer Cells

Since Cx43 expressing level was lower in MDA-231-Gem cells and we showed that miR-218-5p could post-transcriptionally reduce Cx43 expression by directly targeting its 3′-UTR, we established a series of stable cells to investigate the function of miR-218-5p-Cx43 axis in gemcitabine sensitivity and other biological processes of breast cancer cell.

Stable HA-Flag–tagged Cx43-overexpressing cells MDA-231-Cx43 and its mock control MDA-231-HF were established through lentivirus transduction. The same approach was applied to construct stable cell lines, denoted as MDA-231-miR-218-5p and MDA-231-Control. Western blot was adopted to verify the successful construction of the above stable cell lines (Figure 3A and B). Immunofluorescence assay was carried out to further identify sub-cellular localization and expression of Cx43 in indicated cells (Figure 3C and D). Not only higher expression level of Cx43 in MDA-231-Cx43 and MDA-231-Control, presented by Western blot, but also more intensively red fluorescence appeared at the cell–cell border line, representing significant expression at the cell membrane.

Although Cx43 was associated with gemcitabine sensitivity, whether miR-218-5p-Cx43 axis also involved or not? The dose–response curves originated from the cytotoxicity assay of MDA-231-HF, MDA-231-Cx43, MDA-231-Control and MDA-231-miR218-5p was generated. And IC50 value of MDA-231-Cx43 (2.29nM) (Figure 3E) was less than that of MDA-231-HF cell (6.03nM) (Figure 3F), while IC50 value of MDA-231-miR-218-5p (9.12nM) (Figure 3G) was much higher than MDA-231-Control cells (4.27nM) (Figure 3H). In brief, the cytotoxicity assays indicated that miR-218-5p-Cx43 axis may take part in regulating gemcitabine chemosensitivity in breast cancer cells, because Cx43 increased the susceptibility to Gemcitabine and miR-218-5p may attenuate that effect.

miR-218-5p-Cx43 Axis Modulated Cell Proliferation And Migration In Breast Cancer Cells

In view of the multiple facets of Cx43, we further explored the other aspects of Cx43. We further explored its impact on cancer proliferation and migration. The colony number decreased with increasing gemcitabine concentration in each cell lines. As expected, MDA-231-Cx43 cells exhibited less efficiency in colony formation than MDA-231-HF cells ($p<0.01$) (Figure 4A and B), while MDA-231-miR-218-5p increased colony numbers at each gemcitabine concentration group ($p<0.01$) (Figure 4C and D). Otherwise, cell proliferation assay and transwell migration experiments were adopted to investigate the function of miR-218-5p-Cx43 axis in cell growth and migration. As shown in Figure 4E and F, we can see both Cx43 and miR-218-5p decreased cell proliferation rate. Figure 4G and H showed that Cx43 suppressed breast cancer cells migration while miR-218-5p promoted the migration. All in all, miR-218-5p-Cx43 had a profound effect on breast cancer proliferation and migration.

Discussion

In this study, we found significantly lower expression of Cx43, either of mRNA or protein level, in MDA-231-Gem than MDA-231 cells. Our results demonstrated that Cx43 enhanced chemosensitivity of breast cancer cell to gemcitabine. Furthermore, some other experiments were carried

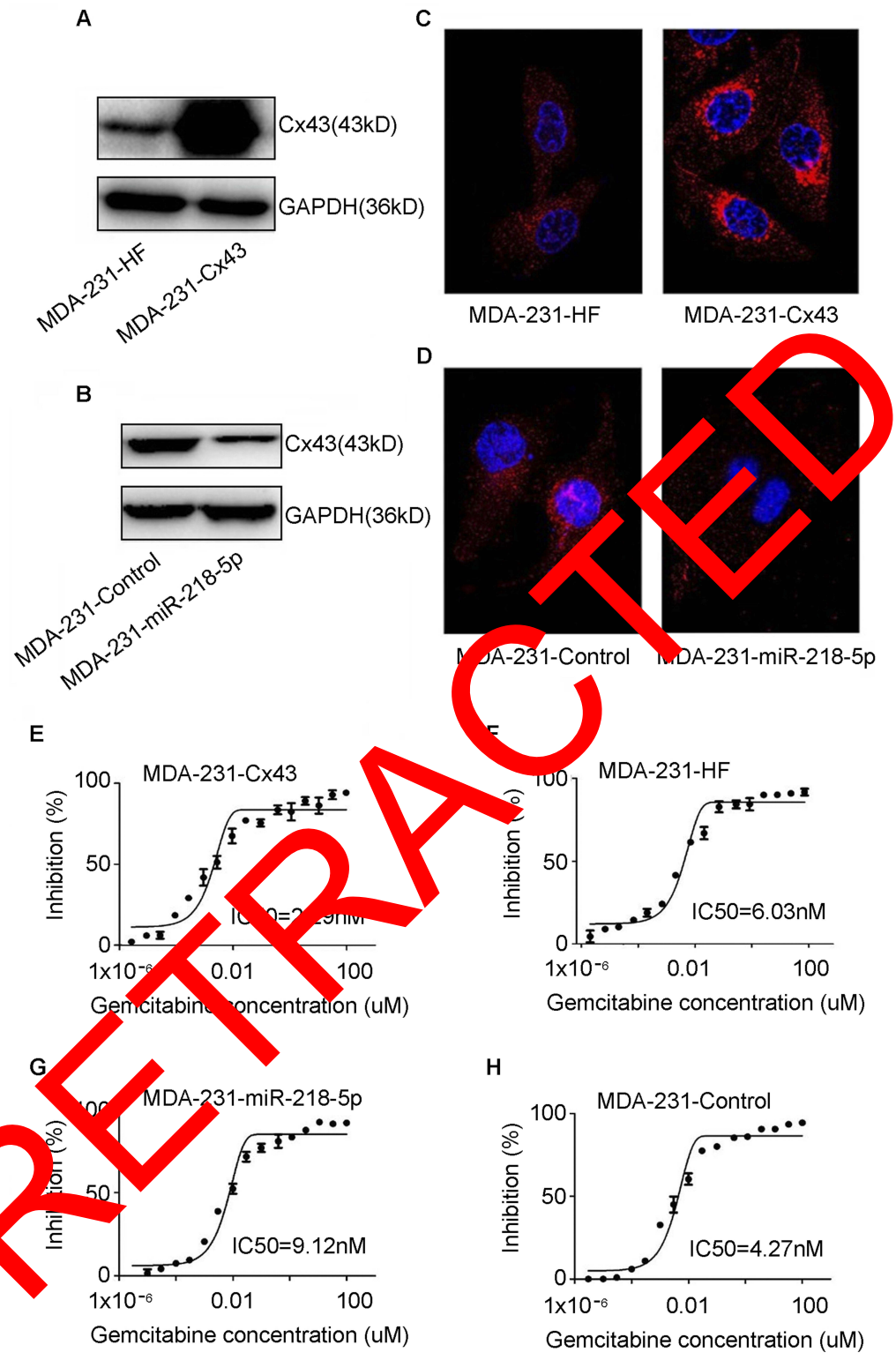

Figure 3 MiR-218-5p-Cx43 axis was involved with gemcitabine resistance. (**A**) Cx43 protein levels were determined by Western blot in Cx43 stable cell and mock control. (**B**) Cx43 protein levels were determined by immunofluorescence assay in Cx43 stable cell and mock control. The red color indicated Cx43. (**C**) Cx43 protein levels were determined by Western blot in miR-218-5p stable cell and control. (**D**) Cx43 protein levels were determined by immunofluorescence assay in Cx43 stable cell and mock control. The red color indicated Cx43. (**E**–**H**) Response of established stable cells to different doses of gemcitabine. The IC50 for each cell line was presented. The assays were performed in triplicate.

out to investigate the role of Cx43 in cell growth and migration. Our experiments proved that Cx43 attenuated the cell proliferation and migration. Interestingly, miR-218-5p was proved to be a post-transcriptional regulator

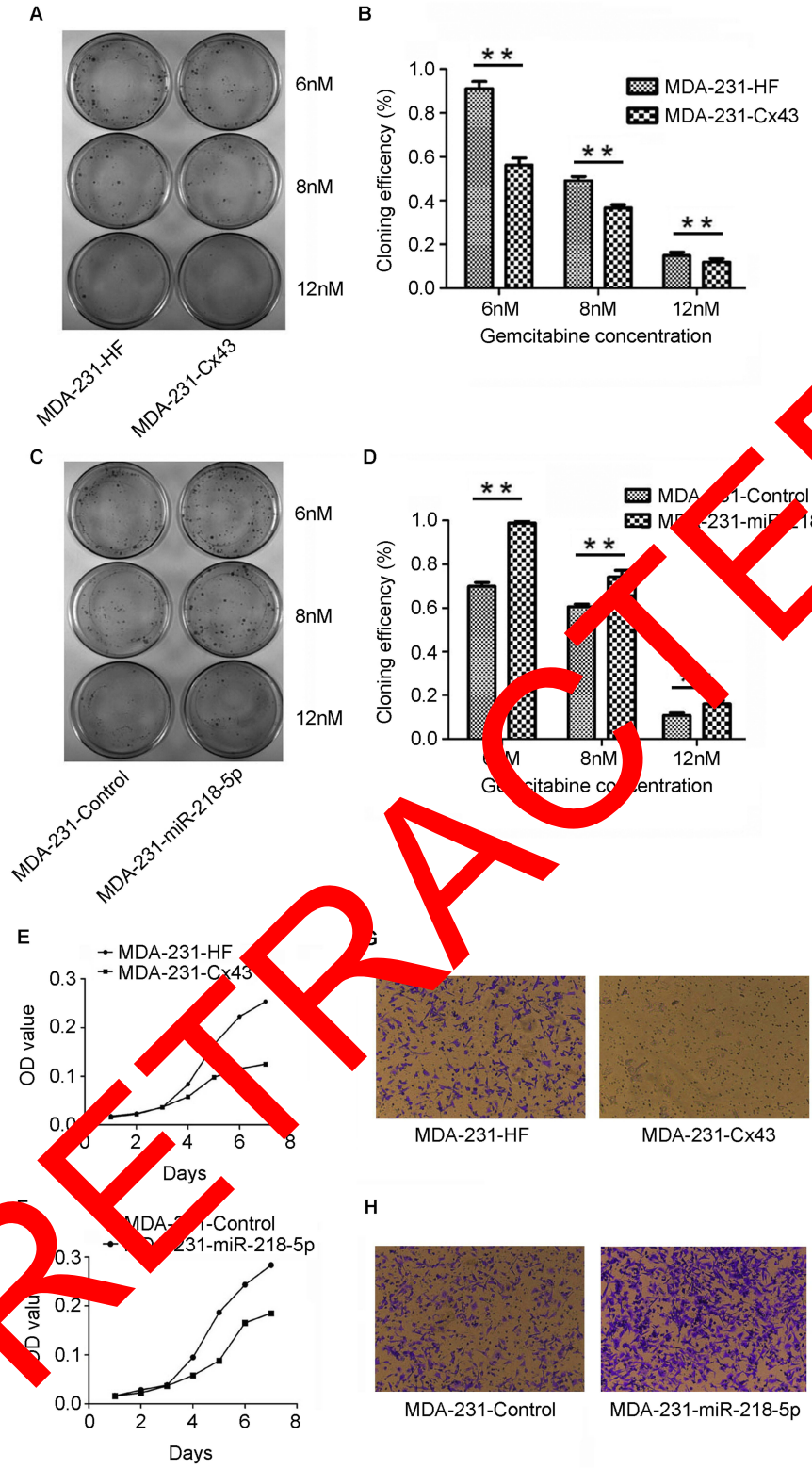

Figure 4 miR-218-5p-Cx43 axis was associated with proliferation and migration. (**A**–**D**) Colony formation assays were conducted to test cell proliferation. (**E** and **G**) CCK-8. (**F** and **H**) Cell migration assay was performed to determine the cell mobility. ***p*<0.01.

to downregulate Cx43 expression by directly targeting its 3′-UTR. Additionally, miR-218-5p counteracted with Cx43 in regulating gemcitabine chemosensitivity, cell growth and migration.

It is well recognized that the role of miRNAs in chemosensitivity of different drugs in various types of cancers has been intensively investigated in the past decades. However, few studies have specifically investigated the association of miRNAs with gemcitabine resistance in breast cancer cells.

Literature indicates that miR-218-5p is a vertebrate-specific intronic miRNA co-expressed with its host genes, tumor suppressor gene SLIT2/3. The mature form of miR-218-5p is generated from two separate loci, miR-218-1 and miR-218-2, which are located on chromosomes 4p15.31 and 5q35.1 within the introns of SLIT2 and SLIT3, respectively.[20] miR-218-5p aberration is associated with multiple drug resistance such as etoposide in breast cancer[21] and gemcitabine in pancreatic cancer.[22] However, we did not find any literature about miR-218-5p affecting the gemcitabine chemoresistance in breast cancer cells.

In our research, on the contrary of most former researches, we found that miR-218-5p enhanced chemoresistance of gemcitabine in a breast cancer cell. miR-218-5p is down-regulated and acts as a tumor suppressor in various cancers such as nasopharyngeal cancer,[23] cervical cancer,[24] breast cancer[25] and so on. Lower miR-218-5p expression level is not only associated with oncogenesis and tumor progression but also with tumor metastasis, such as in prostate cancer.[26] However, the role of miR-218-5p in breast cancer metastasis still remains controversial. Some studies revealed that miRNA-218-5p suppressed breast cancer cell invasion and migration,[27,28] while few studies drew opposite conclusion.[29,30] One study revealed that miR-218-5p expression was much higher in highly metastatic breast cancer cell line MDA-231 than in normal MCF-10A cell and noninvasive MCF-7, and ectopic expression of miR-218-5p promoted metastasis-related molecular properties in MDA-231 breast cancer cells.[30] In our study, we found that ectopic expression of miR-218-5p promoted breast cancer cell migration. This discrepancy might be attributed to the varying downstream genes of miR-218-5p.

Previously, Cx43 is defined as a tumor suppressor gene in breast cancer, which inhibits cell growth, invasion and migration. What is more, Cx43 is proved to be a chemotherapy and radiotherapy sensitizer in multiple cancers through GJIC (Gap Junction Intercellular Communication) dependent or independent manner.[31,32] These findings are greatly in accordance with the increasing evidence.[33–35] Since Cx43 suppresses breast cancer cell growth and miR-218-5p suppresses the expression of Cx43, we assumed that miR-218-5p promoted breast cancer cell growth. In fact, our proliferation assay revealed that Cx43 suppressed breast cancer cell growth; nevertheless, miR-218-5p promoted breast cancer cell growth. Moreover, miR-218-5p counteracted with Cx43 in the aspect of migration.

There are some limitations in current study. The deep underlying mechanisms still need to be further investigated. In addition, the results should be validated both in vitro by using more cell types and in vivo, even in transgenic mouse models.

To conclude, we proved Cx43 to be a direct target of miR-218-5p and inversely correlated with miR-218-5p expression in breast cancer cells. Furthermore, miR-218-5p-Cx43 axis enhanced gemcitabine chemoresistance, suppresses cell proliferation rate and promotes cell migration in breast cancer cells. In a word, the miR-218-5p-Cx43 axis had multiple effects in breast cancer cells, indicating its tremendous potency to treatment and prognosis.

Acknowledgments

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Author Contributions

Chen Xia and Zhigang Zhuang contributed to conception and designed the experiments. Chen Xia, Hong Jiang and Fugui Ye performed the experiments, analyzed the data and wrote the paper. Zhigang Zhuang supervised the entire experimental work. All authors contributed to data analysis, drafting and revising the manuscript, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin*. 2019;69(1):7–34. doi:10.3322/caac.21551
2. Tang Y, Wang Y, Kiani MF, Wang B. Classification, treatment strategy, and associated drug resistance in breast cancer. *Clin Breast Cancer*. 2016;16(5):335–343. doi:10.1016/j.clbc.2016.05.012
3. Si X, Liu Y, Lv J, et al. *ERα Propelled Aberrant Global DNA Hypermethylation by Activating the DNMT1 Gene to Enhance Anticancer Drug Resistance in Human Breast Cancer Cells*. *Oncotarget*. 2016;7(15):20966-80.doi:10.18632/oncotarget.8038
4. Zhang ZM, Wu JF, Luo QC, et al. Pygo2 activates MDR1 expression and mediates chemoresistance in breast cancer via the Wnt/β-catenin pathway. *Oncogene*. 2016. doi:10.1038/onc.2016.10

5. Tang X, Jin L, Cao P, et al. *Microrna-16 Sensitizes Breast Cancer Cells to Paclitaxel through Suppression of IKBKB Expression*. *Oncotarget*.2016;7(17)23668-83.doi:10.18632/oncotarget.8056
6. Cavalcante LDS, Monteiro G. Gemcitabine: metabolism and molecular mechanisms of action, sensitivity and chemoresistance in pancreatic cancer. *Eur J Pharmacol*. 2014;741:8–16. doi:10.1016/j.ejphar.2014.07.041
7. Stephen C, Gilles R, Jens H, et al. Phase III study of gemcitabine plus docetaxel compared with capecitabine plus docetaxel for anthracycline-pretreated patients with metastatic breast cancer. *J Clin Oncol*. 2009;27(11):1753–1760. doi:10.1200/JCO.2007.15.8485
8. Sugarman R, Patel R, Sharma S, Plenker D, Tuveson D, Saif MW. Pharmacokinetics and pharmacodynamics of new drugs for pancreatic cancer. *Expert Opin Drug Metab Toxicol*. 2019;15(7):541–552. doi:10.1080/17425255.2019.1637417
9. Mini E, Nobili S, Caciagli B, Landini I, Mazzei T. Cellular pharmacology of gemcitabine. *Ann Oncol*. 2006;null(undefined):v7–v12.
10. Hu X-C, Zhang J, Xu B-H, et al. Cisplatin plus gemcitabine versus paclitaxel plus gemcitabine as first-line therapy for metastatic triple-negative breast cancer (CBCSG006): a randomised, open-label, multicentre, phase 3 trial. *Lancet Oncol*. 2015;16(4):436–446. doi:10.1016/S1470-2045(15)70064-1
11. Wong A, Soo RA, Yong WP, Innocenti F. Clinical pharmacology and pharmacogenetics of gemcitabine. *Drug Metab Rev*. 2009;41(2):77–88(12). doi:10.1080/03602530902741828
12. Sinyuk M, Mulkearns-Hubert EE, Reizes O, Lathia J. Cancer connectors: connexins, gap junctions, and communication. *Front Oncol*. 2018;8(undefined):646. doi:10.3389/fonc.2018.00646
13. Wu JI, Wang LH. Emerging roles of gap junction proteins connexins in cancer metastasis, chemoresistance and clinical application. *J Biomed Sci*. 2019;26(1):8. doi:10.1186/s12929-019-0555-4
14. Hanna J, Hossain GS, Kocerha J. The potential for microRNA therapeutics and clinical research. *Front Genet*. 2019;10(undefined):478. doi:10.3389/fgene.2019.00478
15. Nakano M, Nakajima M. Most recent findings on microRNA-dependent regulation of drug metabolism. *Nihon Yakurigaku Zasshi Folia Pharmacologica Japonica*. 2019;154(1):28–3 doi: 1254/fpj.154.28
16. Khan S, Ansarullah KA, Jaggi M, Chau SC. microRNAs in pancreatic cancer: micropl ers i b big game. *Cancer Res*. 2013;73(22):6541–6547. d 0.1158/00 5472.CAN-13-1288
17. Liu Y, Cai Q, Bao -P-P, et al. Tum tissue roRNA expression in association with triple-negati breast canc outcomes. *Breast Cancer Res Treat*. 2015;15):183–191. doi:10 07/s10549-015-3460-x
18. Xia L, Zhang D, Du R, al. miR 5b and miR-16 modulate multidrug resistance by targeting in human stric cancer cells. *Int J Cancer*. 2008;12 72–379 i:10.10 ijc.23501
19. Takiuchi D, uchi H, agano Involvement of microRNA-181b in gemcit ne resistan e of pancreatic cancer cells. *Pancreatol*. 201 23. doi:10.1016/j.pan.2013.06.007
20. Shuichi T, Ta C, Kazumori K, et al. miR-218 on the genomic loss region of ch osome 4p15.31 functions as a tumor suppressor in bladder cancer. *I Oncol*. 2011;185(1):13–21.
21. Moitra K, Im K, Limpert K, et al. Differential gene and MicroRNA expression between etoposide resistant and etoposide sensitive MCF7 breast cancer cell lines. *PLoS One*. 2012;7(9):e45268. doi:10.1371/journal.pone.0045268
22. Ruixia L, Kejian C, Shulong Y. microRNA-218 promotes gemcitabine sensitivity in human pancreatic cancer cells by regulating HMGB1 expression. *Chin J Cancer Res*. 2015;27(3):267–278. doi:10.3978/j.issn.1000-9604.2015.04.06
23. Alajez NM, Michelle L, Emma I, et al. MiR-218 suppresses nasopharyngeal cancer progression through downregulation of survivin and the SLIT2-ROBO1 pathway. *Cancer Res*. 2011;71(6):2381–2391. doi:10.1158/0008-5472.CAN-10-2754
24. Litière S, Werutsky G, Fentiman IS, et al. Breast conserving therapy versus mastectomy for stage I–II breast cancer: 20 year follow-up of the EORTC 10801 phase 3 randomised t et Oncol. 2012. doi:10.1016/S1470-2045(12)70042-6
25. Yunhui H, Kun X, Ernesto Y. miR-2 targets survivin nd regulates resistance to chemotherapeutics in brea cancer. *Breas Cancer Res Tr*. 2015;151(2):269–280. doi 1007/s10 -015-337 9
26. Leite KRM, Alberto T, Rei T, et al. Microl A e ression profiles in the progression of pr te cance rom high-g de prostate intraepithelial neoplasia t me asi Urol Onc 2013;31(6):796–801. doi:10.1016/j.uro c.2011.0 2
27. Yang L, Li O ng Q, Zhen Lei Silencing of miRNA-218 promotes m gratio nd invasion breast cancer via Slit2-Robo1 pathway. *Biomed Pha cother*. 2012;66(7):535–540. doi:10.1016/j.biop .04.006
28. L Q, Zhu F, Chen P. miR-7 and miR-218 epigenetically control tumor suppresse genes RASSF1A and Claudin-6 by targeting xB3 in breast ncer. *Biochem Biophys Res Commun*. 2012;424 (8–33. doi:10 016/j.bbrc.2012.06.028
29. Taipa mäki , Farina NH, van Wijnen AJ, et al. Antagonizing miR-218-5p attenuates Wnt signaling and reduces metastatic bone of triple negative breast cancer cells. *Oncotarget*. 2016;7(48):79032–79046. doi:10.18632/oncotarget.12593
30. Hassan MQ, Maeda Y, Taipaleenmaki H, et al. miR-218 directs a Wnt signaling circuit to promote differentiation of osteoblasts and osteomimicry of metastatic cancer cells. *J Biol Chem*. 2012;287(50):42084–42092.
31. Uzu M, Sato H, Yamada R, et al. Effect of enhanced expression of connexin 43 on sunitinib-induced cytotoxicity in mesothelioma cells. *J Pharmacol Sci*. 2015;128(1):17–26. doi:10.1016/j.jphs.2015.04.002
32. Mustapha K, Gerald B. Gap junctions and connexins as therapeutic targets in cancer. *Expert Opin Ther Targets*. 2010;14(7):681–692. doi:10.1517/14728222.2010.487866
33. Aasen T, Leithe E, Graham SV, et al. Connexins in cancer: bridging the gap to the clinic. *Oncogene*. 2019;38(23):4429–4451.
34. Aasen T. Connexins: junctional and non-junctional modulators of proliferation. *Cell Tissue Res*. 2015;360(3):685–699. doi:10.1007/s00441-014-2078-3
35. Sirnes S, Bruun J, Kolberg M, et al. Connexin43 acts as a colorectal cancer tumor suppressor and predicts disease outcome. *Int J Cancer*. 2012;131(3):570–581. doi:10.1002/ijc.26392