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MiR-218 targets survivin and regulates resistance to chemotherapeutics in breast cancer

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Abstract:	Purpose: Multidrug resistance (MDR) remains one of the most significant obstacles in breast cancer treatment, and this process often involves dysregulation of a great number of microRNAs (miRNAs). Some miRNAs are indicators of drug resistance and confer resistance to chemotherapeutic drugs, although our understanding of this complex process is still incomplete. Methods: We have used a combination of miRNA profiling and real-time PCR in two drug resistant derivatives from MCF-7 and Cal51 cells. Experimental modulation of miR expression has been obtained by retroviral transfection. Taxol and doxorubicin IC50 values were obtained by short-term drug sensitivity assays. Apoptosis was determined by flow cytometry after annexin V staining, by caspase 3/7 and caspase 9 activity assays and the levels of apoptosis related proteins bcl-2 and bax by real-time PCR and western blot. Mir target was studied using transient transfection of luciferase constructs with the 3'-untranslated regions (UTR) of target mRNAs. Small interfering RNA-mediated genetic knock-down was performed in multidrug resistance cells and its modulatory effect on apoptosis examined. The effect of miRNA on tumorigenicity and tumor drug response was studied in mouse xenografts. Results: MiRNA profiling of two drug resistant breast cancer cell models indicated that miR-218 was down-regulated in both MCF-7/A02 and CALDOX cells. Ectopic expression of miR-218 resensitized both drug resistant cell lines to doxorubicin and taxol due to an increase in apoptosis. MiR-218 binds survivin (BIRC5) mRNA 3'-UTR and down-regulated reporter luciferase activity. Experimental down-regulation of survivin by RNA interference in drug resistant cells did mimic the sensitization observed when miRNA-218 was up-regulated. In addition, resensitization to taxol was also observed in mouse tumor xenografts from cells over-expressing miR-218. Conclusions: MiR-218 is involved in the development of multidrug resistance in breast cancer cells via targeting survivin and leading to evasion of apoptosis. Targeting miR-218 and survivin may thus provide a potential strategy for reversing drug resistance in breast cancer.
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MiR-218 targets survivin and regulates resistance to chemotherapeutics in breast cancer

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Abstract

Purpose: Multidrug resistance (MDR) remains one of the most significant obstacles in breast cancer treatment, and this process often involves dysregulation of a great number of microRNAs (miRNAs). Some miRNAs are indicators of drug resistance and confer resistance to chemotherapeutic drugs, although our understanding of this complex process is still incomplete.

Methods: We have used a combination of miRNA profiling and real-time PCR in two drug resistant derivatives from MCF-7 and Cal51 cells. Experimental modulation of miR expression has been obtained by retroviral transfection. Taxol and doxorubicin IC50 values were obtained by short-term drug sensitivity assays. Apoptosis was determined by flow cytometry after annexin V staining, by caspase 3/7 and caspase 9 activity assays and the levels of apoptosis related proteins bcl-2 and bax by real-time PCR and western blot. Mir target was studied using transient transfection of luciferase constructs with the 3'-untranslated regions (UTR) of target mRNAs. Small interfering RNA-mediated genetic knock-down was performed in multidrug resistance cells and its modulatory effect on apoptosis examined. The effect of miRNA on tumorigenicity and tumor drug response was studied in mouse xenografts.

Results: MiRNA profiling of two drug resistant breast cancer cell models indicated that miR-218 was down-regulated in both MCF-7/A02 and CALDOX cells. Ectopic expression of miR-218 resensitized both drug resistant cell lines to doxorubicin and taxol due to an increase in apoptosis. MiR-218 binds survivin (BIRC5) mRNA 3'-UTR and down-regulated reporter luciferase activity. Experimental down-regulation of survivin by RNA interference in drug resistant cells did mimic the sensitization observed when miRNA-218 was up-regulated. In addition, resensitization to taxol was also observed in mouse tumor xenografts from cells over-expressing miR-218.

Conclusions: MiR-218 is involved in the development of multidrug resistance in breast cancer cells via targeting survivin and leading to evasion of apoptosis. Targeting miR-218 and survivin may thus provide a potential strategy for reversing drug resistance in breast cancer.

Key words

miR-218, survivin, apoptosis, drug resistance, doxorubicin, taxol

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide [1]. Current treatment strategies for breast cancer include surgery, radiotherapy and a variety of chemotherapeutic approaches using traditional non-targeted drugs, such as epirubicin, hormonal therapy, such as tamoxifen, and targeted drugs, such as herceptin. However, multidrug resistance (MDR) remains a major obstacle to the success of cancer chemotherapy, resulting in relapse and progression in a high proportion of breast cancer patients. MDR involves genetic and epigenetic dysregulation of a wide range of genes and may be caused by drug efflux transporters such as P-glycoprotein [2], breast cancer resistant protein (BCRP) or multiple resistance protein-1 (MRP1). In addition, inactivation by detoxification enzymes, the altered expression of pro-apoptosis and tumor suppressor genes, or the increased activity of DNA repair mechanisms are frequently involved [3,4]. Recent evidence indicates that microRNAs (miRNAs) regulate these processes [5,6].

MiRNAs are a class of highly conserved, short, non-protein-coding small RNAs, usually 18-25 nucleotides in length that negatively regulate gene expression by repressing translation or cleaving RNA transcripts in a sequence-specific manner. MiRNAs regulate the biology of cancer cells, including their chemosensitivity [7,8]. Some miRNAs are indicators of drug resistance and confer resistance to a variety of chemotherapeutic drugs [9,10,11]. MiR-218 is expressed in several carcinomas and plays critical roles in carcinogenesis, cancer proliferation and cancer metastasis. Its down-regulation has been reported in several human malignancies, including cervical, gastric and colon cancer [12-15]. In breast cancer, BRCA1 and HoxB3 mRNAs are direct targets of miR-218 [16,17]. However, the functional role of miR-218 in breast cancer chemosensitivity remains to be characterized.

In this study we present the miRNA signature of multidrug resistant MCF-7 cells and found that miRNA 218 is also down-regulated in drug resistant cells derived from another breast cancer cell line, Cal51. Ectopic expression of miRNA-218 resensitized both drug resistant cell lines to doxorubicin and taxol due to an increase in apoptosis. We show that miRNA-218 binds survivin mRNA 3'-UTR and that experimental down-regulation of survivin by RNA interference in drug resistant cells mimics the sensitization observed when miRNA-218 is up-regulated. Moreover, resensitization to taxol was also observed in mouse tumor xenografts from cells over-expressing miR-218. Targeting miR-218 and survivin may thus provide a potential

strategy for reversing drug resistance in breast cancer cells.

Materials and methods

Cell lines

The human breast cancer cell lines MCF-7 and Cal51 and their drug resistant derivatives, MCF-7/ADR and CALDOX, have been described previously [5, 18]. Cells were cultured in low glucose (1 g/L) Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich, St Louis, MO, USA) supplemented with 10% (v/v) fetal calf serum and 500 units/ml penicillin, 500 μg/ml streptomycin and 10 mM L- glutamine (Sigma Aldrich, St Louis, MO, USA). The drug resistant phenotype was maintained by the addition of 2 μM doxorubicin for MCF-7/A02 and 0.2 μM for CALDOX. Doxorubicin and taxol were purchased from Sigma-Aldrich (St Louis, MO, USA).

Plasmids, oligonucleotides and cell transfection

Lentivirus particles carrying miR-218 cloned into pGLV2-U6-Puro and packaging plasmid mix were purchased from GenePharma (Shanghai, China). Lentiviral particles were filtered through 0.45 μm cellulose acetate filters and supplemented with 8 μg/mL polybrene (Sigma-Aldrich, St Louis, MO, USA) prior to adding to recipient cells. Puromycin (1 μg/ml; Sigma-Aldrich) was added 96 hours after infection to recipient cells for selection of stable transgene expression [18]. A plasmid (pMir-Target) carrying the survivin 3'-UTR downstream from a luciferase gene was purchased from Life Technologies (Grand Island, NY, USA). Cells ($5x10^5$) were transiently transfected with 2 μg pMir-Target and 50 ng phRGTK (expressing *Renilla* luciferase to normalize for transfection efficiency; Promega, Madison, WI, USA) using lipofectamine 3000 (Life Technologies, Grand Island, NY, USA) following manufacturer's recommendations. Luciferase activity was measured 48 h after transfection, in a GloMax™ 20/20 Luminometer (Promega, Madison, WI, USA) essentially as described [19]. A proprietary survivin siRNA was purchased from GenePharma (Shanghai, China). A siRNA (scrambled), comprising the same nucleotides as in survivin siRNA but in randomized order, was used as negative control and was also designed and synthesized by GenePharma. The scrambled sequence have no significant homology to any known human gene sequences in the GenBank database. Cells ($3x10^5$) were transfected with 40 nM siRNA and

lipofectamine 3000 (Life Technologies, Grand Island, NY, USA) following manufacturer's recommendations. The effects of siRNA on survivin expression were examined 48 h after transfection at RNA level and 72 h after transfection at protein level.

Cell viability analysis

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were performed to evaluate the cell growth inhibitory effect in response to drug treatments and were used to determine the concentration of drug that inhibited cell growth by 50% (IC50) after 3 days of treatment [20].

Drug resistance clonogenic assay

Cells (2×10^5 per well of a 6-well plate) were treated with a single dose of doxorubicin (15 μM for MCF-7/A02 and 1 μM for CALDOX), or taxol (0.25 μM for MCF-7/A02 and 2.5 nM for CALDOX) for 1 week. Resistant clones were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet and counted. Crystal violet retained in the cells was quantified by solubilization with 0.5% acetic acid and measurement of optical density at 592 nm [19].

Gene expression analysis

For mRNA detection from cells, 2 μg total RNA (isolated using a miRCURY RNA isolation kit, Exiqon, Vedbaek, Denmark) was reverse transcribed with RNase H^+ MMLV reverse transcriptase (iScript cDNA synthesis kit, Bio-Rad, Hercules, CA, USA) and real-time quantitative PCR was performed on an ABI Prism 7900 detection system (PerkinElmer Life Sciences, Waltham, MA, USA) using SYBR-Green (Promega, Madison, WI, USA) and gene specific primers (RPS14: 5'-TCACCGCCCTACACATCAAACT-3' and 5'-CTGCGAGTGCTGTCAGAGG-3'; survivin: 5'-GGACCACCGCATCTCTACAT-3' and 5'-GACAGAAAGGAAAGCGCAAC-3'; bcl-2: 5'-CAGTTGGGCAACAGAGAACCAT-3' and 5'-AGCCCTTGTCCCCAATTTGGAA-3'; bax: 5'-

CGAGTGGCAGCTGACATGTTTT-3' and 5'-TGAGGCAGGTGAATCGCTTGAA-3'). PCR amplifications included a standard curve for each amplified gene in order to extrapolate transcript amounts from the Ct values. Relative transcript expression was normalized to that of *RPS14* mRNA [20]. For miR-218 detection, 50 ng total RNA was reverse transcribed using a TaqMan MicroRNA Reverse Transcription kit and real-time quantitative PCR was performed using TaqMan miR and *U6* RNA (used as a normalizer) assays (Life Technologies, Grand Island, NY, USA) following the manufacturer's instructions [19]. A customary miRNA expression profiling using a TaqMan® Array Human MicroRNA Cards containing 384 human miRNAs was performed by Life Technologies (Beijing, China).

Protein extraction and western blotting

A modified RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 0.25 % SDS, 1 % Triton X-100, 0.25 % sodiumdeoxycholate, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol) with protease inhibitor cocktail (Sigma, St. Louis, MO, USA) was used for protein isolation. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Cell lysates containing 50 μg of total protein were resolved on 12 % polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA) and blocked with 5 % blotting grade milk (Bio-Rad, Hercules, CA, USA) in PBST (0.1 % Tween 20 in PBS). Then membranes were incubated with primary antibodies (D16H11 for GAPDH, 71G4B7 for survivin, D2E11 for Bax and 50E3 for Bcl-2; all 1:1000) at 4°C overnight, followed by HRP-conjugated secondary antibodies at 1:2000 dilution for 2 h at room temperature. All antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA). Signals were visualized using SuperSignal West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL, USA) according to the manufacturer's instructions.

Annexin V staining

Cell apoptosis was measured using an annexin V-FITC apoptosis detection kit (Becton–Dickinson, San Diego, CA) essentially as described [21]. Briefly, cells (2×10^5) were incubated with taxol (0.25 μM for MCF-7/A02 and 2.5 nM for

CALDOX) for 48 h, washed twice and resuspended in 100 µl binding buffer, followed by staining with both 5 µl annexin V and 5 µl propidium iodide in the dark at room temperature for 15 min. Cells fluorescence was assayed by flow cytometry using a FACSCalibur flow cytometer (Becton–Dickinson, San Jose, CA).

Caspase activity

Cells (1×10^4) were incubated with taxol in 96-well plates for 24 h, and the caspase-3/7 and caspase-9 activities measured using Caspase-Glo® 3/7 Assay and Caspase-Glo® 9 Assay Kits, respectively (Promega, Madison, WI, USA) following the manufacturer's protocol. Fluorescence was measured using a GloMax™ 20/20 Luminometer (Promega, Madison, WI, USA).

In vivo tumor assay

Cells (5×10^6) were resuspended in a total volume of 100 µl containing 50% Matrigel (BD Biosciences, San Jose, CA, USA) in PBS and injected into the mammary fat pad of 4–5 week-old nude mice. Tumor sizes were measured every three days in two dimensions using a caliper, and the tumor volume was calculated with the following formula: tumor volume (mm^3) = $0.5\times ab^2$ (a and b being the longest and shortest diameters of the tumor, respectively). Twenty-one days after cell injection, tumor-bearing mice were randomly divided into different groups (five animals/group) for taxol treatment (2.5 mg/kg) or control (saline). Drug was injected every three days and tumor volume was monitored until the mice were sacrificed in a humane manner. Tumors were collected, RNA isolated using a *mir*Vana miRNA Isolation Kit (Life Technologies, Grand Island, NY, USA) following manufacturer's protocol and gene expression analysis performed by qPCR. All animal studies were performed at the National Institutes of Health (Tianjin cancer hospital) in accordance with guidelines under Institutional Animal Care and Use Committee (IACUC) and approved by the Committee on the Ethics of Animal Experiments of the Tianjin Cancer Hospital.

Statistical analysis

A t test was used when comparing samples. Statistical significance was considered

when $P < 0.05$.

Results

MicroRNA signature of drug resistant breast cancer cells

Two breast cancer cell lines, MCF-7/A02 and CALDOX, were derived from the chemosensitive cell lines MCF-7 and Cal51, respectively, by their resistance to doxorubicin, a common chemotherapeutic used in many cancer treatments [18, 21]. To determine the identity of miRNAs that might contribute to the drug resistant phenotype, a miRNA array was employed to characterize the miRNA signature of MCF-7/A02 cells. We selected those miRNAs differentially expressed at least 5 fold and with P value <0.05 between the sensitive MCF7 and drug resistant MCF-7/A02 cells. A total of 16 miRNAs fullfilled these criteria, 9 being up-regulated and 7 down-regulated in MCF-7/A02 cells (Table 1). Some miRNAs, such as miR-130b, were up-regulated up to 17 fold in drug resistant MCF-7/A02 cells, whereas others, such as miR-7-1* or miR-183* , were down-regulated 20 fold. This drug resistant miRNA signature obtained in MCF-7/A02 cells was tested in CALDOX cells by determining the expression levels of all these miRNAs using qPCR. We found that miR-218 was also down-regulated in CALDOX cells by approximately 3 fold. (Fig. 1A,B).

Over-expression of miR-218 restores drug sensitivity

As miR-218 is down-regulated in drug resistant breast cancer cell lines, we hypothesized that if it would contribute to the drug resistant phenotype, its experimental over-expression might restore drug sensitivity. To test this hypothesis, we generated stable MCF-7/A02 and CALDOX cells over-expressing miR-218 by lentiviral transfection (MCF-7/A02-miR-218 and CALDOX-miR-218, respectively). Cells transfected with the empty vector (MCF-7/A02-miRVec and CALDOX-miRVec) were used as control. MiR-218 expression was up-regulated 6 fold in MCF-7/A02-miR-218 cells and 4 fold in CALDOX-miR-218 cells (Fig. 2A). Drug sensitivity assays indicated that the taxol IC50 values of miR-218 over-expressing cells decreased between 55 and 80% (79.2% in MCF-7/A02-miR-218 cells and 55.6% in CALDOX-miR-218 cells). Equally, miR-218 over-expressing cells were more sensitive to doxorubicin, the IC50 values of miR-218 over-expressing cells decreased between 62% and 70% (69.3% in MCF-7/A02-miR-218 cells and 62.8% in

CALDOX-miR-218 cells) (Fig. 2B, Supplementary Figure S1A.). Moreover, to evaluate the efficacy of miR-218 over-expression on drug resistance, we treated cells with drugs and let them to proliferate for one week. Crystal violet staining was used as a surrogate for cell mass in this assay [19]. MiR-218 restoration slightly decreased cell proliferation in absence of drug. However, it impaired proliferation to a higher extent in the presence of both doxorubicin and taxol (reduction between 64% and 72%, Fig. 2C, Supplementary Figure S1B). Thus, the over-expression of miR-218 restores sensitivity to doxorubicin and taxol in drug resistant breast cancer cells.

MiR-218 targets survivin in breast cancer cells

MiR-218 targets survivin in cervical [22] and nasopharyngeal cancer [23] (Fig. 3A). As survivin has been identified as an anti-apoptosis gene in a variety of cancers [24,25], and may partially lead to drug resistance [26,27], we hypothesized that a negative correlation should exist between miR-218 and survivin expression. Indeed, drug-resistant MCF-7/A02 and CALDOX cells, which express low levels of miR-218 (Fig. 1A), showed abundant survivin expression both at mRNA and protein levels (Fig. 3B). When miR-218 expression was experimentally restored in drug resistant cells (Fig. 2A), a decrease in survivin expression (both mRNA and protein) was observed (Fig. 3C).

To further verify that survivin mRNA was a target of miR-218 in breast cancer cells, we transiently transfected cells over-expressing miR-218 with a luciferase reporter with or without the 3'-UTR of survivin (pMirTarget-survivin and pMirTarget-ev, respectively) together with a *Renilla* luciferase expressing plasmid to normalize for transfection efficiency. We hypothesized that luciferase expression would decrease in cells over-expressing miR-218 in those cells transfected with the reporter plasmid incorporating survivin 3'-UTR but not in the control. Indeed, an approximately 50% reduction in luciferase expression was observed in both MCF-7/A02-miR-218 versus MCF-7/A02-miRVec and CALDOX-miR-218 versus CALDOX-miRVec cells when transfected with pMirTarget-survivin. However, no changes in luciferase expression were observed in the same cells when transfected with the pMiRTarget-ev control vector (Fig. 3D). Thus, miR-218 binds survivin 3'-UTR and decreases its expression in breast cancer cells.

MiR-218 over-expression promotes cell apoptosis, induces caspase activity and modulates apoptosis-related proteins

As survivin inhibits cell apoptosis in prostate, ovarian, and renal carcinomas [28-30], we asked whether miR-218 over-expression could regulate apoptosis in breast cancer cells. For this we used taxol, a widely used microtubule poison known to activate mainly the intrinsic apoptotic pathway [31]. Annexin V staining indicated that in both drug resistant cells ectopic expression of miR-218 increased the percentage of cells undergoing apoptosis after taxol treatment (Fig. 4A, B). As CALDOX cells are more sensitive to taxol than MCF-7/A02 cells (Fig. 2B) the concentration of taxol used was 0.25 μM for MCF-7/A02 and 2.5 nM for CALDOX cells. As the dot plots indicate, approximately 30% of MCF-7/A02-miR-218 cells were in the early stages of apoptosis (positive annexin V and negative propidium iodide), whereas approximately 38% of CALDOX-miR-218 cells were already in the late stage of apoptosis after 48 h taxol treatment (positive for both annexin V and propidium iodide, Fig. 3A).

Measurement of caspase activation confirmed the results obtained with annexin V. Both caspase 9 and caspase 3/7 activities increased between 1 and 2 fold after taxol treatment in drug resistant cells over-expressing miR-218 when compared with the control cells expressing the empty vector (Fig. 3C). As the induction of apoptotic cell death could be partly due to alteration of pro-survival and pro-apoptotic proteins, we evaluated the expression level of bcl-2 (pro-survival) and bax (pro-apototic) both at the mRNA and protein levels. Drug resistant cells over-expressing miR-218 showed decreased mRNA (1-2 fold) and protein expression of bcl-2 (Fig. 4D, E). Although not changed at mRNA level (Fig. 4D), pro-apoptotic bax expression was up-regulated in MCF-7/A02-miR-218 cells whereas CALDOX –miR-218 cells only showed slightly higher protein levels than control cells carrying the empty vector (Fig. 4E). Thus, ectopic expression of miR-218 in drug resistant breast cancer cells restores doxorubicin and taxol sensitivity by promoting apoptosis.

Suppression of survivin mimics the sensitization to taxol by miR-218

To examine whether the phenotype observed after miR-218 over-expression was due to down-regulation of survivin, we knocked-down survivin mRNA in drug resistant

cells by RNA interference. Survivin mRNA was down-regulated by approximately 60% in both drug resistant cells when transfected with a siRNA targeting survivin (Fig. 5A). A similar decrease was observed when survivin protein expression was determined by western blots (Fig. 5B). The decrease in survivin was accompanied by a resensitization of drug resistant MCF-7/A02 and CALDOX cells to taxol with a reduction in the IC50 of approximately 40% in the former and 55% in the latter (Fig. 5C). Measurement of caspase-3/7 and caspase-9 activities indicated an increase after taxol treatement in both drug resistant cells when transfected with siRNA survivin in comparison with siRNA control (Fig. 5C). In addition, knock-down of survivin resulted in down-regulation of bcl-2 and up-regulation of bax mRNAs (Fig. 5E). Thus, suppression of survivin mimics the phenotype obtained by miR-218 over-expression.

MiR-218 suppresses tumor growth and reverses chemoresistance of MCF-7/A02 and CALDOX through targeting survivin *in vivo*

In order to assess whether the resensitization of drug resistant cells observed *in vitro* upon over-expression of miR-218 (Fig. 2) could be reproduced *in vivo*, we used a xenograft model. MCF-7/A02-miRVec, MCF-7/A02-miR-218, CALDOX-miRVec and CALDX-miR-218 cells were injected into the mammary fat pad of nude mice and tumor size were monitored every 3 days from the time when they were apparent (day 6) up to day 48 post-implantation. Compared with the control group, (miRVec), tumor growth in the miR-218 group was significantly reduced (from approximately 600 mm^3 to approximately 400 mm^3). Taxol treatment, that started at day 21 and was repeated every three days, was more effective in tumors derived from cells over-expressing miR-218 than in those from control cells (Fig. 6A). Consistent with tumor volumes, tumor weights of animals sacrificed at day 48 post-implantation were lighter in the miR-218 than miRVec group and decreased further after taxol treatment (Fig. 6B). As expected, tumors generated from miR-218-overexpressed cells had a higher expression of miR-218 and lower expression of survivin than tumors generated from control cells (Fig. 6C). Thus, miR-218 acts as a tumor suppressor and is effective in sensitizing breast cancer drug resistant cells to taxol *in vivo*.

Discussion

Chemotherapeutics are widely used in the treatment of various solid tumors. However, the appearance of drug resistance is a considerable obstacle for effective cancer therapy. Dysregulation of miRNAs has been found in nearly all types of human malignancies [7]. In order to characterize the role of miRNAs in breast cancer drug resistance, we applied high-throughput miRNA microarray analysis to obtain a miRNA signature of drug resistant MCF-7/A02 breast cancer cells. These cells have been generated from MCF-7 cells, an estrogen receptor-positive line, by exposure to doxorubicin and they are resistant to drugs such as taxol, etoposide, vincristine and mitoxantrone [21]. We identified 16 miRNAs differentially regulated (9 up and 7 down) in MCF-7/A02 cells, including miR-130b, miR-19a, miR-222, miR-218, miR-425 and miR-452. Some of these miRNAs have been associated with chemoresistance in several carcinomas. MiR-222 confers dexamethasone resistance in human multiple myeloma and tamoxifen resistance in breast cancer by targeting CDKN1B (alias p27, Kip1) [32], miR-19a regulates drug resistance in gastric and breast cancer cells through targeting PTEN [33,34], and deletion of miR-15a contributes to drug resistance in chronic lymphocytic leukemia, by targeting Mcl1 [35,36], and osteosarcoma through down-regulation of CCND1 [32].

In order to test the functionality of the drug resistance signature, we measured the expression of these 16 miRNAs in CALDOX cells. These drug resistant cells are derived from Cal51, a triple negative breast cancer cell line, by exposure to a single concentration of doxorubicin (0.4 μM) and show decrease sensitivity to doxorubicin, etoposide and mitoxantrone [18]. Only miR-218 was also down-regulated in these drug resistant cells. Since MCF-7/A02 and CALDOX have different resistance profiles and are derived from two different type of breast cancer cells, it is very likely that miR-218 down-regulation plays a role in drug resistance.

MiR-218 dysregulation may be a common occurrence in tumorigenesis [37], and plays a critical function in tumor invasion and metastasis progression in a variety of malignancies, including breast cancer [38]. In addition, miR-218 has been found to modulate chemosensitivity in human gastric cancer cells [39], and restoration of miR-218 expression increases cisplatin sensitivity, and induces apoptosis, in cisplatin-resistant MCF-7 cells by targeting BRCA1 [17]. We find that experimental over-expression of miR-218 in both MCF-7/A02 and CALDOX cells restores short-term sensitivity and long-term proliferation in the presence of doxorubicin and taxol.

MiRNAs bind the 3'-UTR of target mRNAs repressing their translation and leading to mRNA degradation. As binding to target sequences is not very stringent, a single miRNA may regulate the expression of many different mRNAs. MiR-218 binds to a variety of targets, such as IKK-β, ECOP, BMI1, Rictor, BRCA1, Robo1, SLIT2, and PXN in different cell systems [12, 23]. In nasopharayngeal and cervical cancinoma, miR-218 targets survivin and contributes to cell migration and invasion [22,23]. Our results indicate that in both estrogen receptor-positive and triple negative breast cancer cells, miR-218 also targets survivin.

Survivin (encoded by *BIRC5*) is the smallest member of the inhibitor of apoptosis (IAP) protein family which comprises eight members. Although survivin was initially discovered as an anti-apoptotic protein due to its caspase inhibition activity, it also serves as an indispensable regulator of cell mitosis [40]. Dysregulation of survivin is a key signature of many cancers, where it is over-expressed, and is undetectable in normal differentiated tissues [41,42]. Survivin carries out its anti-apoptotic function through inhibition of caspase-9, for which the presence of a helper protein, hepatitis B X-interacting protein (HBXIP), is necessary [24], and thus plays an important role in cancer drug resistance. As a consequence, survivin is a very promising biomarker for drug resistance [43]. Survivin dysregulation in human cancers can be the result of epigenetic mechanisms due to promoter methylation [44]. In addition, several survivin-targeting miRNAs have been described in different cancers [45], including miR-218 in nasopharyngeal cancer [23]. Here we present data indicating that miR-218 targets survivin in breast cancer cells leading to an increase in apoptosis, and restoration of drug sensitivity in drug-resistant cells.

MiR-218 is down-regulated in many tumors, including breast cancer, and is involved in cancer initiation and development. It is thus considered as a tumor suppressor. Our xenograft results confirm that miR-218 acts as a tumor suppressor and modulator of drug response, via survivin, in breast cancer. As lung cancer patients with low miR-218 expression have shorter overall survival rates than patients with high miR-218 expression [46], the potential clinical applications of miR-218 in breast cancer deserve further investigation. For survivin, YM155, an agent identified after a chemical library screening, has a potent suppressive activity against the survivin promoter [47], and it is the only small molecule specifically blocking survivin in humans [48]. Targeting miR-218 and survivin may thus provide a potential strategy for reversing drug resistance in breast cancer cells. Further studies using preclinical

animal models are necessary to confirm whether or not the proposed approach has potential value for clinical trials.

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Conflicts of interest

The authors declared no conflict of interest.

Ethical approval

All procedures performed in studies involving animals performed at the National Institutes of Health (Tianjin Cancer Hospital) in accordance with guidelines under the Institutional Animal Care and Use Committee (IACUC) and approved by the Committee on the Ethics of Animal Experiments of the Tianjin Cancer Hospital. All applicable national and institutional guidelines for the care and use of animals were followed.

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Fig 1 MiR-218 is down-regulated in drug resistant breast cancer cells. MiR-218 expression was determined by qPCR in pairs of drug-sensitive and resistant A) MCF-7, B) Cal51 cells. MiR expression was normalized to that of *U6* RNA. Data represent the average ± SD of three independent experiments. * $P<0.05$

Fig 2 MiR-218 over-expression restores drug sensitivity. Drug resistant MCF-7/A02 and CALDOX cells were stably transfected with a lentivirus carrying an expression vector for miR-218, generating MCF-7/A02-miR-218 and CALDOX-miR-218 cells, respectively. Control cells, MCF-7/A02-miRVec and CALDOX-miRVec, were transfected with the empty vector. (A) Expression of miR-218 was determined by qPCR and was normalized to *U6* RNA expression. (B) Doxorubicin, *left panels,* and taxol, *right panels,* IC50 values. (C) Cells were treated with doxorubicin (15 μM for MCF-7/A02 and 1 μM for CALDOX) and taxol (0.25 μM for MCF-7/A02 and 2.5 nM for CALDOX) for seven days and the cells stained with crystal violet. Dye was solubilized and the OD592 nm determined. Data represent the average ± SD of three independent experiments. * $P < 0.05$

Fig 3 Identification of survivin as a direct target of miR-218 in breast cancer cells. (A) Annealing of miR-218 to survivin (BIRC5) 3'-UTR according to the microRNA.org database [49]. (B) Survivin expression at mRNA (qPCR; *upper panel*) and protein levels (western blot; *lower panel*) in sensitive and drug resistant breast cancer cells. (C) Expression level of survivin expression in drug resistant cells after stable expression of miR-218 or miRVec vector control at mRNA (qPCR; *upper panel*) and protein levels (western blot; *lower panel*). (D) MiR-218 targets survivin mRNA. Normalized firefly luciferase activity from the reporter with the survivin 3'-UTR (*pMirTarget-survivin*) or the empty vector (*pMirTarget-ev*) after transient transfection into cells over-expressing miR-218 or control (miRVec) cells. In all cases cells were co-transfected with a *Renilla* luciferase expression vector to normalize for transfection efficiency. Numerical data show the average ± SD of at least three experiments (* $P < 0.05$) and the immunoblot shows a representative of at least three replicates

Fig 4 MiR-218 restores drug sensitivity by activating apoptosis in breast cancer cells. Cells were treated with taxol (0.25 μM for MCF-7/A02 series and 2.5 nM for CALDOX series) for 48 h. A) Detection of annexin V / propidium iodide staining by

flow cytometry. Cells in the *upper right* (UR) quadrant are at the late apoptotic stage, whereas those in the *lower right* (LR) quadrant are early apoptotic. Plots shown are representative of three independent experiments. B) Quantification of cell death by flow cytometry. Data show the average percentage of annexin V positive cells ± SD of three independent experiments. (C) Caspase-3/7 (*upper histograms*) and caspase-9 (*lower histograms*) activity of drug resistant cells after taxol treatment. (D, E) Expression levels of bcl-2 and bax in transfected drug resistant cells were determined by qPCR (D) and western blotting (E). Numerical data represent mean ± SD based on three independent experiments. *$P < 0.05$ and the immunoblot shows a representative of three replicates

Fig 5 Down-regulation of survivin mimics miR-218 overexpression.
Drug resistant MCF-7/A02 and CALDOX cells were transiently transfected with siRNAs targeting survivin (*siSurvivin*) or a scrambled siRNA (*siControl*) and survivin expression determined by qPCR (A) after 2 days and western blotting (B) after 3 days. (C) Taxol IC50 in both sets of cells. (D) Caspase 3/7 (*upper panels*) and caspase 9 (*lower panels*) were determined in transfected cells after 24h of treatment with taxol. (E) Bcl-2 and bax mRNA analysis determined by qPCR. Numerical data represents the average ± SD of three independent experiments. The immunoblot is a representative of three independent experiments

Fig 6 MiR-218 acts as a tumor suppressor and resensitizes breast cancer drug resistant cells to taxol in tumor xenografts. Drug resistant breast cancer cells stably expressing miR-218 or miRVec control were subcutaneously injected into the fat pad of nude mice (five per group). Twenty-one days after implantation, mice were randomly split in two groups and treated either with vehicle or taxol (2.5 mg/kg) every three days until they were sacrificed 48 days after implantation, when xenografts were removed. (A) Size of MCF-7/A02 or CALDOX xenograft tumors after treatment with saline or taxol. Data are mean tumor size ± SD of five tumors per group. (B) Tumor weight 48 days after implantation in mice treated with saline or taxol. Data indicates tumor weight in individual mice (*dots*) and the mean values (*line*) in five mice per group. (C) RNA was isolated from tumors 48 days post-implantation and miR-218 and survivin mRNA expression determined by qPCR. The individual tumor expression data (*dots*) and the mean values (*line*) are indicated

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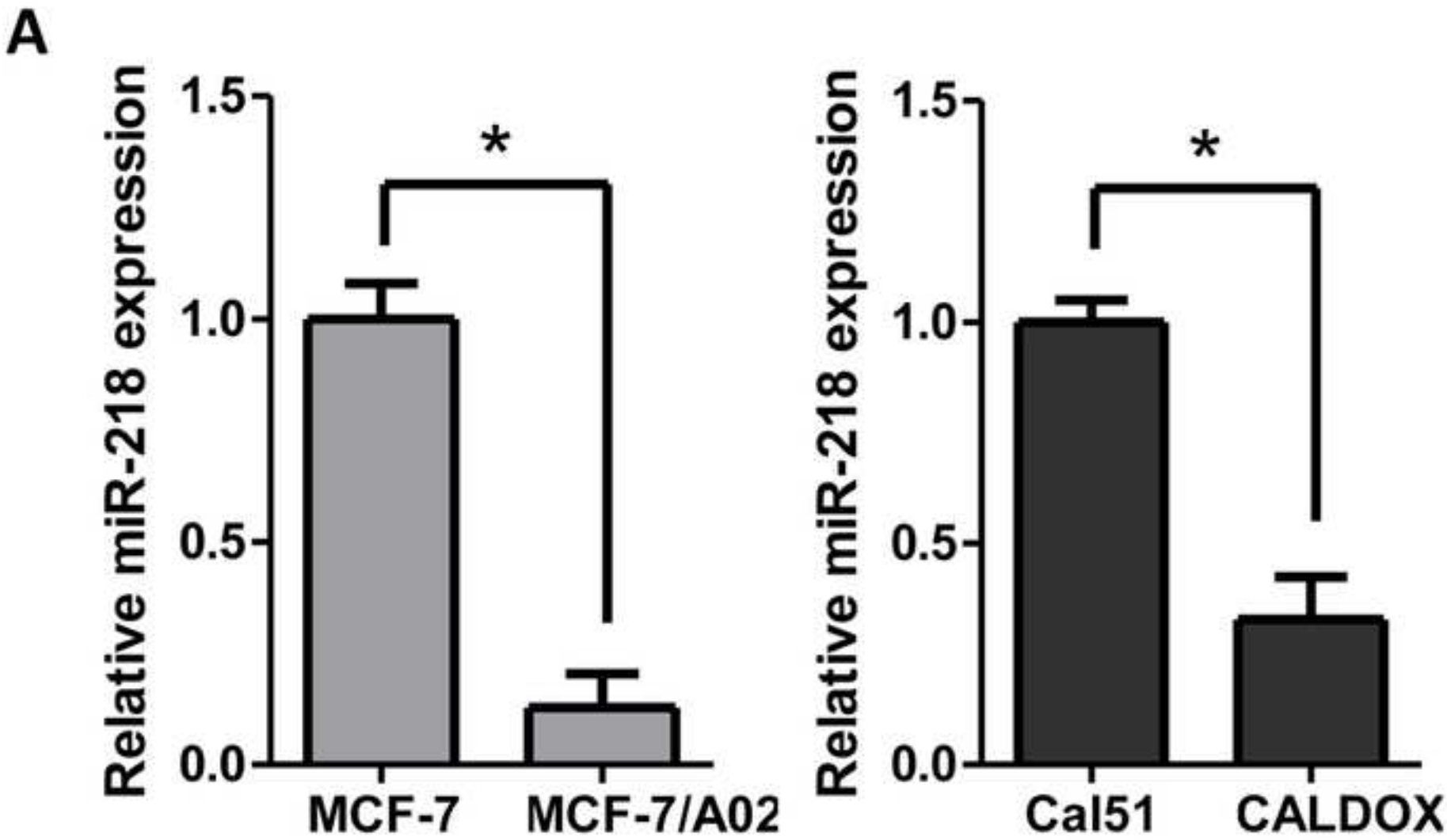

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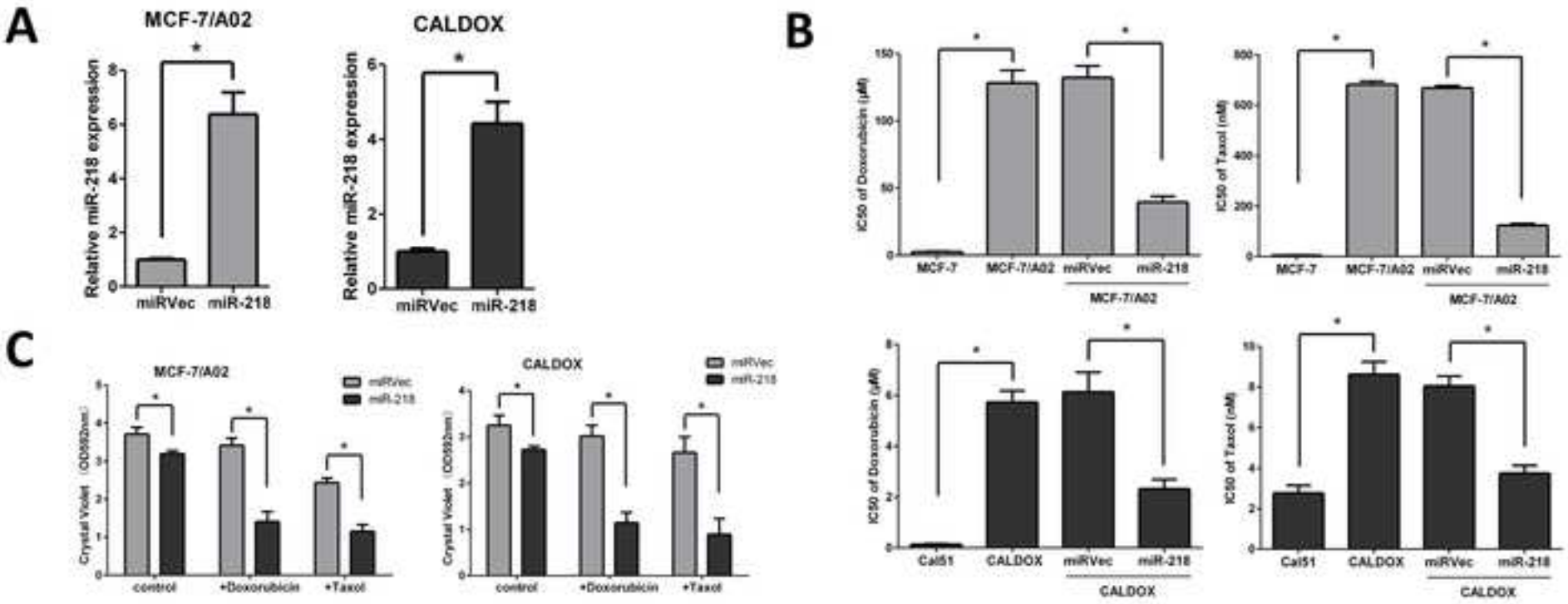

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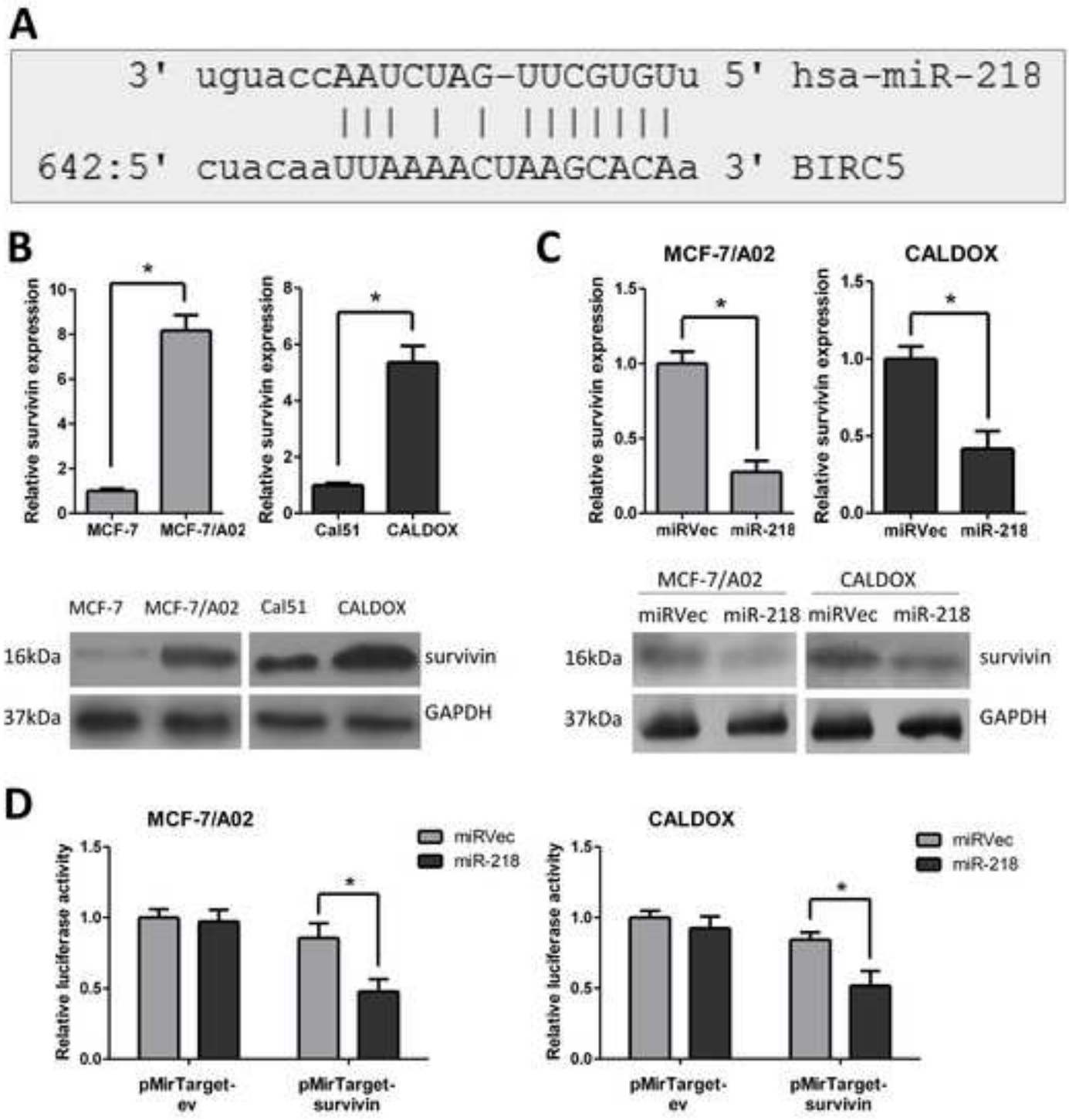

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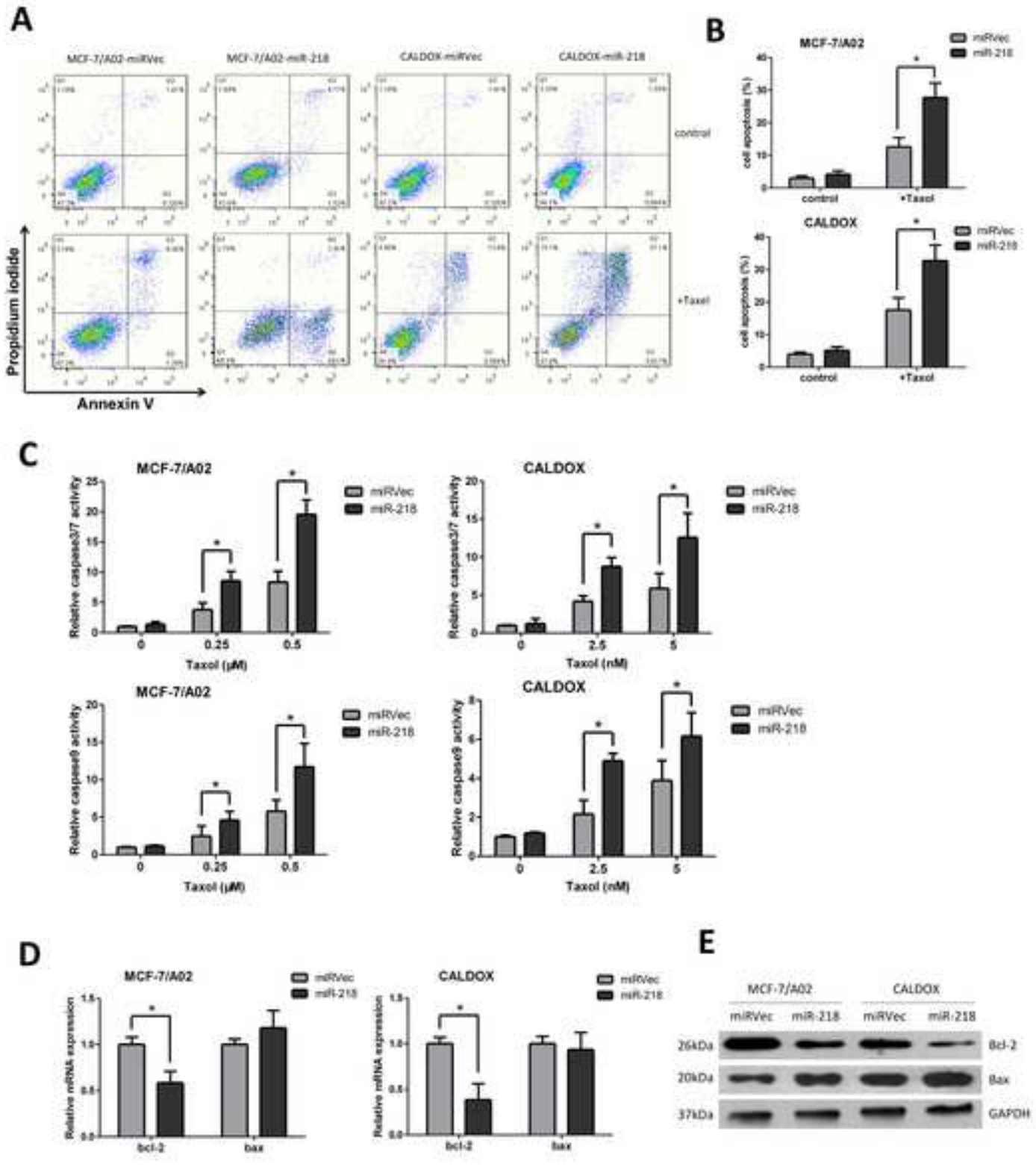

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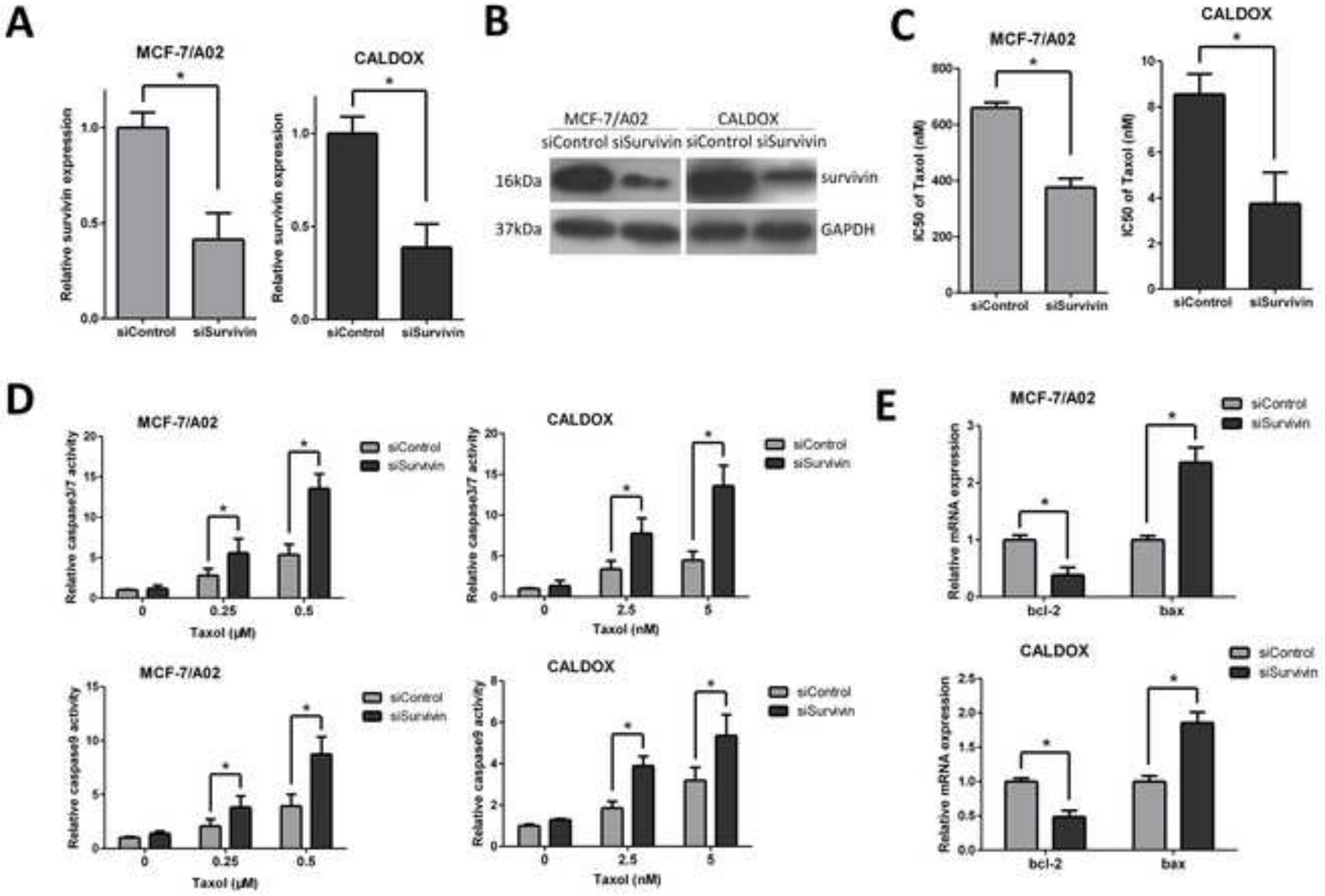

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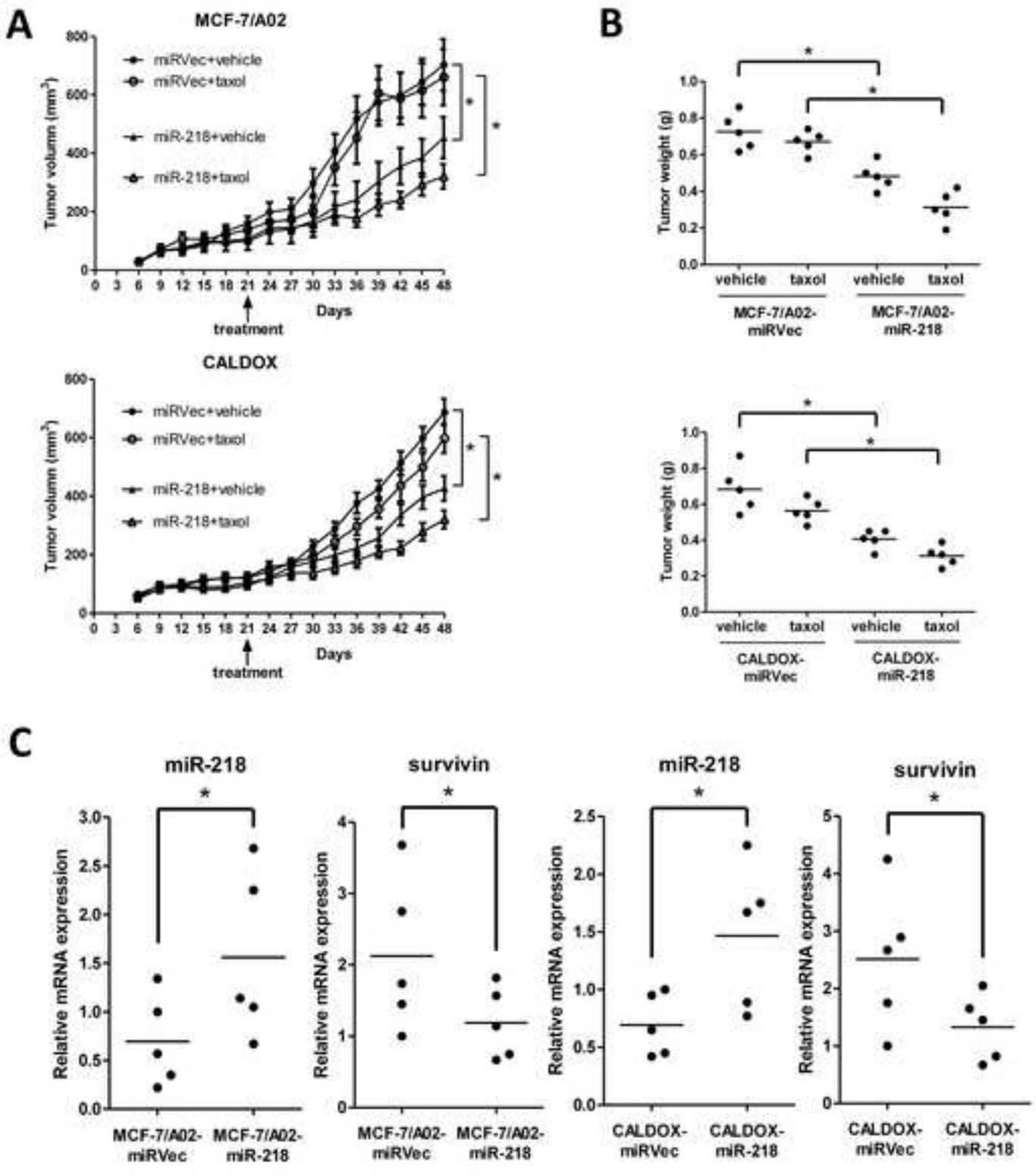

Table
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Table 1 MiRNA drug resistance signature of MCF-7/A02 cells

Gene name	Fold change[a]
hsa-miR-130b	**17.495**
hsa-miR-135a*	**17.081**
hsa-miR-942	**15.526**
hsa-miR-19a	**13.377**
hsa-miR-9	**12.962**
hsa-miR-222	**12.362**
hsa-miR-20a*	**6.883**
hsa-miR-572	**6.506**
hsa-miR-126*	**5.421**
hsa-miR-7-1*	**0.057**
hsa-miR-183*	**0.067**
hsa-miR-15a	**0.078**
hsa-miR-425	**0.086**
hsa-miR-99b*	**0.104**
hsa-miR-218	**0.137**
hsa-miR-452	**0.174**

[a]ratio of expression between MCF-7/A02 and MCF-7 cells