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Doxorubicin resistance in breast cancer xenografts and cell lines can be counterweighted by microRNA-140-3p, through PD-L1 suppression

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Summary. Background. Doxorubicin, a first-line chemotherapeutic drug for breast cancer, kills cancer cells by inducing DNA-crosslinking damage. Dysregulated micro-RNA (miRNA) is associated with the drug resistance of tumors. However, little is known about the effect of miRNA-140-3p on DOX resistance of breast cancer.

Methods. The miRNA microarray was used to sequence the transcripts of DOX-chemoresistant breast cancer tissues and DOX-chemosensitive tissues. Then, the breast cancer tissue chip in the GEO database was also analyzed to screen the target gene. Flow cytometry, in situ hybridisation (ISH), immunohistochemistry (IHC), Western blot, cell proliferation assay, real-time PCR analyses (qRT-PCR), and pull-down assay were used to explore the effects of miRNA-140-3p and programmed death ligand-1 (PD-L1) on the chemoresistance of DOX-resistant breast cancer cells treated with DOX. *In vivo*, the DOX-resistant breast cancer cell lines treated with miRNA-140-3p overexpression were injected subcutaneously into mice to construct breast cancer subcutaneous xenograft tumor models.

Results. Based on miRNA microarray, GEO database, and bioinformatics analysis, it was found that miRNA-140-3p and PD-L1 are the core molecules in the DOX resistance regulatory network in breast cancer, and lower miRNA-140-3p and higher PD-L1 expression levels were observed in DOX-resistant breast cancer tissues and cells. IHC results showed that compared with breast cancer tissues with high miRNA-140-3p expression, PD-L1 protein expression levels in breast cancer tissues with low miRNA-140-3p were significantly higher (P<0.01). Moreover, compared with DOX-sensitive tissues, the levels of PD-L1 protein expression in DOX-resistant tissues were significantly higher (P<0.01). In *in vitro* and *in vivo* experiments, the introduction of miRNA-140-3p decreased PD-L1 expression. Mechanically, we found that the MCF-7/DOX and HS598T/DOX cells pretreated with miRNA-140-3p inhibitor or exosomes containing PD-L1 have higher stemness and lower apoptosis rate, which can be abrogated by co-treating cells with anti-PD-L1 antibody or miRNA-140-3p mimic.

Conclusions. MiRNA-140-3p can suppress PD-L1 expression in breast cancer cell-derived exosomes, thereby attenuating the chemoresistance induced by DOX in breast cancer.

Key words: Breast cancer, Programmed death ligand-1, Doxorubicin, Chemoresistance

Introduction

Global Cancer Statistics 2020 estimated that there were 2,261,419 new cases of breast cancer and 684,996 deaths from breast cancer (Sung et al., 2021). Doxorubicin (DOX) is regarded as the most effective chemotherapeutic medication used for breast cancer treatment (Zheng et al., 2021); however, DOX effectiveness is negatively affected by multidrug resistance in breast cancer cells during chemotherapy. About 30-50% of metastatic breast cancer patients are responsive to DOX treatment (Zangouei et al., 2021).

Programmed death ligand-1 (PD-L1) is expressed on the surface of a variety of tumor cells and is related to the immune escape of tumors (Kudo, 2020). PD-L1 is the ligand of the programmed death 1 (PD-1) receptor molecule, and PD-1 is an important immunosuppressive molecule (Kudo, 2020). Immunomodulation targeting

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Abbreviations. DOX, Doxorubicin; GEO, Gene Expression Omnibus; IHC, Immunohistochemistry; ISH, In situ hybridisation; miRNA, Micro-RNA; PD-1, Programmed death 1; PD-L1, Programmed death ligand-1; qRT-PCR, Real-time PCR analyses.

PD-L1 is of great significance for anti-tumor, anti-infection, anti-autoimmune diseases, and organ transplant survival (Majidi et al., 2021; Paver et al., 2021). However, tumor cells can produce PD-L1, which can bind to the PD-1 protein on the surface of T cells, making T cells unable to recognize and kill tumor cells, thereby promoting tumor progression (Shigeta et al., 2020).

Studies have pointed out that the abnormal regulation of micro-RNA (miRNA) can promote the occurrence and development of tumors and can also promote the resistance of tumor cells to anti-tumor drugs (Han et al., 2020; Liu et al., 2020). Several studies demonstrated that miRNA-140-3p may serve as a tumor suppressor (Flamini et al., 2017; Kapodistrias et al., 2017; Jiang et al., 2021). After performing correlation analysis, expression analysis, survival analysis, and luciferase activity assays, Jiang et al. (2021) disclosed that miRNA-140-3p was decreased in different cancers (adrenocortical carcinoma, breast invasive carcinoma, lung squamous cell carcinoma, thyroid carcinoma, pancreatic adenocarcinoma, rectum adenocarcinoma, etc.) and was correlated with poor prognosis in a pan-cancer analysis. Flamini et al. (2017) has also shown that miRNA-140-3p had a lower expression in NSCLC, and its overexpression reduced the migration and invasion properties of NSCLC cells. Furthermore, Naba et al. (2020) demonstrated that miR-140-3p expression is downregulated in DOX-treated HCT116 cells, resulting in increased PD-L1 expression. However, the effect and underlying mechanism of miRNA-140-3p on DOX-induced breast cancer chemotherapy resistance have not been reported.

In this study, based on miRNA microarray and bioinformatics methods, we identified that miRNA-140-3p was dramatically downregulated in DOX-resistant breast cancer cells. We also confirmed that miRNA-140-3p enhances the sensitivity of DOX-resistant breast cancer cells to DOX by inhibiting PD-L1. *In vivo* experiments further showed that miRNA-140-3p exerted marked anti-tumor effects through targeting PD-L1. Our work broadens our knowledge of the pathogenesis of DOX-induced chemoresistance in breast cancer and provides potential treatments for this disease.

Materials and methods

Clinical specimen collection

Informed consent was obtained from each patient, and the study conforms to The Code of Ethics of the Declaration of Helsinki and the Clinical Medical Research Ethics Committee of The Xianning Central Hospital (Approval no.: 2019-A017R1). Forty-six paired breast cancer and adjacent tissues were obtained from breast cancer patients at Xianning Central Hospital. 46 breast cancer patients were divided into chemoresistant patients (n=23) and chemosensitive patients (n=23), according to the guidelines for clinical diagnosis and treatment of advanced breast cancer in China (National Cancer Center for Quality Control Breast Cancer Expert Committee et al., 2020).

DOX resistant cells

DOX-resistant cells (HS598T/DOX and MCF-7/DOX) were established by exposing HS598T and MCF-7 cells to gradually increasing doses of DOX (0.1-2.0 μg/mL) for 5 months. Two weeks after the withdrawal of the DOX treatment, the biological characteristics of the resistant strains were observed. One month after the withdrawal of the DOX, the DOX-resistant cell lines can still maintain drug resistance. Compared with the half maximal inhibitory concentration (IC_{50}) when the DOX was withdrawn, there was no statistical difference in the IC50 change at 1 month after the DOX was withdrawn ($P>0.05$), indicating that the DOX-resistant cell lines have better drug resistance stability. Cell culture was performed as described before (Naba et al., 2020). Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640, Gibco, CA, USA) supplemented with 10% fetal bovine serum (Gibco, CA, USA) and 1% penicillin/streptomycin (Solarbio, Beijing, China). The cells were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Oligonucleotide transfection

Synthetic miRNA-140-3p mimic, inhibitor, and their negative control oligonucleotides were purchased from Aibosi Biological Co., Ltd. (Shanghai, China). Target cells were transfected with the above oligonucleotides using Lipofectamine® 3000 reagent (Invitrogen, Carlsbad, CA, USA).

Construction of miRNA-140-3p knockout (KO) breast cancer cells

CRISPR/Cas9 lentivirus LV-VMP1-sgRNA-miRNA-140-3p (miRNA-140-3p KO) and negative control virus sgRNA-CON251 (U6-sgRNA-EF1a-Cas9-FLAG-P2A-EGFP) were purchased from Shanghai Jikai Gene Chemical Technology Co., Ltd., China. HS598T/DOX and MCF-7/DOX cells in logarithmic growth phase were collected and seeded in 96-well plates, and the number of cells per well was about 5×10^3 (100 μl). After 72 hours, HS598T/DOX and MCF-7/DOX cells transfected with CRISPR/Cas9 lentivirus and negative control virus were collected by centrifugation. PCR amplification was performed using miRNA-140-3p primer. The digestion products were identified by electrophoresis in a 20 g/L agarose gel, and the mutation efficiency of CRISPR/Cas9-induced miRNA-140-3p was estimated by Image J software. Then, HS598T/DOX and MCF-7/DOX cells were collected after LV-VMP1-sgRNA-miRNA-140-3p transfection for 72h. The cell density was adjusted to 10

cells/ml, and 100 µl/well (1 cell per well) was seeded in a 96-well cell culture plate. When the clones formed by single cells reach more than 70% confluence in the 96-well plate, 50 µl of each single cell clone was taken into an EP tube, and continued to culture the remaining cells. QuickExtract™ DNA extraction reagent (product of Lucigen, USA) was used to extract genomic DNA from each EP tube as a template. Using miRNA-140-3p upstream and downstream primers for PCR amplification, single-cell clones with shorter PCR product fragments than wild-type HS598T/DOX and MCF-7/DOX cells were screened.

miRNA microarray

In the miRNA microarray, we included a total of 8 DOX-chemosensitive patients and 16 DOX-chemoresistant patients (including 8 patients with stage T1-T2 and 8 patients with stage T3-T4). miRNA microarray analysis was performed with a total of 1,366 represented miRNAs (G4872A-031181, Agilent Technologies, Palo Alto, California, USA) following the manufacturer's instructions. Using Mann-Whitney U Test, a miRNA is considered as differentially expressed when $|logFC|> 1$ and $P<0.05$.

Bioinformatics analysis

The differentially expressed genes related to DOX resistance in breast cancer were entered into the STRING database to search the interaction relationship between the proteins online, and the combined score>0.4 was used as the screening condition. The GSE162187 breast cancer tissue chip was selected using "breast cancer" AND "Doxorubicin" as the search keywords. The chip included 22 patients with locally advanced breast cancer who received DOX-based chemotherapy and were finally clinically evaluated 6 months after the end of treatment. We used the limma R package to analyze the differences in the expression profiles of the two groups of patients and screened out the differential genes related to DOX resistance in breast cancer.

In situ hybridisation (ISH) and immunohistochemistry (IHC)

Forty-six breast cancer patients, including chemotherapy-resistant patients (n=23) and chemotherapy-sensitive patients (n=23), underwent ISH and IHC testing. ISH and IHC were performed as previously described (Han et al., 2020), using PD-L1 antibody (1:500, Thermo Fisher Scientific Cat# 13-9971-85, RRID: AB_467011) and a probe specific for miRNA-140-3p on breast cancer tissue. The results of ISH and IHC were independently scored by two independent observers (Xia Zhang and Chao Wang). The intensity of staining was divided into four grades (intensity scores): negative (0), weak (1), moderate (2) and strong (3).

Real-time PCR analyses (qRT-PCR)

A parallel qRT-PCR analysis was performed in this study. The SYBR-Green I Premix Ex Taq was purchased from Beijing Kangwei Century Biotechnology Co., Ltd (RR820A). The primer sequences used for qRT-PCR were 5'-AAATGGAACCTGGCGAAAGC-3' (upstream) and 5'-GATGAGCCCCTCAGGCATTT-3' (downstream) for PD-L1; 5'-TACCACAGGGTAGAACCACGG-3' for miRNA-140-3p; 5'-ACACCAATCCCATCCACACT-3' (upstream) and 5'-GCAAACTTCCTGCAAAGCTC-3' (downstream) for SOX2; 5'-TCCCGGGTTCAAGTGATTCT-3' (upstream) and 5'-ATGGTCGTTTGGCTGAATAC-3' (downstream) for OCT4; 5'-AGTAGAGACGGGGTTTCAC-3' (upstream) and 5'-AGAGACGGCAGCCAAGGTTA-3' (downstream) for Nanog; 5'-CTCGCTTCGGCAGCACA-3' (upstream) and 5'-AACGCTTCACGAATTTGCGT-3' (downstream) for U6; and 5'-TGGTGAAGACGCCAGTGGA-3' (upstream) and 5'-GCACCGTCAAGGCTGAGAAC-3' (downstream) for GAPDH. The relative level was calculated by the $2^{-\Delta Ct}$ (U6 or GAPDH as control) or $2^{-\Delta\Delta Ct}$ (The control group as control) method.

Western blot

Mouse anti-PD-L1 (1:1000, Abcam Cat# ab205921) and GAPDH (1:5000, Abcam Cat# 2251-1) antibodies were added overnight at 4°C. The secondary antibody (1:5000, Covance Cat# SMI-5030C-2000) was then added. Blot densitometric analysis was done by ImageJ software (NIH, USA).

Flow cytometry assay

Transfected or untransfected HS598T/DOX and MCF-7/DOX cells (1×10^5 per well) in 24-well plates in quadruplicate were collected for apoptotic analysis using Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis assay kit (Solarbio). In brief, the treated cells were resuspended in binding buffer and then stained with Annexin V-FITC and PI for the indicated time point. A flow cytometry (Countstar, Shanghai, China) was used to assess the apoptotic cells, and the apoptotic rate was expressed as the percentage of cells at the upper right and lower right quadrants in the total number of cells.

Dual-luciferase reporter gene system

PD-L1 3'UTR containing miRNA-140-3p binding site was constructed into pGL3 vectors (Wuhan Jinkairui Biological Co., Ltd. Wuhan, China) to form wild-type (WT)-PD-L1. Mutant type (MuT)-PD-L1 was generated through GeneArt™ Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA, USA). Then, they were co-transfected with pRL-TK vectors (Wuhan Jinkairui Biological Co., Ltd. Wuhan, China) and miRNA-140-3p

mimic or correlated negative control into HS598T/DOX and MCF-7/DOX cells. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After incubation for 30h at 37°C, reporter gene expression was detected by the Dual-Luciferase Assay System (Promega, Madison, WI, USA).

Pull-down assay of biotin-labeled PD-L1

A pull-down assay was performed as described before (Liu et al., 2020). The biotin-labeled probe for the 3' untranslated region (3' UTR) of PD-L1 was designed and synthesized by Shanghai Walan Biotechnology Co., Ltd. (Shanghai, China). The pull-down assay was conducted to assess its binding ability with miRNA-140-3p. Briefly, the cells were fixed, lysed, and centrifuged, the supernatants were used as input, and the above probes were incubated with the supernatants overnight at room temperature. After that, the lysis buffer and Proteinase K were used to reverse the formaldehyde crosslinking. qRT-PCR was conducted to examine miRNA-140-3p levels.

Isolation and purification of exosomes

Approximately 2×10^6 cells (HS598T, MCF-7, HS598T/DOX, and MCF-7/DOX) were cultured in RPMI−1640 medium containing 10% exosome−free FBS (Cat#: abs993, Aibixin (Shanghai) Biotechnology Co., Ltd.). Then, approximately 4 ml of cell culture medium was collected after 72h and centrifuged at 2000 g for 20 min and 25,000×g for 40 min at 4°C. The supernatant was filtered with a 0.22 μm filter and centrifuged at 120,000×g for 80 min at 4°C. The number and morphology (cup-shaped) of exosomes were examined using a NanoSight NS300 microscope (Malvern Instruments Ltd., UK) and a Philips CM120 BioTwin transmission electron microscope (FEI Company, USA), respectively. About 10^8-10^9 exosomes can be obtained from 1 ml of cell supernatant. For cell processing, the number of recipient cells was controlled to 2×10^5 and exosomes (10^8-10^9) and recipient cells were incubated for 72h.

Establishment of xenograft mice models

Experiments were performed under a project license (NO.: C201901A-003) granted by the Ethics Committee of The Xianning Central Hospital, in compliance with The Xianning Central Hospital's guidelines. Twelve BALB/c nude mice (20-22 g, 4-6 weeks old, female:male=1:1) were obtained from Shanghai Jie Sijie Laboratory Animal Co., Ltd. (Shanghai, China).

Twenty-four mice were divided into four groups. 5×10^6 of HS598T/DOX cells, miRNA-140-3p overexpressing HS598T/DOX cells, MCF-7/DOX cells, miRNA-140-3p overexpressing MCF-7/DOX cells, miRNA-140-3p KO HS598T/DOX cells, miRNA-140-3p KO MCF-7/DOX cells were subcutaneously injected into nude mice for 14 days. After the model was successfully established, DOX was injected intraperitoneally, once a day, at a dose of 3 mg/kg, for a total of 5 times.

Statistical analysis

R version 3.6.1 (81 megabytes, 64 bit), Cytoscape version 3.7.2 (http://www.cytoscape.org/), and GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA) were used. One-way ANOVA was used for the comparison among multiple groups if the variance was homogeneous. $P<0.05$ was considered significant.

Results

Screening of DOX resistant-related miRNAs in breast cancer

We analyzed 1,366 human miRNAs between DOX-chemoresistant (n=16) group and DOX-chemosensitive (n=8) group. A total of 13 candidate human miRNAs were identified for further testing via qRT-PCR, including miRNA-21, miRNA-29b-3p, let-7g-5p, miRNA-126-3p, miRNA-4286, miRNA-634, miRNA-4310, miRNA-817-3p, miRNA-3679-3p, miRNA-140-3p, miRNA-129-3p, miRNA-151a-3p, and miRNA-361-5p, as is shown in the heatmap (Fig. 1A). Next, we detected the expression levels of the above 13 miRNAs in two DOX-resistant cell lines. The qRT-PCR results showed that the expression of miRNA-817-3p and miRNA-140-3p was significantly reduced in the HS598T/DOX and MCF-7/DOX cell lines, while the expression of miRNA-126-3p was significantly increased ($P<0.05$, Fig. 1B). Moreover, we further verified the expression of miRNAs in tumor tissues of 46 breast cancer patients. The results showed that compared with DOX-chemosensitive patients, the levels of miRNA-140-3p in tumor tissues of DOX-chemoresistant patients were significantly lower, while the expression of miRNA-126-3p and miRNA-817-3p was not statistically significant between the two groups of patients (Fig. 1C). Finally, we measured miRNA-140-3p expression in breast cancer patients' tissues using ISH. miRNA-140-3p was significantly downregulated in DOX-chemoresistant tissues compared with DOX-chemosensitive tissues ($P<0.01$, Fig. 1D).

Intracellular PD-L1 was targeted and inhibited by overexpressing miRNA-140-3p in HS598T/DOX, MCF-7/DOX cells, and breast cancer tissues

Then, we searched the interactions between differentially expressed genes related to DOX resistance through the STRING database. Finally, we screened the 10 differentially expressed genes (PD-L1, MZB1, CXCL9, NKG7, OLR1, NFIL3, LOGR, CCL5, ADIPOQ, MMP13) with the highest degree as core

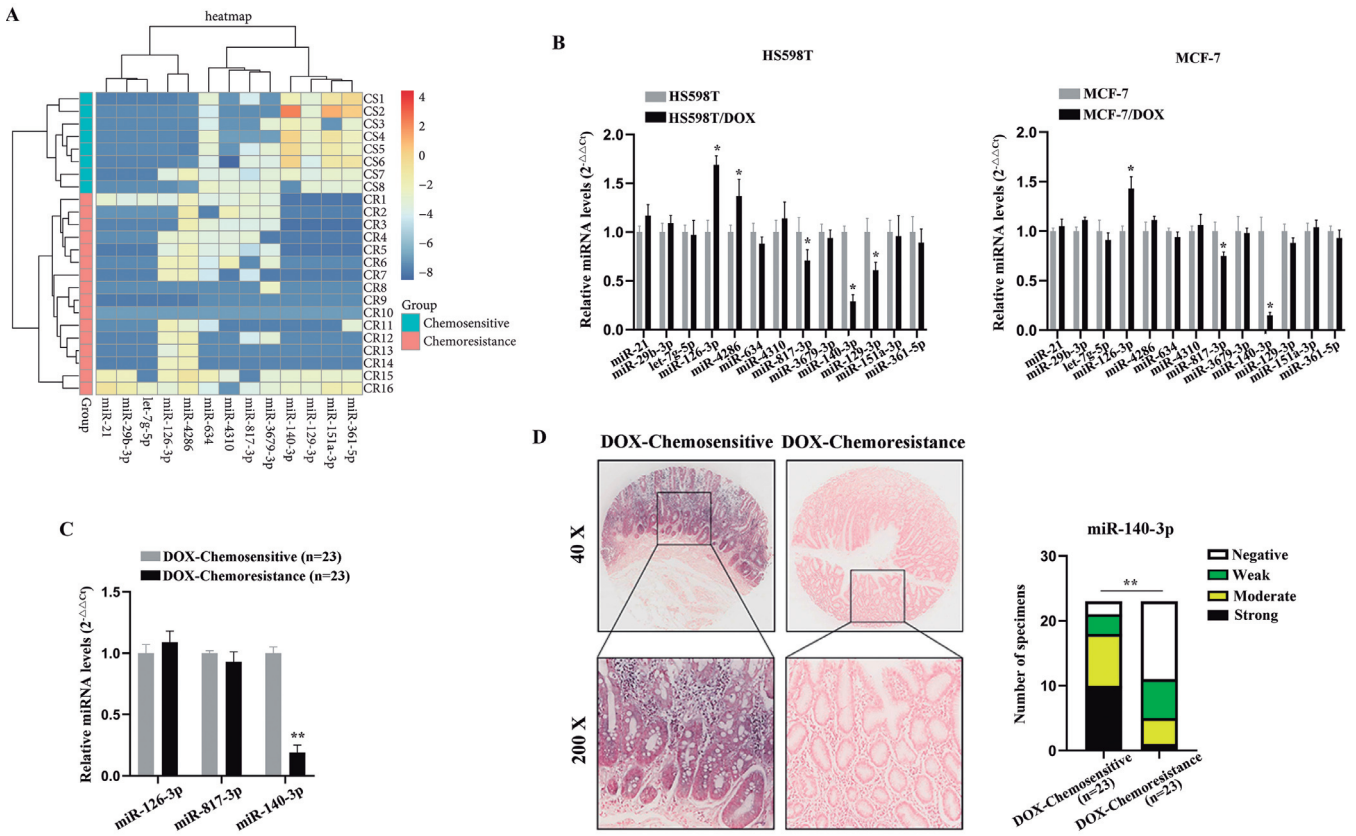

Fig. 1. MiRNA-140-3p was decreased in DOX-resistant breast cancer cells and tissues. **A.** Heatmap showing differential miRNA expression in DOX-chemoresistant tissue samples compared with DOX-chemosensitive tissue samples. **B.** Expression of miRNA-21, miRNA-29b-3p, let-7g-5p, miRNA-126-3p, miRNA-4286, miRNA-634, miRNA-4310, miRNA-817-3p, miRNA-3679-3p, miRNA-140-3p, miRNA-129-3p, miRNA-151a-3p, and miRNA-361-5p were determined by qRT-PCR in the HS598T/DOX and MCF-7/DOX cell lines. U6 RNA was used as an internal control. *: Compared with control cell line, and $P<0.05$. Three independent experiments were performed, each with two technical replicates. **C.** Expression of miRNA-126-3p, miRNA-817-3p, and miRNA-140-3p was determined by qRT-PCR in the DOX-chemoresistant and DOX-chemosensitive tissues. U6 RNA was used as an internal control. **: Compared with DOX-chemosensitive tissue group, and $P<0.01$. Three independent experiments were performed, each with two technical replicates. **D.** Representative images and analysis of ISH staining for miRNA-140-3p in 23 DOX-chemoresistant tissues and 23 DOX-chemosensitive tissues. **: Compared with DOX-chemosensitive tissue group, and $P<0.01$.

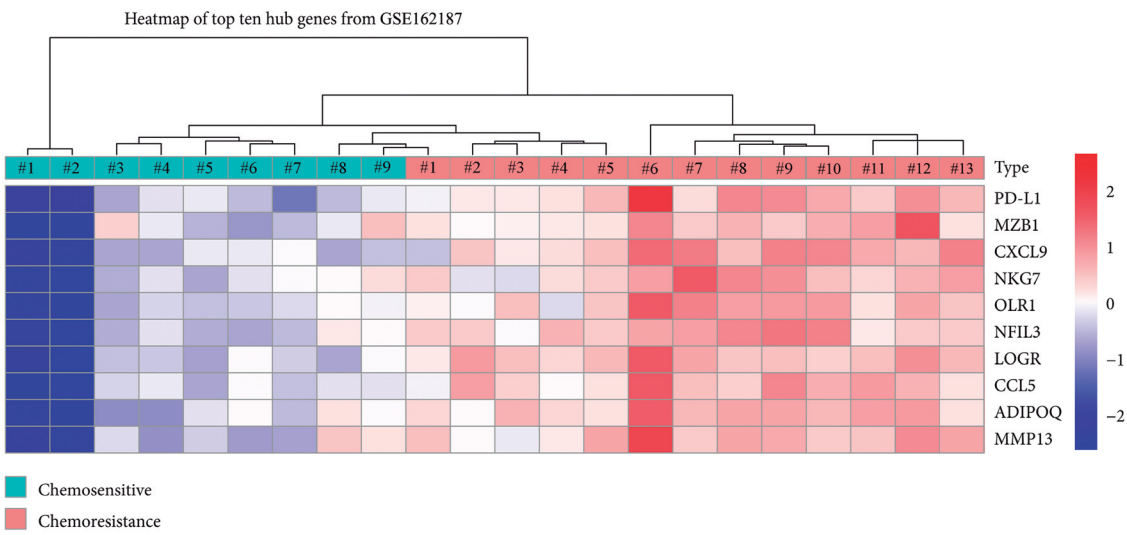

Fig. 2. Comprehensive analysis of the expression of core genes in DOX-resistant breast cancer patients (from GEO GSE162187 data).

genes (Fig. 2). Subsequently, the pheatmap package was used to perform bi-clustering analysis on the 10 core genes, and PD-L1 possessed a high diagnostic value in DOX-resistant breast cancer patients (Fig. 2).

The protein PD-L1 can be sponged and inhibited by miRNA in multiple cancers, further enhancing the efficacy of cancer immunotherapy (Dou et al., 2020; Yao et al., 2020; Zhang et al., 2020). The online TargetScan software (https://www.targetscan.org/vert_72/) predicted the binding sites of miRNA-140-3p and the 3' UTR of CD274 (PD-L1) mRNA (Fig. 3A), and the dual-luciferase reporter gene system validated these binding sites (Fig. 3B). Similarly, the PD-L1 probe pull-down assay results verified that miRNA-140-3p could be enriched by the probes of 3' UTR region of PD-L1 mRNA in HS598T/DOX and MCF-7/DOX cells (Fig. 3C).

The expression levels of miRNA-140-3p and PD-L1 in DOX-sensitive and -resistant breast cancer tissues

We found that miRNA-140-3p expression was significantly downregulated in breast cancer tissues, while the PD-L1 mRNA level was upregulated, compared to adjacent tissues (P<0.01, Fig. 4A,B). Further analysis indicated that miRNA-140-3p expression was negatively correlated with PD-L1 mRNA level in breast cancer tissue (r=-0.495, P<0.001, Fig. 4C). Similarly, miRNA-140-3p (Fig. 4D) was downregulated, while the PD-L1 (Fig. 4E) mRNA level was upregulated in DOX-resistance tissues compared to in DOX-sensitive tissues. IHC results showed that compared with breast cancer tissues with high miRNA-140-3p expression, PD-L1 protein expression levels in breast cancer tissues with low miRNA-140-3p were

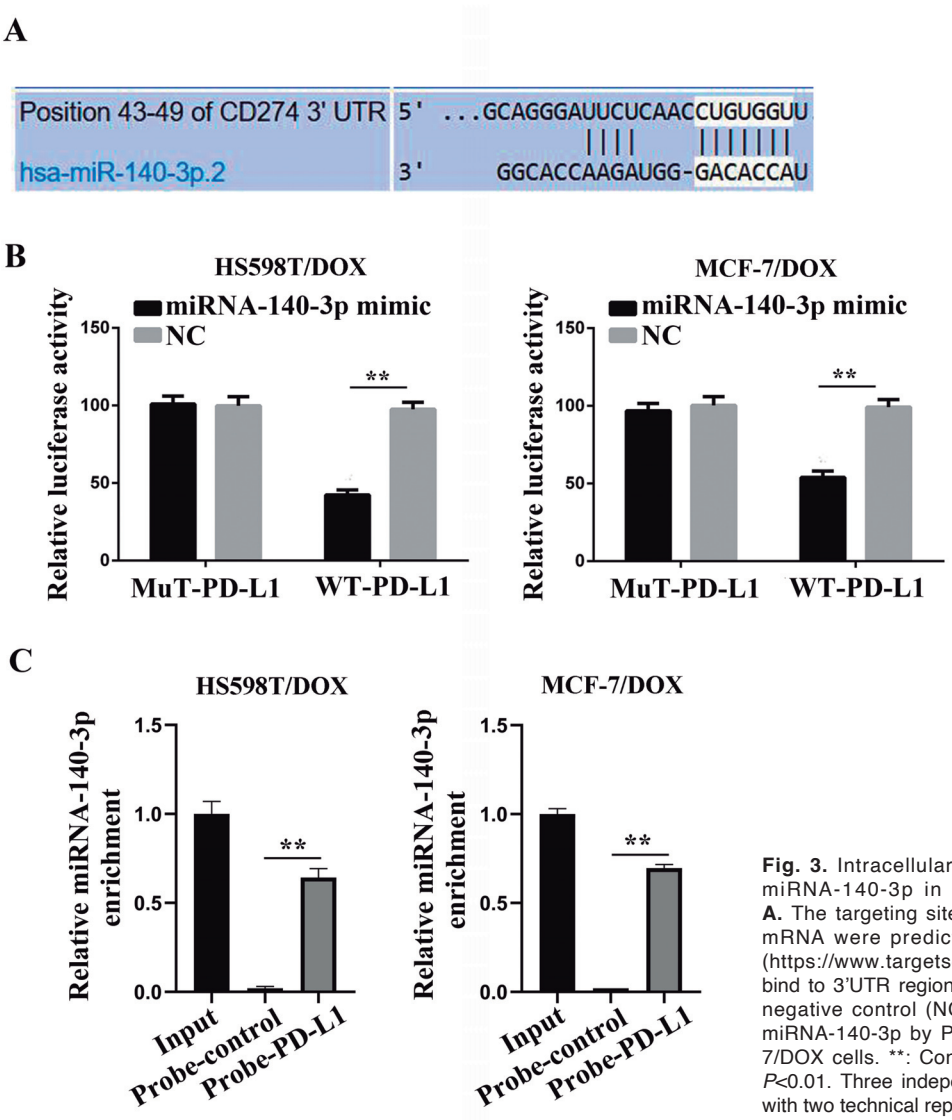

Fig. 3. Intracellular PD-L1 can be negatively regulated by miRNA-140-3p in HS598T/DOX and MCF-7/DOX cells. **A.** The targeting sites of miRNA-140-3p and CD274 (PD-L1) mRNA were predicted by the online TargetScan software (https://www.targetscan.org/vert_72/). **B.** MiRNA-140-3p can bind to 3'UTR regions of PD-L1 mRNA. **: Compared with the negative control (NC) group, and P<0.01. **C.** Enrichment of miRNA-140-3p by PD-L1 probes in HS598T/DOX and MCF-7/DOX cells. **: Compared with the probe-control group, and P<0.01. Three independent experiments were performed, each with two technical replicates.

significantly higher (P<0.01, Fig. 4F). Moreover, compared with DOX-sensitive tissues, the levels of PD-L1 protein expression in DOX-resistant tissues were significantly higher (P<0.01, Fig. 4G).

MiRNA-140-3p inhibits DOX-induced chemoresistance by down-regulating the levels of PD-L1 in HS598T/DOX and MCF-7/DOX cell-derived exosomes

Then, the effect of breast cancer cell-derived exosomes on breast cancer cell chemoresistance was investigated. By co-culturing the breast cancer cell-derived exosomes with DOX-sensitive breast cancer cells, the PD-L1 mRNA levels in HS598T and MCF-7 cells were significantly upregulated (Fig. 5A), suggesting that exosomal PD-L1 invaded the cytoplasm of the above breast cancer cells (Fig. 5B). Besides, the mRNA expression levels of the stemness molecular markers (OCT4, Nanog, SOX2, and ALDH1) in HS598T/DOX and MCF-7/DOX cells were all increased by exosomes or miRNA-140-3p inhibitor treatment, compared to the control and NC groups, but anti-PD-L1

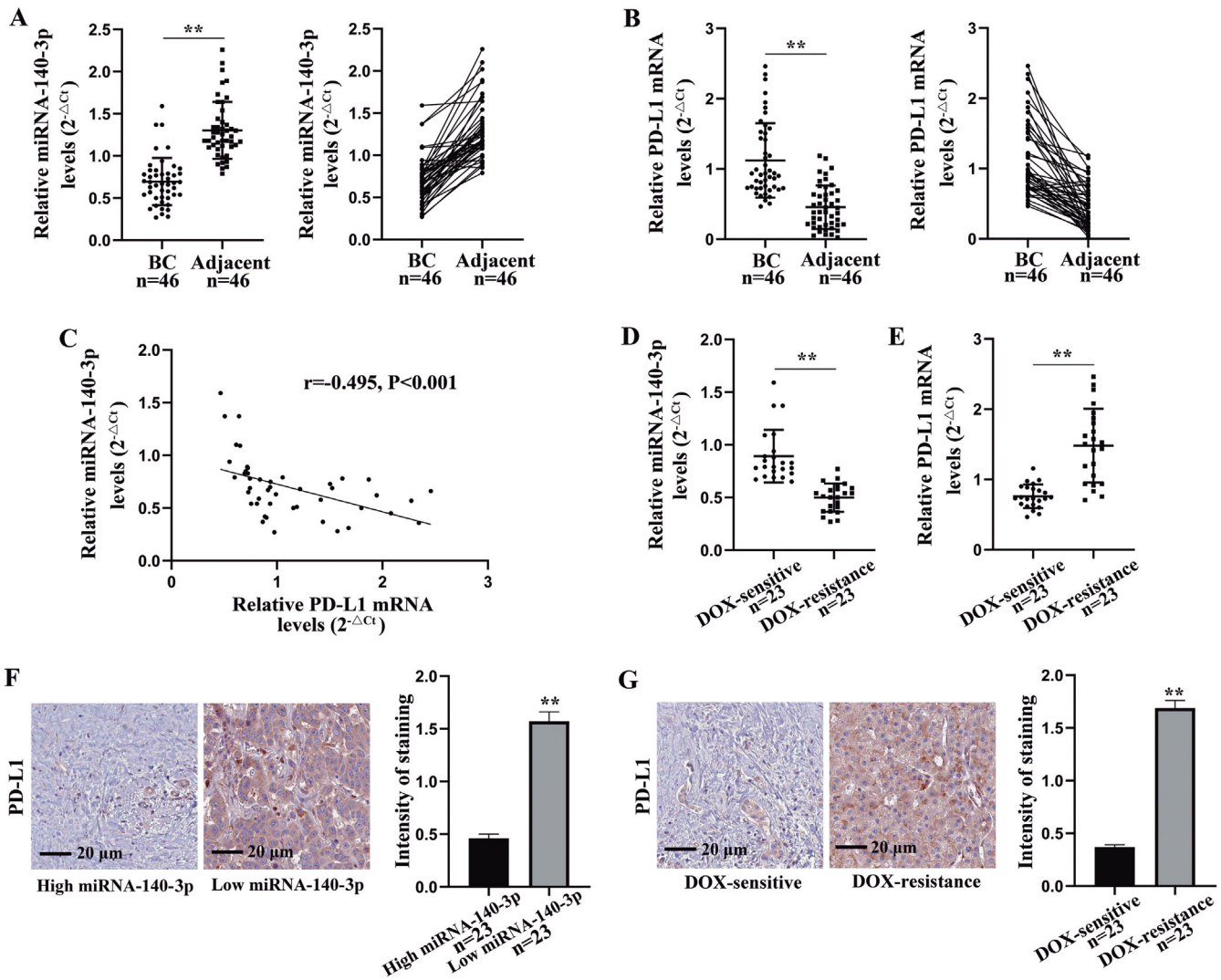

Fig. 4. The expression of miRNA-140-3p and PD-L1 in DOX-sensitive and -resistant breast cancer tissues. **A.** Compared to adjacent tissues, miRNA-140-3p expression was significantly downregulated in breast cancer tissues. **: Compared with adjacent tissues, and P<0.01. **B.** Compared to adjacent tissues, PD-L1 expression was significantly upregulated in breast cancer tissues. **: Compared with adjacent tissues, and P<0.01. **C.** MiRNA-140-3p expression was negatively correlated with the PD-L1 mRNA level in breast cancer tissue. **D, E.** Compared to DOX-chemosensitive tissues, miRNA-140-3p was downregulated, while the PD-L1 mRNA level was upregulated in DOX-chemoresistant tissues. **: Compared with DOX-chemosensitive tissue group, and P<0.01. **F.** Compared with breast cancer tissues with high miRNA-140-3p expression, PD-L1 protein expression levels with low miRNA-140-3p were significantly higher. **: Compared with the high miRNA-140-3p group, and P<0.01. **G.** Compared with DOX-chemosensitive tissues, the levels of PD-L1 protein expression in DOX-chemoresistant tissues were significantly higher. **: Compared with DOX-chemosensitive group, and P<0.01. Three independent experiments were performed, each with two technical replicates.

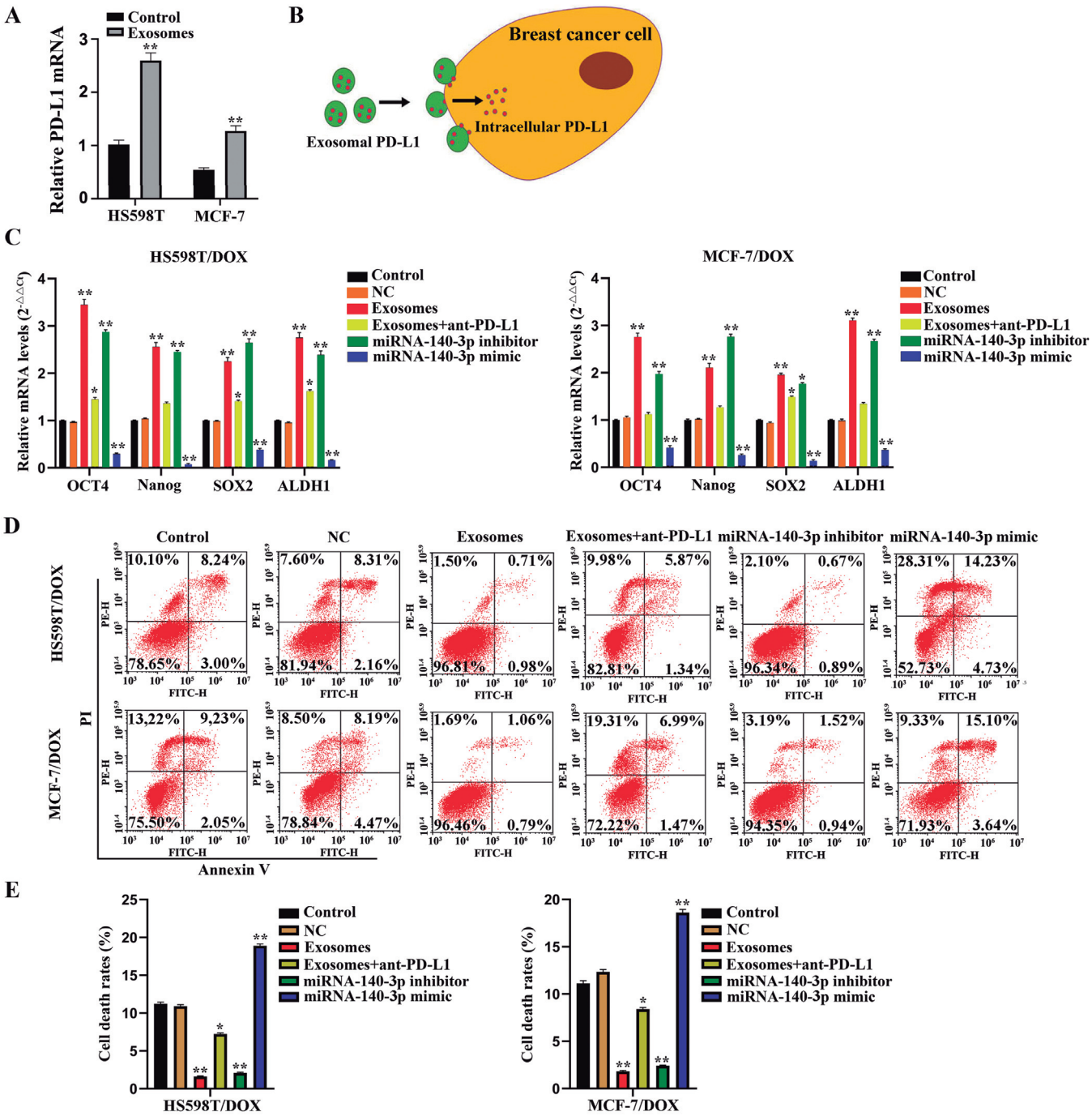

Fig. 5. MiRNA-140-3p inhibits DOX-induced stemness by down-regulating the levels of PD-L1 in HS598T/DOX and MCF-7/DOX cell-derived exosomes. **A.** The HS598T/DOX and MCF-7/DOX cell-derived exosomes were purified and incubated with HS598T and MCF-7 cells. qRT-PCR was used to detect the mRNA expressions of PD-L1 in HS598T and MCF-7 cells. **: Compared with the control group, and $P<0.01$. **B.** A schematic diagram of the transfer of exosomes PD-L1 from exosomes to breast cancer cells. **C.** The stemness molecular markers (OCT4, Nanog, SOX2, and ALDH1) were examined in HS598T/DOX and MCF-7/DOX cells by using qRT-PCR. *: Compared with the control group and the NC group, and $P<0.05$. **: Compared with the control group and the NC group, and $P<0.01$. **D, E.** The HS598T/DOX and MCF-7/DOX cells pretreated with exosomes or miRNA-140-3p inhibitor possessed a lower apoptosis rate, which was abrogated by co-treating cells with anti-PD-L1 antibody or miRNA-140-3p mimic. *: Compared with the control group and the NC group, and $P<0.05$. **: Compared with the control group and the NC group, and $P<0.01$. Three independent experiments were performed, each with two technical replicates.

antibody or miRNA-140-3p mimic treatment abrogated the effects of HS598T/DOX and MCF-7/DOX cell-derived exosomes on cell stemness molecular markers (Fig. 5C). In addition, HS598T/DOX and MCF-7/DOX cells pretreated with exosomes or miRNA-140-3p inhibitor possessed lower apoptosis rates compared with control and NC groups, which was abrogated by co-treating cells with anti-PD-L1 antibody or miRNA-140-3p mimic (Fig. 5D,E). Notably, after exosomes were treated with TRiton and proteinase K, the inhibitory effect of exosomes on apoptosis was attenuated. When exosomes were treated only with proteinase K, the inhibitory effect of exosomes on apoptosis was significantly higher than that of combined treatment with TRiton and proteinase K, which confirmed the presence of PDL1 in exosomes (Fig. 6).

MiRNA-140-3p enhances the efficacy of DOX in HS598T/DOX and MCF-7/DOX breast cancer xenograft nude mouse models

In vivo experiments further confirmed the results of cell experiments. The anti-tumor effects of DOX on miRNA-140-3p up-regulated xenograft mice were significantly better than the control group, indicating that nude mice with high miRNA-140-3p expression are more sensitive to DOX (Fig. 7A). IHC (Fig. 7B) and Western blot (Fig. 7C) results showed that the expression of PD-L1 protein in tumors of mice overexpressing miRNA-140-3p was significantly reduced. Furthermore, The anti-tumor effect of DOX on miRNA-140-3p KO xenograft mice was significantly poorer than the control group, indicating that nude mice with low miRNA-140-3p expression are less sensitive to DOX (Fig. 8).

Discussion

It is worth noting that for patients with advanced breast cancer who have recurrence and metastasis that are not suitable for local treatment, DOX-based systemic treatment is often used (Zheng et al., 2021). DOX resistance, whether it is inherent resistance or acquired resistance, will seriously affect the effectiveness of treatment. In response to this situation, we have launched a study on how to improve the sensitivity of DOX in breast cancer.

In this study, we screened some miRNAs related to DOX resistance in breast cancer by using miRNA microarray, ISH, online bioinformatics software, and qRT-PCR analysis. Among these predicted candidates, we chose miRNA-140-3p because it was found to have a tumor suppressor effect earlier (Jiang et al., 2021). Furthermore, based on our PPI network analysis, GEO tissue microarray data, qRT-PCR, and western blot

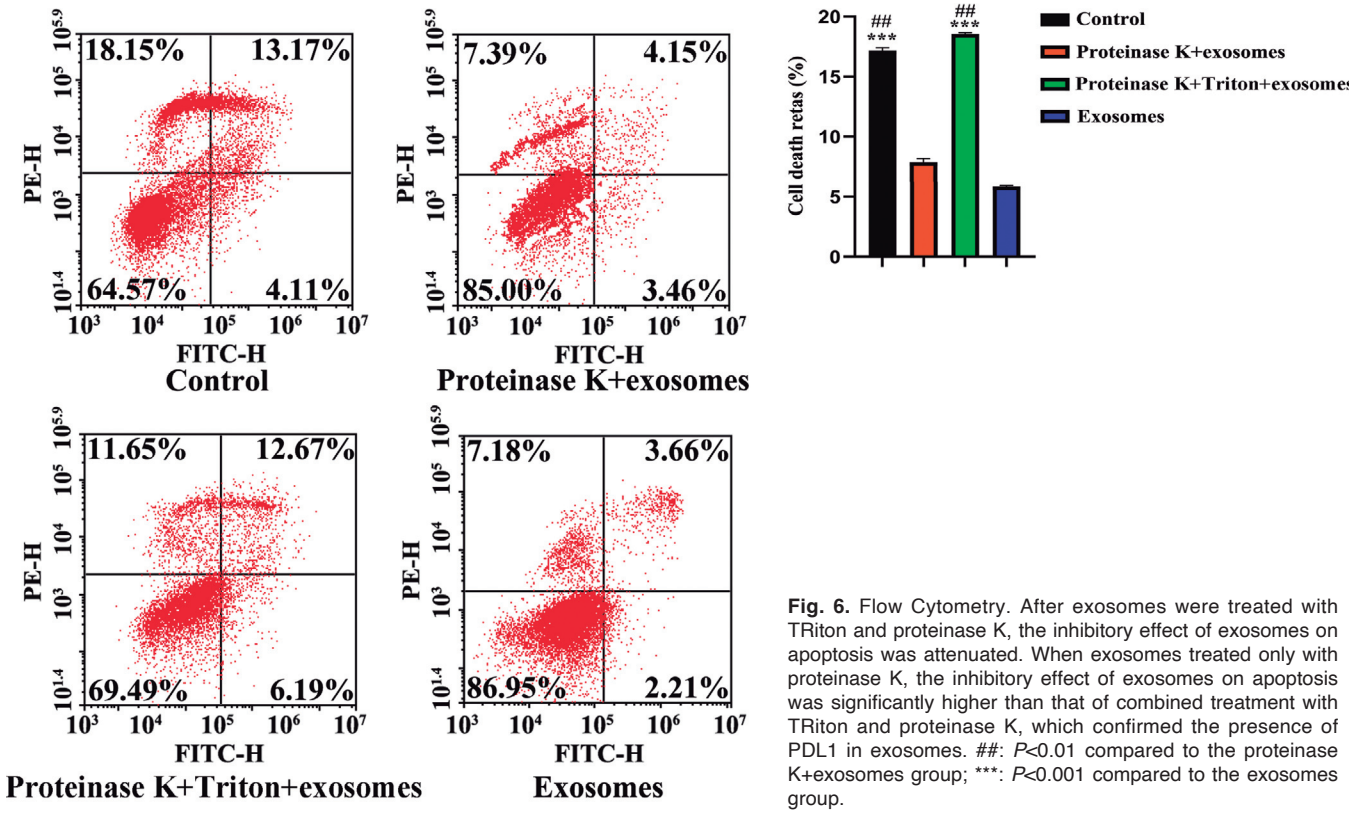

Fig. 6. Flow Cytometry. After exosomes were treated with TRiton and proteinase K, the inhibitory effect of exosomes on apoptosis was attenuated. When exosomes treated only with proteinase K, the inhibitory effect of exosomes on apoptosis was significantly higher than that of combined treatment with TRiton and proteinase K, which confirmed the presence of PDL1 in exosomes. ##: $P<0.01$ compared to the proteinase K+exosomes group; ***: $P<0.001$ compared to the exosomes group.

analyses, the PD-L1 protein was finally determined. Luciferase reporter assay and molecular analysis indicated that PD-L1 was a direct target of miRNA-140-3p for influencing the malignancy of breast cancer cells. Clinically, we found that compared to adjacent tissues, miRNA-140-3p expression in breast cancer tissue was significantly downregulated, and was negatively correlated with PD-L1 mRNA level. Interestingly, compared to DOX-sensitive tissues, miRNA-140-3p was downregulated, while the PD-L1 mRNA level was upregulated in DOX-resistant tissues. Similarly, compared with breast cancer tissues with high miRNA-140-3p expression, PD-L1 protein expression levels in breast cancer tissues with low miRNA-140-3p were significantly higher. Interestingly, several studies demonstrate that miRNA-140-3p may serve as a tumor suppressor (Flamini et al., 2017; Jiang et al., 2021). The above results disclosed that miRNA-140-3p is involved in regulating the sensitivity of breast cancer cells to DOX from a clinical perspective. Next, we also uncovered the regulating mechanisms of miRNA-140-3p and PD-L1 in two breast cancer cell lines. The gain-and-loss of function experiments validated that the introduction of miRNA-140-3p attenuated DOX-induced breast cancer cell chemoresistance, and promoted cell apoptosis by regulating PD-L1 *in vitro*. Besides, *in vivo* experiments further confirmed the results of cell experiments. The anti-tumor effects of DOX on miRNA-140-3p up-regulated xenograft mice were significantly better than the control group, indicating that nude mice with high miRNA-140-3p expression are more sensitive to DOX. The above results were in accordance with the previous data (Jiang et al., 2021), and indicated that overexpression of miRNA-140-3p inhibited breast cancer progression by inhibiting PD-L1. Furthermore, PD-L1 is expressed in macrophages and plays a major role in inhibiting immune surveillance. Li et al. (2016) dissected the mechanisms by which cancer cells initiate T-cell immunosuppression by inducing PD-L1 stabilization. They also demonstrated a novel

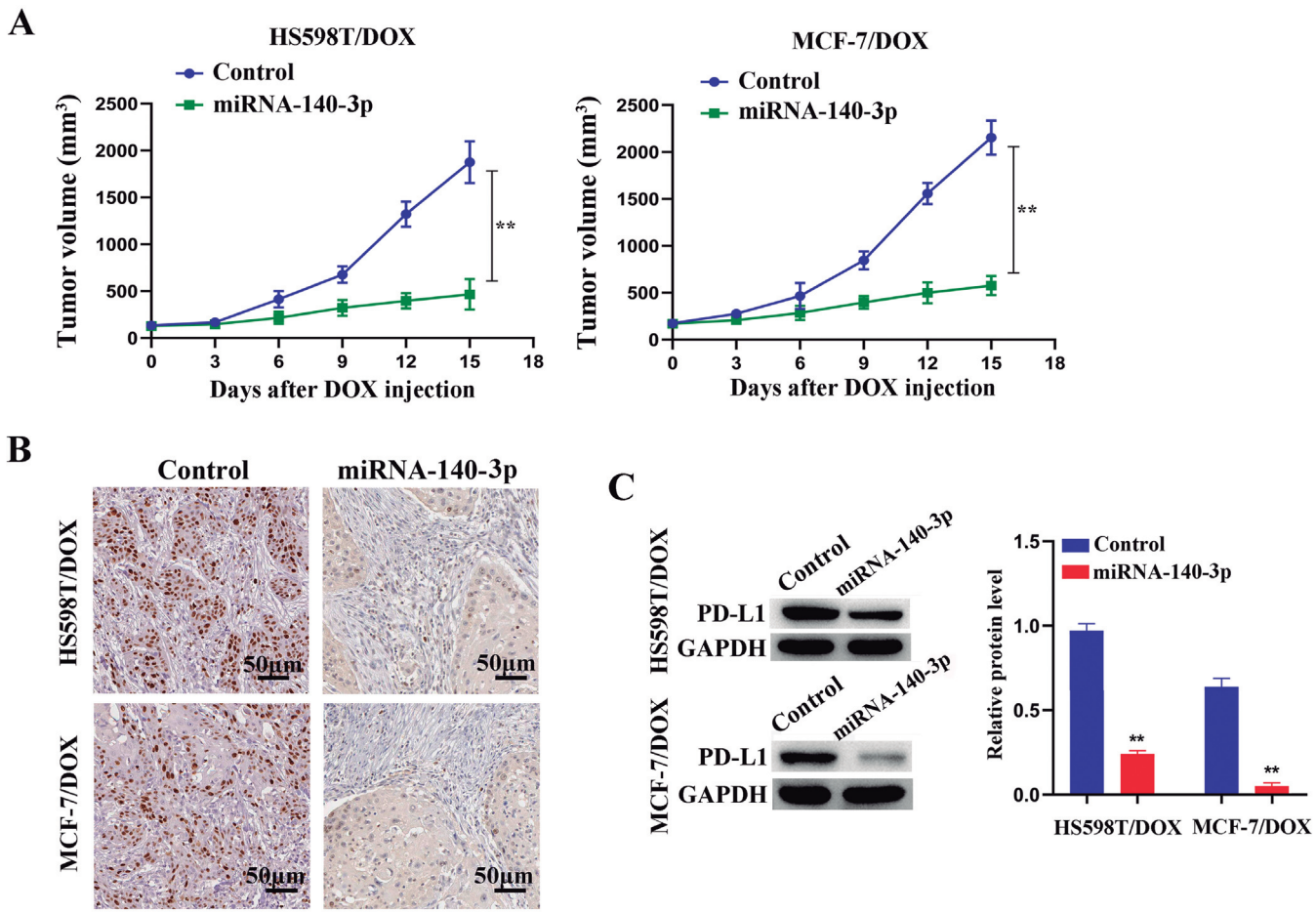

Fig. 7. MiRNA-140-3p enhances the efficacy of DOX in HS598T/DOX and MCF-7/DOX breast cancer xenograft nude mouse models. **A.** Tumor-bearing mice with high miRNA-140-3p expression are more sensitive to DOX. **B, C.** The expression of PD-L1 protein in tumor-bearing mice with overexpressing miRNA-140-3p was significantly reduced. Three independent experiments were performed, each with two technical replicates. **: Compared with the control group, $P<0.01$.

interchange between glycosylation and phosphorylation regulating ubiquitination and degradation of PD-L1 (Li et al., 2016). This regulatory event is critical for basal-like breast cancer cells that escape immune surveillance via PD-L1/PD-1 interaction. However, for *in vivo* experiments, we did not explore the effect of applying exosomes purified from miRNA-140-3p knockout breast cancer cells to treat tumors in mice. This is a shortcoming of this study, which needs to be further explored in the future.

Currently, the resistance of breast cancer cells to chemotherapeutic drugs seriously limits their therapeutic efficacy in the clinic (Amawi et al., 2019; Mehraj et al., 2021), and the discovery of novel therapeutic agents to improve the sensitivity of chemoresistant breast cancer cells is urgent. Previous data showed that PD-L1-containing exosomes from cancer cells greatly contributed to drug resistance by inducing cancer stem cells (Li et al., 2019; Poggio et al., 2019; Vautrot et al., 2021). In terms of mechanism, chemotherapy-resistant cancer cells survive and proliferate under long-term stimulation of chemotherapy drugs (Vautrot et al., 2021). Then, the survivingal cancer cells are resistant to chemotherapy. Interestingly, the above results were also validated in breast cancer cells, and we found that breast cancer cell-derived exosomes increased resistance of breast cancer cells to DOX treatment, which were all abrogated by treating with anti-PD-L1 antibody, indicating that breast cancer cell-derived PD-L1 exosomes self-regulated cell stemness to increase the resistance of breast cancer cells to DOX, which were in accordance with the previous studies (Yang et al., 2018; Li et al., 2019; Poggio et al., 2019; Dou et al., 2020).

To sum up, miRNA-140-3p was extremely lowly expressed in breast cancer, especially in DOX-resistant breast cancer patients. MiRNA-140-3p ultimately reduces the carcinogenicity of breast cancer cells by down-regulating the expression of intracellular and extracellular PD-L1. Therefore, the miRNA-140-3p/PD-L1 axis may be a valuable therapeutic target for the prevention of breast cancer in the future.

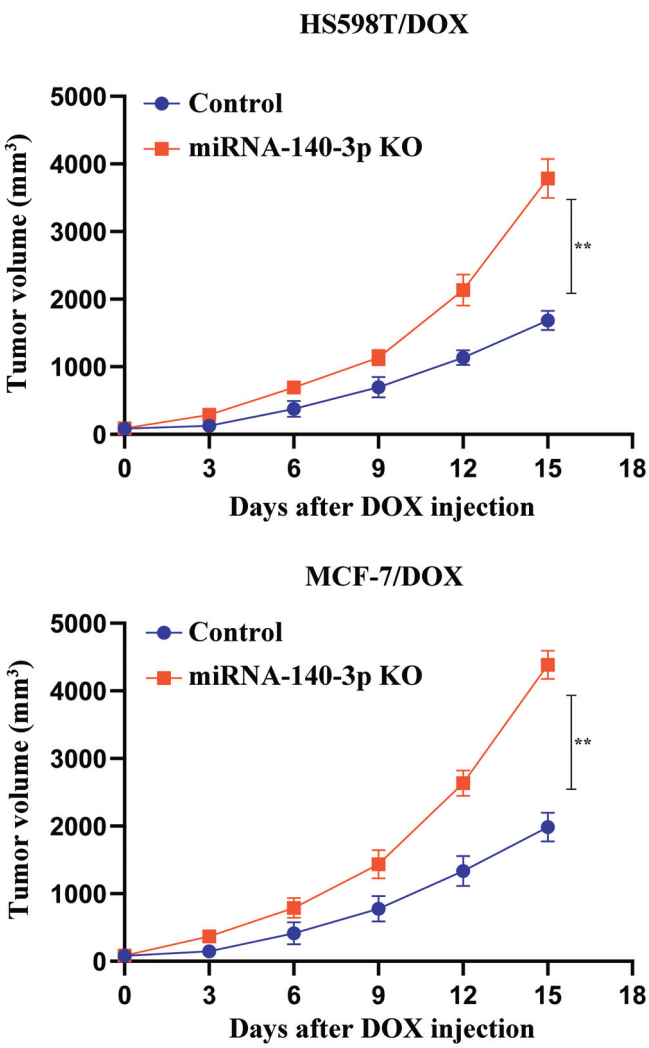

Fig. 8. MiRNA-140-3p KO inhibits the efficacy of DOX in HS598T/DOX and MCF-7/DOX breast cancer xenograft nude mouse models. ** Compared with the control group, $P<0.01$.

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Conflict of interest statement. The authors declare that they have no competing interests.

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