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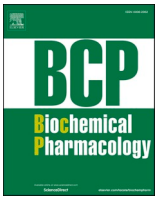

Activation of the γ-secretase/NICD-PXR/Notch pathway induces Taxol resistance in triple-negative breast cancer

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ABSTRACT

Triple-negative breast cancer (TNBC) is currently the only subtype lacking efficient targeted therapies. Taxol is the primary chemotherapeutic agent for TNBC. However, Taxol resistance often develops in the treatment of TNBC patients, which importantly contributes to high mortality and poor prognosis in TNBC patients. Recent preclinical studies have shown that the inhibition of Notch pathway by γ-secretase inhibitors can slow down the progression of TNBC. Our studies in bioinformatic analysis of breast cancer patients and TNBC/Taxol cells *in vitro* showed that there was high correlation between the activation of Notch pathway and Taxol resistance in TNBC. Increased γ-secretase activity (by the overexpression of catalytic core PSEN-1) significantly reduced Taxol sensitivity of TNBC cells, and enhanced biological characteristics of malignancy *in vitro*, and tumour growth *in vivo*. Mechanistically, increased γ-secretase activity led to the accumulation of NICD in the nucleus, promoting the interaction between NICD and PXR to activate PXR, which triggered the transcription of PXR downstream associated drug resistance genes. Furthermore, we showed that pharmacological inhibition of γ-secretase with γ-secretase inhibitors (Nirogacestat and DAPT) can reverse Taxol resistance *in vivo* and *in vitro*. Our results for the first time demonstrate that the activation of γ −secretase/NCD-PXR/Notch pathway is one of important mechanisms to cause Taxol resistance in TNBC, and the blockades of this pathway may represent a new therapeutic strategy for overcoming Taxol resistance in TNBC.

1. Introduction

Breast cancer is one of the cancers with the highest incidence rate and is showing a trend of youth [1]. The rate of triple-negative breast cancer (TNBC) accounts for approximately a quarter of all breast cancer patients [2]. Due to its high heterogeneity and lack of effective therapeutic targets, TNBC has a higher risk of distal metastasis and recurrence [3]. Taxanes, represented by Taxol, are the most commonly agents used for chemotherapy in the clinic. However, due to the development of drug resistance, taxanes are unable to control the progression of the disease in many TNBC cases.

Currently, lots of mechanisms related to TNBC resistance have been proposed, including: 1) The activation of ABC transporter proteins promotes the efflux of chemotherapy agents from tumour cells [4]; 2) Cancer stem cells (CSCs) undergo self-renewal which may increase the ability to rebuild tumours after Taxol treatment, leading to

Abbreviations: TNBC, Triple-negative breast cancer; BC, Breast cancer; NICD, Notch intracellular domain; PXR, Pregnane X receptor; DAPT, N-[2-(3,5-Difluorophenyl) acetyl]-L-alanyl-L-(2-phenyl) glycine *tert*-butyl ester; PSEN-1, Presenilin-1; BCRP, Breast cancer resistance protein; MDR-1, Multidrug-resistant protein 1; CYP3A4, Cytochrome P450 3A4 enzyme; EMT, Epithelial-mesenchymal transition; CSCs, Cancer stem cells; Nirogacestat, Niro; OE, Overexpression; HES-1, Hes family bHLH transcription factor 1.

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chemoresistance in TNBC [5,6]; 3) Mutations in DNA repair enzymes alter the sensitivity of tumour cells to chemotherapy drugs [7]; 4) The changes in apoptosis-related genes may prevent chemotherapy-induced apoptosis of tumour cells [8]; 5) TGF-β activation promotes EMT and acquires CSCs characteristics, thereby inducing chemoresistance in TNBC [9]; 6) The activation of NF-κB pathway promotes tumour drug resistance [10]; 7) Hyperactivation of the PI3K/Akt/mTOR pathway due to PTEN deficiency induces chemoresistance in TNBC [11]. However, therapeutic strategies based on these mechanisms have not achieved satisfactory efficacy. Therefore, there is an urgent need to elucidate the molecular mechanisms of Taxol resistance and to discover new targets of Taxol resistance and therapeutic agents.

The Notch pathway is associated with tumor and Alzheimer's disease (AD) [12].The evolutionarily highly conserved Notch signalling pathway consists of four Notch-1/-2/-3/-4 receptors and five canonical ligands Jagged-1/-2 and Delta like-1/-3/-4. The activation of Notch signalling requires a three-step enzymatic process [13]. Notch receptors are cleaved by Furin protease in the Golgi to form mature Notch receptors; Mature Notch receptors bind to the ligands and are cleaved into extracellular and intracellular fragments by matrix metalloproteinases on the cell surface; the intracellular segments adhered to the cell membrane are sheared by activated γ-secretase to release the Notch intracellular domain (NICD); Subsequently, the activated NICD undergoes nuclear translocation and activates nuclear CSL transcription factor, which in turn activates the expression of related genes, such as HES and PXR [14]. γ-secretase is a transmembrane protein complex localized in the cytoplasmic membrane and consists of four subunits: anterior pharynx-defective (APH-1, supporting the proteolytic activity of the γ-secretase), nicastrin (NCSTN, maintains presenilin stability and provides docking sites for γ-secretase substrates), presenilin (homologous proteins 1 and 2, PSEN-1/-2, provides the catalytic subunit), and presenilin enhancer (PEN-2, facilitates internal decomposition of presenilin and substrate catalysis) [15]. Notch pathway is activated by γ-secretase, preclinical studies have shown that blocking Notch pathway by inhibiting the activity of γ-secretase can slow down the progression of TNBC [16]. NICD is a major product of the hydrolysis of Notch receptors by γ-secretase, and its accumulation in the nucleus promotes tumour growth, inhibits apoptosis and DNA damage [17]. In addition, NICD is involved in the regulation of double-strand break repair and genes associated with drug resistance [18].

The pregnane X receptor (PXR) is a nuclear receptor responsible for regulating the expression of a variety of metabolic enzymes closely related to physiological processes such as drug metabolism [19]. We have shown there is an interaction between NICD and PXR; however, the mechanisms by which γ-secretase regulates the intranuclear accumulation of NICD to activate PXR and induce TNBC resistance are unclear. Our data reported here demonstrate that the activation of γ −secretase/Notch pathway may be a new mechanism to induce Taxol resistance in TNBC, and inhibition of this pathway may expand our armamentarium for revising Taxol resistance in TNBC.

2. Materials and methods

2.1. Materials

Nirogacestat [20] (PF-03084014, CAS: 1290543–63-3, Selleckchem, TX, USA); DAPT (CAS: 208255–80-5, Selleckchem, TX, USA); Taxol (Cat. No.: MB1178, Meilunbio, Dalian, China); Sulforhodamine B (SRB, Sigma, USA); Hoechst 33,342 kit and mitochondrial membrane potential (JC-1) kit (Beyotime, Shanghai, China); All-in-One First-Strand cDNA Synthesis Kit (Transgene, Beijing, China).

Anti-Notch1 (Rabbit mAb #3608, CST, MA, USA); anti-NICD (Rabbit mAb #4147, CST, MA, USA); anti-Hes1 (Rabbit mAb #11988, CST, MA, USA); anti-BCRP (ABCG2, Rabbit mAb #42078, CST, MA, USA); anti-PXR (Rabbit mAb #44646, CST, MA, USA); anti-presenilin1(Rabbit mAb #5643, CST, MA, USA); anti-MDR1 (ABCB1, Rabbit mAb #13342, CST, MA, USA); anti-CYP3A4 (Rabbit mAb #13384, CST, MA, USA); anti-β-actin (Proteintech, Rosemont, USA).

The mono-clone antibodies conjunct with HRP (Horseradish Peroxidase) against HA and FLAG tags were purchased from Abcam Corporation, UK. The full-length sequences of NICD with a HA tag, FLAG tag in C-terminal or PXR with a HA tag in C-terminal was cloned into pcDNA3.1 plasmid. All of the vectors were confirmed by DNA-sequencing.

2.2. The construction of TNBC/Taxol resistant cell lines

Human triple negative breast cancer MDA-MB-231 and BT-20 cell lines were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Construct TNBC resistant cell lines using Taxol stepwise induction. MDA-MB-231 and TB-20 cells were cultured in L-15 medium s upplemented with 10 % fetal bovine serum, 100U/mL penicillin streptomycin, and 5 nM Taxol at 37℃ and humidity saturation in a 5 % CO_2 incubator. After one week, when the cell viability was not affected by the drug, the Taxol concentration was gradually increased in the culture medium, in the order of 6 nM, 7 nM, 8 nM, 9 nM, 10 nM, 15 nM, 20 nM, 30 nM, 40 nM, 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, 100 nM, 120 nM, 160 nM, 200 nM, 240 nM, 280 nM, and 320 nM. Each concentration lasts for 1–2 weeks, with a construction period of about 10 months, and MDA-MB-231/Taxol and TB-20/Taxol cells can be obtained. Determine the IC_{50} values of parental cells and drug-resistant cells through MTT assay, calculate the resistance index, and verify whether the drug-resistant cells have been successfully constructed. Drug resistance index = IC_{50} (resistant cells)/IC_{50} (parent cells).

2.3. SRB analysis

The TNBC/Taxol cells were treated with Taxol or Niro for 48 h and fixed with a 50 % trichloroacetic acid (TCA) solution. The cells were stained with 0.4 % sulforhodamine B (SRB) staining solution, and the dye bound to the cellular proteins was dissolved using unbuffered Tris-base (10 nM, pH = 10.5). The dissolved solution was placed on a horizontal shaker for 20 min, and optical density (OD) values at 540 nm were measured by a microplate reader (BMG LABTECH GmbH, Germany) [21]. Experiments were performed in triplicate.

2.4. Colony formation and cell scratching experiments

2.4.1. Colony formation

TNBC parents and TNBC/Taxol-resistant cells were inoculated into a 6-well plate and treated with Taxol (100 nM) for 48 h. Afterward, replace the complete culture medium and continue cultivation for 9 days. The formed cell colonies were stained with a 0.1 % crystal violet solution, and the optical density (OD) value was measured using a microplate reader at 570 nm.

2.4.2. Scratch assay

TNBC parents and TNBC/Taxol-resistant cells were inoculated into a 6-well plate, forming a monolayer. Subsequently, scratches were induced using a 200 μL pipette tip. After 24 h of treatment with Taxol (100 nM), floating cells were washed away with phosphate buffer solution (PBS). The cells were observed, and images were captured using a DM4B microscope (Leica, Germany). These images were imported into Image-Pro Plus software, and the wound area was measured to evaluate the healing effect. Experiments were performed in triplicate.

2.5. Western blotting

Cell samples (lysed with lysis buffer) or tissue samples (lysed with RIPA lysis solution) were processed to obtain total protein. The total protein samples were denatured by boiling at 99 °C for 10 min. The quantification of the total protein was performed using the BCA

(Bicinchoninic acid) method. Subsequently, a series of operations were completed, including SDS-PAGE electrophoresis, protein transduction into a membrane, closure of BSA (Bovine serum albumin) protein sites, antibody incubation, and membrane washing. The PVDF (Polyvinylidene fluoride) bands were detected using a chemiluminescence detector (BIO-RAD) in combination with an ECL detection kit (Wanleibio, Shenyang, China), and statistical analysis was conducted using Image J software.

2.6. Rnai transfection

The cells in the log growth phase were collected, and centrifuged at 800 r/min for 5 min, the supernatant was discarded. The cells were then resuspended and inoculated into a 6-well plate at a density of 1×10^5/cm^2. After 24 h, the cells were transfected with either control siRNA or PSEN-1 siRNA (Hs00997789_m1, ID:105003, Thermo Fisher Scientific Inco.). The transfection was performed using lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) with a final concentration of 20 nM, following the manufacturer's instructions. After 48 h of transfection, cells were subjected to the SRB assay, and the expression of specific proteins was measured by western blot. The efficacy of RNAi transfection was confirmed by reducing mRNA expression of target genes by more than 70 %.

2.7. Measurement of the mitochondrial membrane potential

Cell apoptosis was assessed by monitoring the mitochondrial membrane potential in TNBC/Taxol cells with stable knockdown or overexpression of PSEN-1, as well as in TNBC parental cells. JC-1, a fluorescent probe, was used. Cells were washed with PBS buffer once, stained with JC-1 staining working solution (1 mg/mL), and incubated at 37 °C for 30 min. Subsequently, cells were washed with JC-1 staining buffer and observed by a fluorescence microscope.

2.8. Cell morphology analysis

The apoptosis of TNBC/Taxol cells by DAPT in combination with Taxol was detected by Hoechst 33,342 staining. TNBC/Taxol cells were treated with DAPT (5 μM) and Taxol (100 nM) for 48 h, and then stained with Hoechst 33,342 fluorescent dye. The samples were washed three times with PBS and photographed using a fluorescence microscope.

2.9. Differential analysis of drug resistance genes in TNBC cell lines

Screening for drug resistance genes associated with the MDA-MB-231 cell line was performed using two drug sensitive gene databases of GDSC1 and GDSC2 (Genomics of Drug Sensitivity in Cancer, https://www.cancerrxgene.org/), through which we can analyze the correlation between drug sensitive genes and Notch-1 & 2 receptors. Data were downloaded and processed based on database categories and receptor subtypes. Using OriginPro-2017 data mapping software, the data were integrated and analyzed to generate four scatter plots. Drug resistance genes with significant differences in each dataset were marked in the plots.

2.10. Co-Immunoprecipitation (Co-IP)

MDA-MB-231 cells were transfected with FLAG, FLAG-NICD, and HA-PXR, and then harvested for immunoprecipitation. The protein complex was immunoprecipitated using beads (FLAG-beads) connected with anti-FLAG monoclonal antibodies. The presence of FLAG-NICD or HA-PXR in the protein complex was examined using anti-FLAG or anti-HA monoclonal antibodies.

2.11. Luciferase reporter gene

The PXR binding element 5 (direct repeat element-3, DR-3 element) and 5 sequences (everted repeat element-6, ER-6 element) of 5 polymers were obtained by chemical synthesis. The sequences of the PXR response element and vector were connected by T4 ligase to ensure that the PXR response element sequences were cloned into the Pgl-3Promoter vector. These constructs were named DR-3-Luc and ER-6-Luc reporter genes after sequencing and verification. Then, the constructed reporter genes were transfected into TNBC/Taxol cells to interfere the expression of γ-secretase. Luciferase reporter gene assays were used to determine the effect of inhibitors on PXR transcription factor activity in TNBC/Taxol cells by measuring the activity of the PXR response element reporter genes DR3-Luc and ER6-Luc.

2.12. Microarray and gene expression analysis

The total RNA of MDA-MB-231/Taxol cells after knocking down PSEN-1 was extracted by TRIzol Reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. The RNA was hybridized to an array (Clariom D assay) and the hybridized array was scanned with an Affymetrix Gene-chip Scanner. The raw microarray data of CELL files were normalized by Robust Multichip Average (RMA) assay.

2.13. Quantitative real-time PCR analysis

Total RNA from TNBC/Taxol cells was extracted according to the RNA extraction kit (Transgene, Beijing, China) instructions, and the RNA concentration was measured with a spectrophotometer. The total RNA was transformed to cDNA using an All-in-One First-Strand cDNA Synthesis Kit (Transgene, Beijing, China). The primary sequence for the target genes, including PSEN-1, HSE-1, HCRP, MDR-1, CYP3A4 and β-actin, were present in Table 1. PCR amplification was performed by a real-time quantitative PCR instrument using a two-step method. Predenaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, and annealing at 60 °C for 30 s. Repeat 40 cycles to obtain the lysis curves. Based on the CT values of gene amplification, the relative expression of mRNA of PSEN-1, HES-1, BCRP, MDR-1 and CYP3A4 genes was calculated using the $2^{-\Delta\Delta CT}$ method with β-actin as an internal housekeeper gene.

2.14. Human tumor xenografts in nude mice

The effect of PSEN-1 overexpression (OE) on tumor growth and the *in vivo* effect of γ-secretase inhibitor in combination with Taxol were assessed by xenograft tumor models, respectively. The MDA-MB-231 cell suspension (5×10^6 cells/mL) was subcutaneously transplanted into the middle of shoulder blades of BALB/c Nude mice (weight 15–18 g, 5-week-old mice, female, Vital River Laboratory Animal Technology Co.,

Table 1
Name and sequence of PCR primers.

Gene	Sequence primer	Primer length (bp)
PSEN-1	F: 5′-GCCAAACATGTGATCATGCTTT-3′	22
	R: 5′-ACTGGCTGTTGCTGAGGCTT-3′	20
HSE-1	F: 5′-TGATTTTGGATGCTCTGAAGAAAGATA-3′	27
	R: 5′-GCTGCAGGTTCCGGAGGT-3′	18
BCRP	F: 5′-TTATCCGTGGTGTGTCTGGA-3′	20
	R: 5′-CCTGCTTGGAAGGCTCTATG-3′	20
MDR-1	F: 5′-CAGCATTAACCACCTTTGC-3′	19
	R: 5′-TCGCTTCCCTTCCCATA-3′	17
CYP3A4	F: 5′-ATCCGATATGGAGATCAC-3′	18
	R: 5′-GAAGAAGTCCTTGTCTGC-3′	18
β-actin	F: 5′-TCGTGCGTGACATTAAGG-3′	18
	R: 5′-AAGGAAGGCTGGAAGAGT-3′	18

Ltd., Beijing). After 7–10 days, nude mice that met the inclusion criteria were grouped using the balanced random grouping method. The Nirogacestat (30 mg/Kg, intragastric) and Taxol (5 mg/Kg, intraperitoneal) were co-administered for 14 days. At the end of the study, the mice were euthanized, the tissue samples were harvested and stored at −80∘C and 4 % paraformaldehyde for western blot and histological analysis, respectively. The experimental protocols were conducted in accordance with the ARRIVE guidelines and followed the regulations of the Animal Ethics Committee of Shenyang Medical College, approval number: SYYXY2023101901.

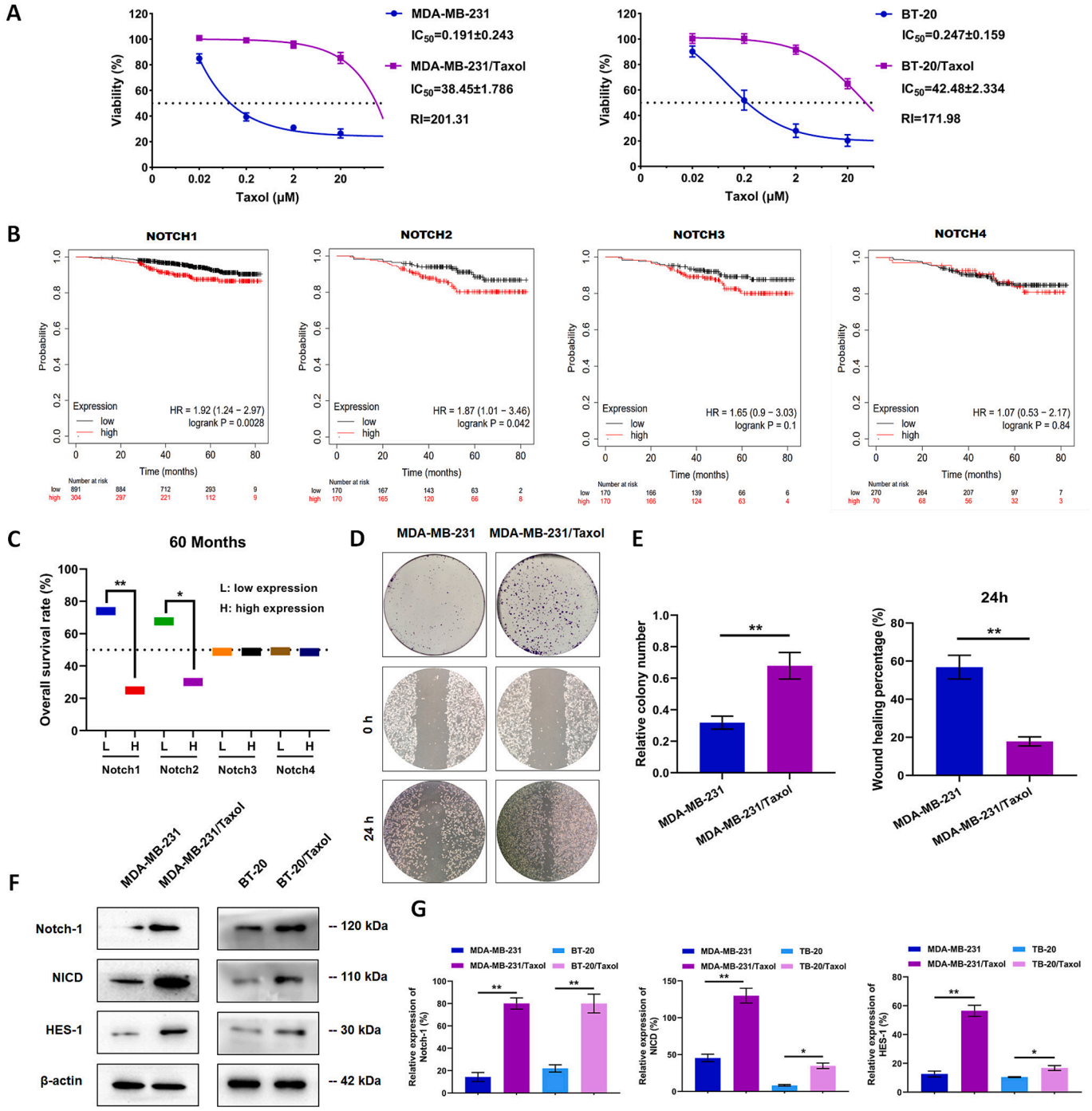

Fig. 1. The dysregulation of the Notch pathway in TNBC/Taxol cells. (A) The effects of Taxol on cell viability in TNBC/Taxol cells; The calculation of IC_{50} and resistance index (RI) of Taxol in TNBC/Taxol cells. **(B&C)** Bioinformation analysis on Notch gene expression in TNBC patients after chemotherapy associated with clinical overall survival from the Kaplan-Meier Plotter database showed that total survival rate in the patients with breast cancer is negatively correlated with the expression of Notch 1&2 receptor but not Notch 3& 4 receptor. **(D&E)** The photographs and histograms of colony formation assay, wound healing assay in MDA-MB-231 and MBA-MB-231/Taxol cells. The photographs were taken at magnifications of × 200. Scale bar = 50 μm. $^{*}p < 0.05$, $^{**}p < 0.01$, *vs.* parental cells. **(F)** The representative images of protein expression of the Notch pathway molecules Notch-1, NICD, and HES-1 in TNBC and TNBC/Taxol cells. **(G)** Quantitation of Notch molecules Notch-1, NICD and HES-1 in TNBC and TNBC/Taxol cells. Data was expressed mean ± SEM, n = 3. $^{**}p < 0.01$, $^{*}p < 0.05$, *vs.* parental cells. All statistical results include data from three parallel experiments.

2.15. Color Doppler ultrasound

At the end of the experiment, the animals were placed in the abdomen-up position and anesthetized continuously using an anesthesia machine (RWD Life Science Co., Ltd) equipped with 3 % isoflurane. Doppler color ultrasound (ZS3 SCI, Myriad Animal Care, Shenzhen, China) was used to detect the morphological changes of subcutaneous tumors and some major organs.

2.16. Statistical analysis

Statistical analyses were performed using SPSS v.26.0 (SPSS). Data in all graphs are represented as mean ± SD of biological triplicates. Statistical significance was determined by Student's *t* test, one-way ANOVA or two-way ANOVA. For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

3. Results

3.1. The activation of Notch signaling pathway is associated with Taxol-resistance in TNBC cells

To investigate Taxol resistance, we determined IC_{50} (50 % of Taxol concentration to inhibit tumour cell proliferation) and resistance index (RI, the calculation formula for RI = IC_{50} of resistant cells / IC_{50} of parental cells) in two TNBCs (MDA-MB-231 and TB-20)/Taxol cells. IC_{50} of two TNBCs/Taxol was significantly higher than correspondent TNBCs (Fig. 1 **A**). RI is an index for the tolerance of tumour cells to chemotherapeutic drugs, with a grading of 1 ~ 5 indicating low resistance; 5 ~ 15 is moderate resistance; and 15 or above is considered high resistance. RI in TNBC/Taxol cell was greater than 15 (Fig. 1 **A**), indicating a successful cell model of drug resistance. We used the Kaplan-Meier Plotter database (https://kmplot.com/analysis/) to analyse the correlation between Notch gene expression and clinical prognosis in breast cancer patients receiving chemotherapy. After chemotherapy, the overall survival rate of tumour patients was negatively correlated with Notch expression. We speculate that the change in overall survival of patients after chemotherapy may reflect a relationship between was Notch receptor and drug resistance. It is worth noting that there were also significant differences in the survival rate of breast cancer patients among Notch receptor subtypes. Specifically, the mRNA expression of Notch-1 and Notch-2 was significantly upregulated in breast cancer patients (all $p < 0.001$)), whereas the mRNA expression of Notch-3 and Notch-4 did not show statistical significance, as depicted in Fig. 1 **B&C**. These results suggest that higher expression of Notch-1/-2 may be an index of poor prognosis and drug resistance in TNBC chemotherapy. Subsequently, we analysed the biological characteristics of TNBC parental and TNBC/Taxol cells. The clone formation and cell scratch assays revealed that the proliferation and migration of TNBC/Taxol cells were significantly increased compared to TNBC cells (Fig. 1 **D&E**). These results suggest that the chemoresistance of TNBC/Taxol cells may be related to the dysregulation of the Notch pathway.

The activation of the Notch pathway caused by the over-cleavage of the Notch receptor by γ-secretase may increase NICD release and accumulation in the nucleus, which binds to the transcription factor CSL to promote transcription of downstream target genes. HES-1 is considered a direct target gene for Notch pathway activation. We analysed the expression of Notch-1 receptor, NICD and the HES-1, TNBC/Taxol cells had higher expression of Notch-1, NICD, and HES-1 compared to TNBC parental cells (Fig. 1 **F&G**). These results further support the notion that the Notch pathway plays the role in TNBC/Taxol drug resistance.

3.2. Genetic manipulation of PSEN-1 gene significantly affects Taxol resistance in TNBC cells in vitro and xenograft tumor growth in BALB/c nude mice ex vivo

PSEN-1 is a corn catalytic component of γ-secretase. To elucidate the role of γ-secretase activation of Notch pathway in Taxol resistance, we designed two shPSNE-1 sequences (#1 and #2) to knock down PSEN-1 gene, both of shPSNE-1 sequences reduced the protein expression of PSEN-1 by over 80 % and improved inhibitory effect of Taxol on cell viability in TNBC/Taxol cells (Fig. 2 **A**). In contrast, the transfection of PSEN-1 significantly increased the protein expression of PSEN-1 by 260 % and attenuated inhibitory effect of Taxol on cell viability in TNBC/Taxol cells (Fig. 2 **B**). These results suggest that the modification of γ −secretase by genetic manipulation of PSEN-1 can affect Taxol resistance in TNBC/Taxol cells.

To further confirm the role of γ-secretase in Taxol resistance, we conducted mitochondrial apoptosis assays with stable knockdown or overexpression of PSEN-1. The ratio of apoptosis in TNBC/Taxol cells after PSEN-1 knockdown significantly increased. In contrast, the apoptotic proportion of TNBC cells after PSEN-1 overexpression was substantially reduced (Fig. 2 **C**). DAPT is a specific inhibitor of γ-secretase. DAPT at low dose is considered low cytotoxic. To investigate whether γ −secretase inhibitor can reverse Taxol resistance, we added low dose (5 μM) of DAPT to Taxol regimen in TNBC/Taxol cell. The combination of DAPT with Taxol significantly increased the sensitivity of TNBC/Taxol cells to Taxol **(**Fig. 2 **D)**. Cytomorphological staining further confirmed that the combination of DAPT with Taxol significantly increased the apoptotic ratio of TNBC/Taxol cells (Fig. 2 **E**).

To investigate effect of γ-secretase on tumour growth and Taxol resistance *in vivo*, MDA-MB-231 cells were transfected with either an empty vector (EV) or PSEN-1 overexpression plasmid (OE). Then, we subcutaneously injected these cells into BALB/c nude mice according to experimental protocols described in Fig. 2 **F**. Overexpression of PSEN-1 attenuated Taxol inhibition of Xenograft tumour growth (Fig. 2 **G**). While the injection of MDA-MB-231 cells with overexpressing PSEN-1 into mice significantly promoted xenograft tumour growth as compared with the injection of empty vector cells (Fig. 2 **H**), the results indicate that an increase in the activity of γ-secretase promotes tumour growth. Furthermore, in MDA-MB-231 cells without overexpression of PSEN-1, the inhibition of tumour growth by Taxol was increased by 67.71 % compared to control group; the overexpression of PSEN-1 reduced the inhibition of tumours by Taxol to 11.50 % compared to the correspondent control group (Fig. 2 **I**). Thus, the overexpression of PSEN-1 in MAD-MB-231 cells significantly attenuated Taxol inhibition of Xenograft tumour compared to empty vector-transfected MDA-MB-231 cells, indicating that γ-secretase plays an important role in Taxol resistance in Xenograft tumor.

3.3. NICD regulates transcriptional activity of drug resistance genes by binding to nuclear receptor PXR

It has been shown that the activation of PSEN-1 can promotes NICD into the nucleus, which increases the formation of the Notch ternary complex with nuclear transcription factors CSL and MAML, resulting in increased expression of target genes. We screened Notch-related resistance genes in TNBC by GDSC database (containing two sub-databases of GDSC1 and GDSC2). There was a total of 18 Notch-related resistance gene upregulated and 21 genes downregulated in GDSC1 database, among the upregulated genes, NAMPT (Nicotinamide phosphoribosyl transferase) ranked top 1 among the up-regulated genes, and PXR ranked third. It has been reported that NAMPT may be involved in regulating stress response and aging related process, while PXR is a gene highly associated with drug resistance. We screened the GDSC2 database for resistance genes associated with Notch1 and the GDSC database for resistance genes associated with Notch2, and no statistical difference in upregulated genes was found in two GDSC databases. These results

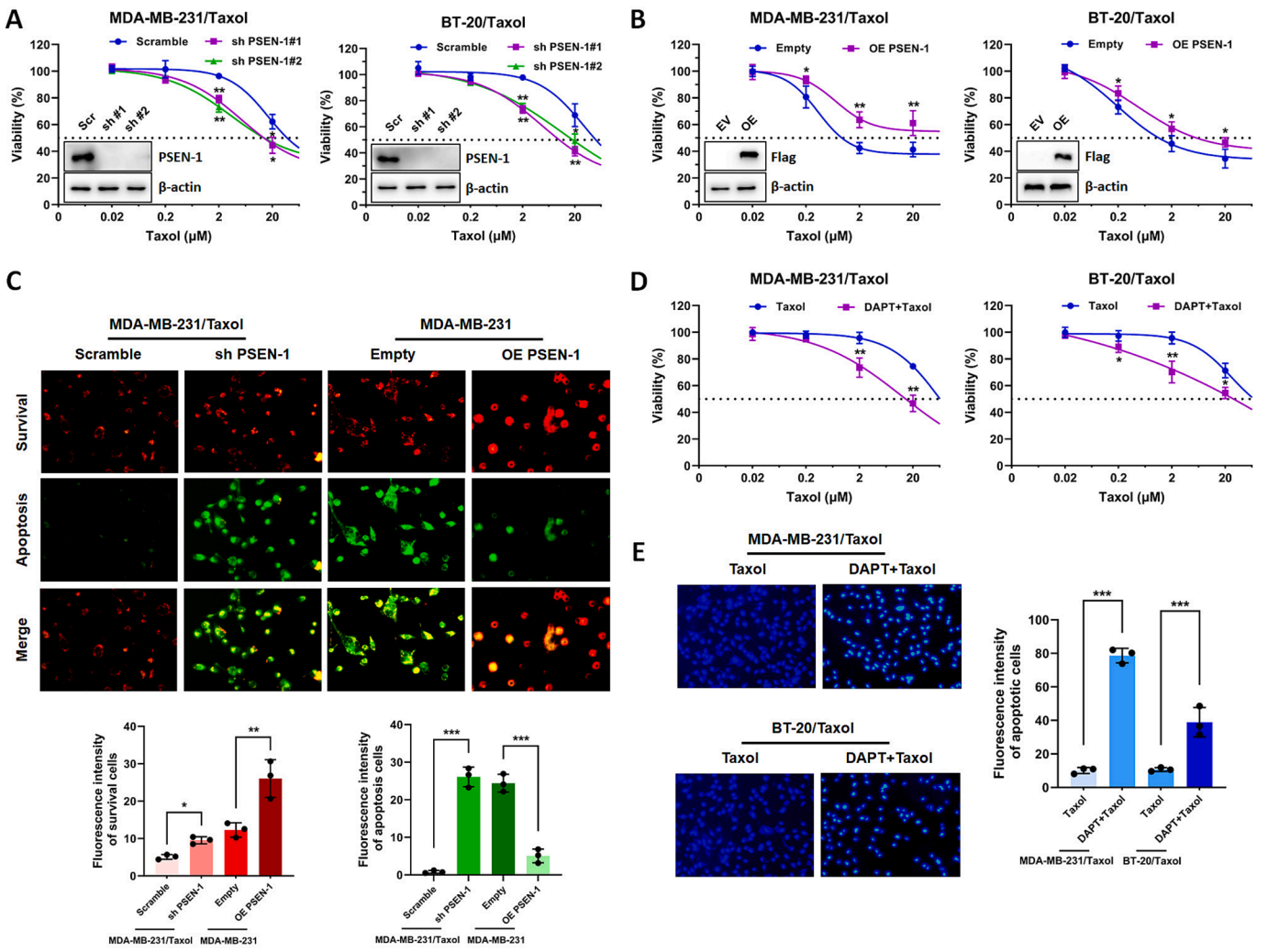

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Fig. 2. The modification of the γ-secretase activity with genetic manipulation of PSEN1 gene expression affected Taxol resistance in TNBC cells and xenograft tumor growth in BALB/c nude mice. (A) Knockdown of PSEN-1 gene significantly reduced PSEN-1 expression and improved Taxol inhibitory effect on cell viability in TNBC/Taxol cells. $^{*}p < 0.05$, $^{**}p < 0.01$, *vs.* scramble group. **(B)** Overexpression of PSEN-1 increased protein expression of PSEN-1 and attenuated Taxol inhibitory effect on cell viability in TNBC/Taxol cells. $^{*}p < 0.05$, $^{**}p < 0.01$, *vs.* scramble group. **(C)** The representative images of the apoptosis ratio in MDA-MB-231/Taxol cells transfected with PSEN-1 shRNA or PSEN-1 overexpression plasmid. Cell apoptosis was assessed by JC-1 assay. Red fluorescence and green fluorescence indicate a normal and decreased mitochondrial membrane potential, respectively. The photographs were taken at the magnifications of × 200. Scale bar = 100 μm. **(D)** The combination of Taxol with DAPT (a specific inhibitor of γ-secretase) increased Taxol inhibition of cell viability in MDA-MB-231/Taxol cells. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* Taxol group. **(E)** The representative images of positive apoptosis fluorescence cells in MDA-MB-231/Taxol cells treated with DAPT. Cell apoptosis was assessed by Hoechst 33,342 fluorescence staining. **(F)** Schematic view of experimental protocols in MDA-MB-231 xenograft nude mice. MDA-MB-231 single-cell suspension was inoculated into the armpit of the right upper limb of BALB/c nude mice. Taxol was administered as indicated by triangles (i.p., intraperitoneal). **(G)** the representative images of Xenograft tumor growth in BALB/c nude mice treated with MDA-MB-231 cells overexpressing PSEN-1. **(H&I)** The quantitation of relative tumor growth in MDA-MB-231 xenograft nude mice treated with Taxol or vehicle. $^{**}p < 0.01$ *vs.* empty group; $^{\#\#}p < 0.01$ *vs.* PSEN-1 overexpression group. All statistical results include data from three parallel experiments.

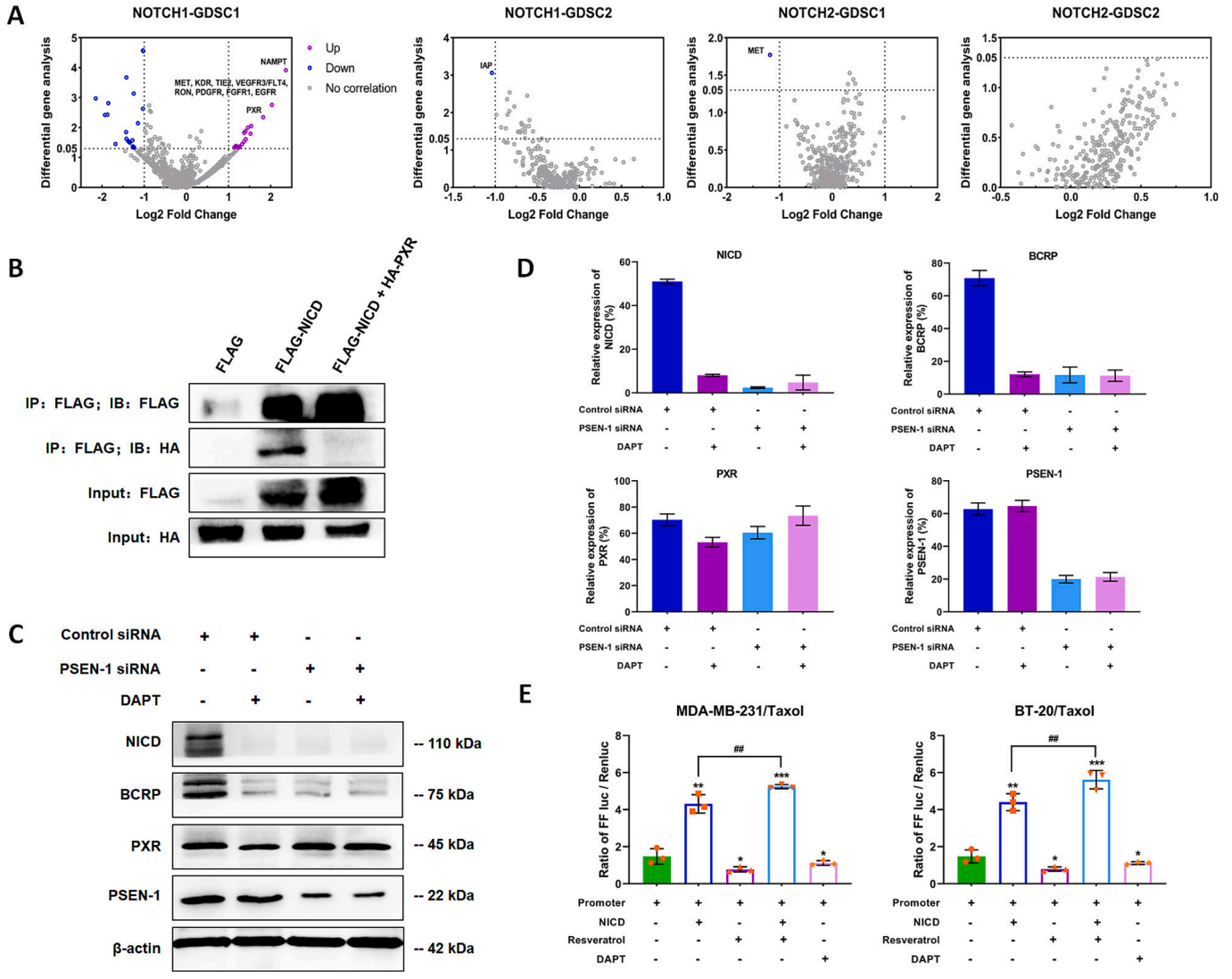


Fig. 3. The NICD is involved in the transcriptional activation of drug resistance gene PXR. (A) Notch signaling pathway related drug resistance genes. The title of each volcano plot represents the name of the Notch gene and the GDSC database. When Log2 Fold change < -1, the larger its absolute value, the stronger the drug sensitivity of the gene corresponding to that point; When Log2 Fold change > 1, the larger its value, the greater the drug resistance of the gene corresponding to that point; Taking the dotted line P-value = 0.05 as the boundary, the right side of the drug-resistant genes were labelled as purple circles, and the screened drug-resistant genes pointed to the corresponding data points. **(B)** MDA-MB-231/Taxol cells were transfected with FLAG, FLAG-NICD and HA-PXR for Co-IP experiments. Detection of FLAG-NICD and HA-PXR in protein complexes with monoclonal antibodies against anti-FLAG or anti-HA. **(C)** MDA-MB-231/Taxol cells were transfected with control siRNA or PSEN-1 siRNA, and cells were collected for Western blotting to detect the protein expression levels of PSEN-1, NICD, PXR and BCRP. **(D)** Quantitative results of protein expression of NICD, BCRP, PXR and PSEN-1 in MDA-MB-231/Taxol cells. Each value is average (±SEM) of triplicate samples. **(E)** PXR SV2 promoter-excited luciferase activity after overexpression of NICD, treatment of TNBC/Taxol cells with PXR inhibitors or γ-secretase inhibitors. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *vs.* promotor group. $^{\#\#}p < 0.01$, *vs.* NICD overexpression group. All statistical results include data from three parallel experiments.

suggest a significant correlation between our PXR and Notch-1 receptor (Fig. 3 **A**).

We used Co IP to investigate the binding and interaction between NICD and PXR in MDA-MB-231/Taxol cells. NICD was labelled with FLAG (denoted as FLAG NICD) and PXR was labelled with HA (HA-PXR). Cells were transfected with FLAG-NICD and HR-PXR vectors, the control group was only transfected with FLAG without protein labelling. The samples were then immunoprecipitated with anti-FLAG antibody, so only proteins bound to NICD can be detected with IB. As shown in Fig. 3 **B**, the image bands of the FLAG-NICD + HA-PXR group using anti-FLAG antibodies for IB were higher than those of the FLAG-NICD group, indicating that some HA-PXR proteins bind to FLAG-NICD in the FLAG-NICD + HA-PXR group, while the image band using anti-HA antibody for IB can be detected in the FLAG-NICD group, indicating that the FLAG-NICD protein bind with some HA-PXR proteins. These results indicate that there is indeed an interaction and binding activity between NICD and PXR in MDA-MB-231/Taxol cells. PXR is a nuclear receptor that regulates the transcription of various metabolism-related genes, including breast cancer resistance protein (BCRP), an important signalling molecule in breast cancer cells. To clarify whether γ-secretase affects the expression of BCRP, we inhibited γ-secretase with knocked down PSEN-1 gene or chemical inhibitor DAPT in MDA-MB-231/Taxol cell, either the knockdown of PSEN1 or DAPT significantly reduced the protein expression of NICD and BCRP, but did not significantly affect the protein expression of PXR (Fig. 3 **C&D**). The results concomitantly with the data in Fig. 4 C strongly suggest that γ-secretase mediates BCRP expression via regulating NICD and PXR interaction.

To identify the role of NICD in PXR activation, we treated TNBC/Taxol cells with Resveratrol, a potent PXR inhibitor, then overexpressed NICD in these cells. NICD overexpression significantly increased PXR SV2 promoter activity, while inhibiting γ-secretase activity with DAPT resulted in a significant decrease in PXR SV2 promoter activity (Fig. 3 **E**).

3.4. γ-secretase mediated Taxol resistance through NICD-PXR/Notch pathway

To elucidate the potential mechanism by which γ-secretase mediates Taxol resistance in TNBC, we conducted gene microarray analysis in MDA-MB-231/Taxol cells transfected with or without PSEN-1 shRNA. The knockdown of PSEN-1 downregulated expression of 331 genes in MDA-MB-231/Taxol cells (Fig. 4 **A**). Compared with MDA-MB-231 cells, MDA-MB-231/Taxol cells upregulated 6900 genes, an enrichment analysis revealed there were 151 overlap genes between up-regulated genes in MDA-MB-231/Taxol cell and down-regulated genes in PSEN-1-shRNA-transfected MDA-MB-231/Taxol cell (Fig. 4 **B**). Pathway analysis demonstrated significant enrichment in the Cytokine pathway, Notch pathway, and JAK-STAT pathway (Fig. 4 **C**), suggesting that loss of function of γ-secretase is associated with Taxol resistance in breast cancer.

Next, we synthesized two sequences of PSEN1 (PSEN1 #1 & #2) to know down the PSEN-1 gene and investigated the protein expression of Notch pathway-related molecules, including PSEN-1, Notch-1, HES-1, MDR-1, CYP3A4 in two types of MDA-MB-231/Taxol cells, all these genes are associated with drug resistance. The knockdown of PSEN-1 with both of PSEN #1 and #2 significantly reduced the protein expression of PSEN1, HER-1, MDR-1 and CYP3A4, but did not significantly affect Notch-1 expression in MDA-MB-231/Taxol cells (Fig. 4 **D&E**). To confirm the results, we further examined the mRNA levels of these drug resistance-related genes, consistent with the findings in the protein expression of these molecules, knockdown of PSEN-1 also markedly reduced the mRNA expression of above mention molecules. These results further confirm the regulatory role of the γ-secretase/Notch pathway on PXR (Fig. 4 **F**).

We further investigated effects of overexpressed NICD and PXR on the efficacy of Taxol in PSEN-1 knockdown MDA-MB-231/Taxol cells. The overexpression of either NICD or PXR increased cell viability in PSEN-1 knockdown TNBC/Taxol cells (Fig. 4 **G&H**), suggesting overexpression of two genes increased Taxol resistance. These findings strongly support γ-secretase-mediated activation of the NICD-PXR/Notch signaling pathway promotes Taxol resistance.

3.5. Pharmacological inhibition of γ-secretase sensitizes TNBC/Taxol cells to Taxol in vitro and in vivo

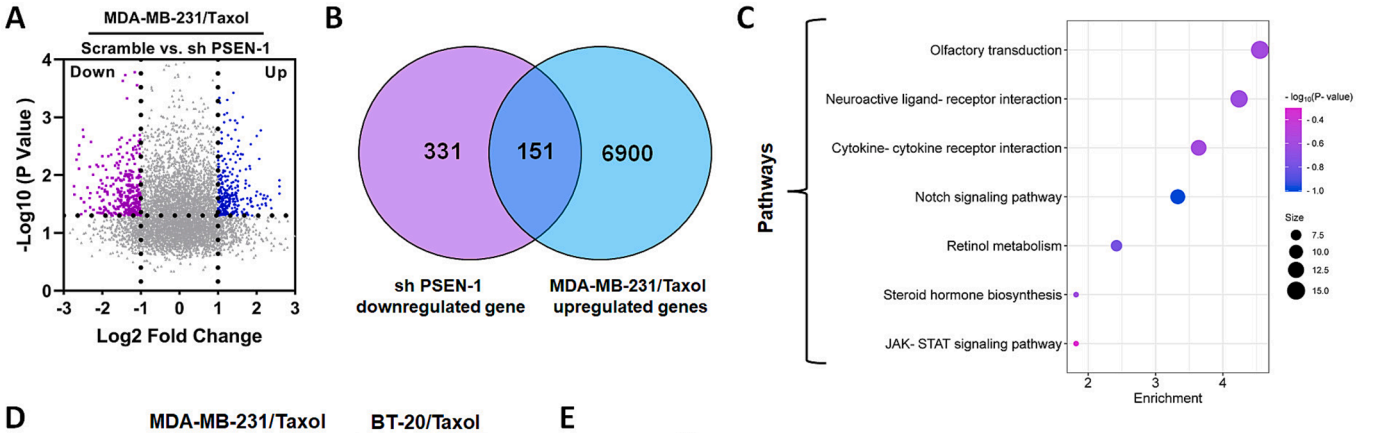
Because we have shown that the activation of γ-secretase reduces the efficacy of Taxol in TNBC/Taxol cells in the above-mentioned studies. Accordingly, we hypothesize that pharmacological inhibition of γ-secretase might serve as a potential therapeutic strategy to overcome TNBC/Taxol resistance. Nirogacestat (Niro, PF-3084014) is a potent and selective γ-secretase inhibitor, its chemical structure is depicted in Fig. 5 **A**. We combined Taxol with Nirogacestat to treat two types of TNBC/Taxol cells, Nirogacestat dose-dependently reduced cell viability in MDA-MB-231/Taxol cells and BT20/Taxol cells (Fig. 5 **B**), while the combination therapy of low dose of Nirogacestat with different dose of Taxol can significantly enhance inhibitory effects of Taxol on cell viability, suggest improvement of Taxol resistance (Fig. 5 **C**).

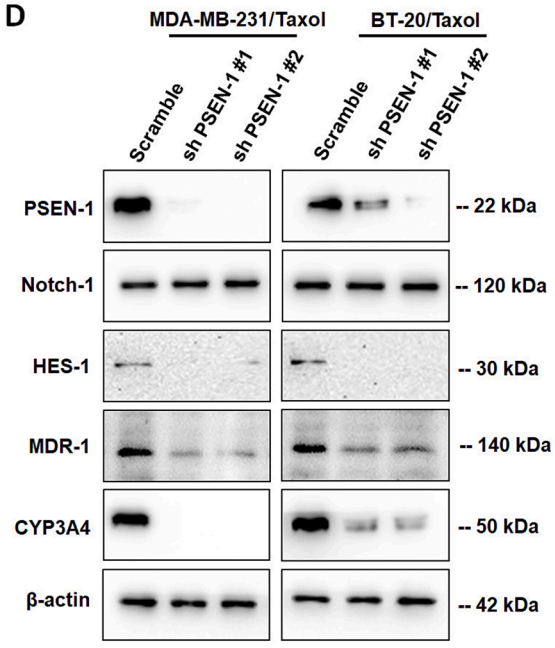
To validate these *in vitro* findings, we employed the MDA-MB-231/Taxol xenograft model to assess the inhibitory effect of targeted γ-secretase *in vivo* (Fig. 5 **D**). The combination therapy of Taxol with Nirogacestat significantly decreased tumour volume (Fig. 5 **E&F**) as demonstrated by Doppler ultrasound, and reduced tumour weight (Fig. 5 **G**), compared with Taxol monotherapy. It must be pointed out that the combination therapy did not increase the liver and kidney morphological injury (Fig. 5 **H**). We further examined the protein expression of PXR downstream molecules MDR-1, CYP3A4 and BCRP, the combination of Taxol and γ-secretase significantly downregulated the expression of these proteins compared with Taxol alone (Fig. 5 **I&J**). These results suggest that addition of γ-secretase inhibitor effectively reverse Taxol resistance but did not bring any additional side effects. Therefore, the inhibition of the NICD-PXR/Notch signalling activation by γ −secretase inhibitor Nirogacestat may be a novel strategy to mitigate or revers Taxol resistance in the MDA-MB-231/Taxol xenograft tumour model.

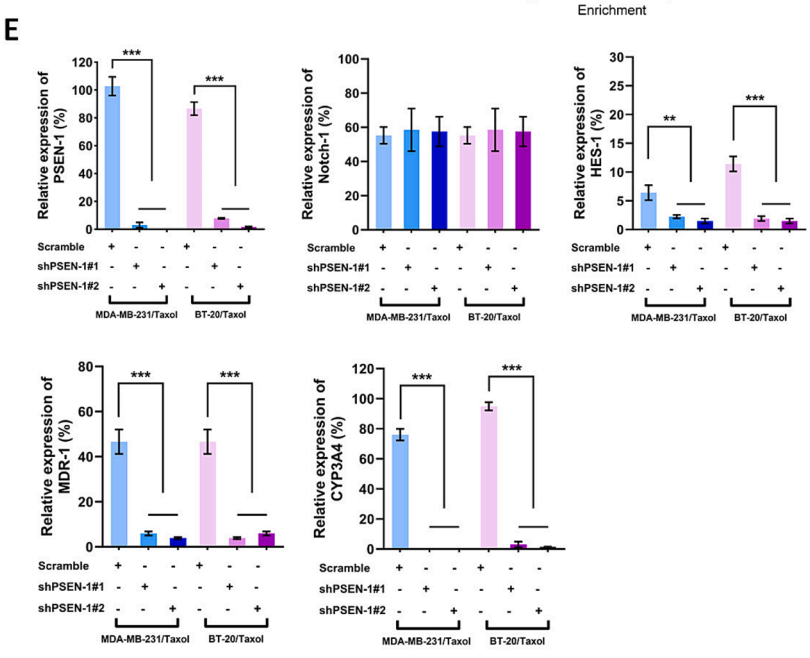
4. Discussion and Conclusion

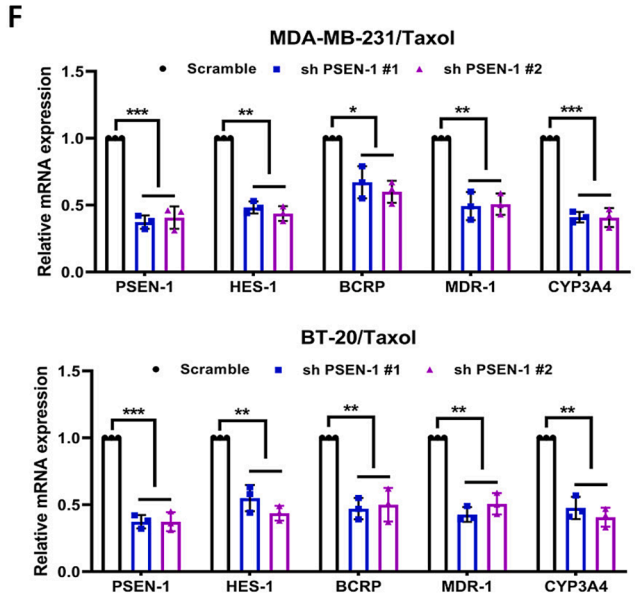
TNBC is characterized with a poor clinical treatment response, strong lesion metastasis, and susceptibility to drug resistance [22]. As the primary agent for the treatment of TNBC, Taxol is often used in combination with Trastuzumab or Pertuzumab due to the heterogeneity of TNBC. However, the neoadjuvant strategy combining chemotherapy and targeted therapy has not achieved the expected clinical goal [23]. In this study, we provide solid evidence showing that dysregulation of γ −secretase/Notch pathway is associated with Taxol resistance in TNBC cell, the modification of γ −secretase activity with either genetic manipulation of PSEN-1 gene or γ-secretase inhibitor affects Taxol resistance, the mechanisms underlying may involve in promoting interaction of NICD-PXR and Notch pathway, which induces the expression of drug resistance-related gene. More importantly, we show that inhibition of γ −secretase/Notch pathway with γ −secretase inhibitor can increase the sensitivity of Taxol to TNBC cells and the therapeutic effect of Taxol.

It has been shown that aberrant activation of Notch signaling pathway caused by γ-secretase is associated with tumor resistance to chemotherapy and radiotherapy. Notch signaling regulates cell proliferation, apoptosis, differentiation, stem cell regeneration and DNA repair. The Notch receptor is activated by hydrolysis, releasing the intracellular domain of Notch (NICD), and the NICD translocated across the membrane into the nucleus, where it binds to the transcription factor CSL to turn on the transcription of target genes, triggering a series of biological behaviors. Hes-1 is located downstream of the Notch pathway and is a direct target gene of Notch. The expression and mRNA









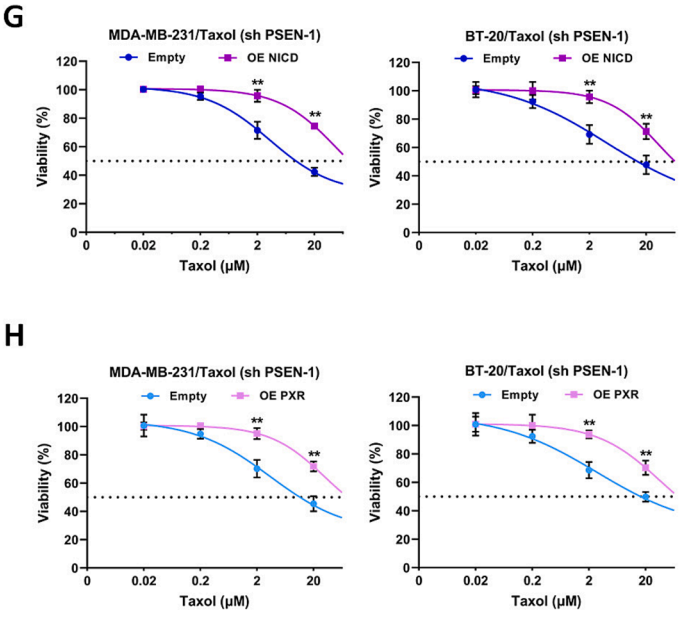


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Fig. 4. Knockdown of PSEN-1 inhibits the NICD-PXR/Notch pathway in TNBC/Taxol cells. (A) Volcano plot of gene microarray data illustrating upregulated and downregulated genes of interest ($-1 >$ fold change > 1, $p < 0.05$) in MDA-MB-231/Taxol cells transfected with PSEN-1 shRNA compared with scramble. **(B)** The Blue circle represents the up-regulated genes of MDA-MB-231/Taxol-resistant cells compared to the parental cells; the purple circle represents the down-regulated genes of MDA-MB-231/Taxol cells shPSEN-1 compared to MDA-MB-231/Taxol cells. Venn diagram showing overlapping genes between upregulated genes and downregulated genes. **(C)** Bubble diagram of Enrichment pathway analysis based on the differentially expressed genes in MDA-MB-231/Taxol cells transfected with PSEN-1 shRNA compared with scramble. **(D&E)** The expression level and statistical graphs of Notch pathway-related proteins in TNBC/Taxol cells transfected with PSEN-1 shRNA or scramble. **(F)** The mRNA expression levels of genes related to the Notch pathway in TNBC/Taxol cells transfected with PSEN-1 shRNA or scramble. **(G)** SRB results showing the efficacy of Taxol in PSEN-1 knockdown TNBC/Taxol cells transfected with NICD overexpression (OE) or empty plasmid for 48 h. **(H)** SRB results showing the efficacy of Taxol in PSEN-1 knockdown TNBC/Taxol cells transfected with PXR overexpression (OE) or empty plasmid for 48 h. $^{*}p < 0.05$, $^{**}p < 0.01$, *vs.* empty group. All statistical results include data from three parallel experiments.

transcription level of Hes-1 directly reflects the activation degree of the Notch pathway. It has been shown that *NOTCH1* and *NOTCH2* are involved in the biological regulation of chemotherapy resistance in gastric cancer [24]. In this study, we analyzed the correlation between Notch receptor expression and overall survival rate in TNBC patient with chemotherapy in database. The expression of Notch 1&2 is negatively correlated with chemotherapy survival rate of TNBC patients. TNBC/Taxol cell exhibits higher cell proliferation and metastasis and an increase in the expression of Notch signaling molecules Notch1, NICD and HES-1. These data support the notion that activation of the Notch signaling pathway is involved in Taxol resistance in TNBC cells.

Activation of the Notch pathway requires three enzymatic cleavages. The C-terminal cleavage product of Notch, in the transmembrane region, is sheared by γ-secretase at the S3 site, and releasing NICD [25], γ-secretase activity is positively correlated with the release and intranuclear accumulation of NICD, which is critical for the recruitment of transcription factors and activation of Notch target genes. γ-secretase is a transmembrane protein complex, PSEN-1 is the catalytic core of γ-secretase, providing the catalytic subunit for γ-secretase to perform its enzymatic function. In this study, increased γ −secretase activity by overexpression of PSEN-1 reduced the sensitivity of TNBC cells to Taxol. In contrast, knocking down PSEN-1 or treatment with γ −secretase inhibitor DAPT enhances the sensitivity of TNBC/Taxol cells to Taxol. Similarly, we showed in *in vivo* experiments that increasing activity of γ −secretase can attenuate Taxol inhibitory effects on Xenograft tumour growth, because the injection of MDA-MB-231 cells with overexpressing PSEN-1 into nude mice significantly promoted xenograft tumour growth. Our results clearly indicate that γ −secretase play an important role in the regulation of TNBC cell resistance to Taxol.

Increasing evidence shows that the γ-secretase/Notch pathway plays an important role in the development of tumor drug resistance. The activation of γ −secretase/Notch pathway can induce tumor drug resistance through two pathways: one is to promote the expression of tumor resistance genes by activating Notch receptor, and the other is to activate Notch related drug resistance signaling pathways, such as CSCs, EMT, TME [25], etc. We screened Notch-related resistance genes in TNBC by GDSC database and found that PXR is the gene with the most significant changes among these resistance genes, is closely related to Notch1. PXR is a nuclear receptor that mediates the expression of drug metabolizing enzymes (such as CYP3A4), thereby accelerating the drug clearance and inducing drug resistance. In breast cancer cell lines, upregulation of PXR triggered the expression of downstream target genes, including multidrug resistance gene (MDR-1) and breast cancer resistance protein (BCRP), which enhanced the tolerance of breast cancer cells to chemotherapeutic agents [26].

NICD is an important hub of Notch pathway, the activation of γ −secretase can release NICD and promote NICD nuclear translocation where NICD to induce the expression of resistance genes. We hypothesize the activation of γ −secretase/Notch induce the expression of drug resistance genes by promoting the interaction of NICD and PXR. Using Co-IP technology, we found that there is indeed an interaction between NICD and PXR in MDA-MB-231/Taxol cells. the activation of γ −secretase by overexpression of PSEN-1 activating secretase can promote the binding of NICD and PXR and increase the expression of resistance genes, while silencing PSEN- gene can reduce the expression of NICD and resistance genes. Using PXR SV2 promoter-excited luciferase assay, we further show that overexpression of NICD increases PXR V2 promoter luciferase activity, while inhibition of γ −secretase by DAPT reduced it luciferase activity. These results clearly indicate that the activation of γ −secretase/Notch pathway induce drug resistance through promoting interaction of NICD and PXR, NICD increases PXR promoter activity to activate PXR and induce drug resistance gene expression in MDA-MB-231/Taxol cells.

Currently, many small molecule components that target γ-secretase to inhibit the Notch pathway have been developed. It has been shown that γ-secretase inhibitors (GSIs), such as DAPT, PF-03084014 and RO4929097, can reverse tumor resistance [27–29]. and the inhibition of the γ-secretase/Noch pathway reverses drug resistance in the patients with prostate cancer, the mechanisms may involve [30–32]. In the study, we used Nirogacestat (a potent and selective γ-secretase inhibitor) to treat TNBC/Taxol cells, Nirogacestat at low dose enhances Taxol inhibition of TNBC proliferation. Furthermore, we show that Nirogacestat downregulates the expression of some tumor resistance genes and enhances Taxol inhibitory effects on xenograft tumor growth in mice without addition side effects. Our results suggest that inhibition of γ −secretase may be a feasible and new strategy for reversing TNBC resistance.

It is worth mentioning that with the continuous advancement of high-throughput activity screening technology, some natural sources of γ-secretory enzyme inhibitors have been discovered, which possess some unique pharmacological structures and functional characteristics, as well as superior inhibitory activity of γ −secretase. Among them, a few natural products have entered clinical trials. They provide a template for the development and study of TNBC-resistant therapeutic and adjuvant agents. Therapeutic and safety perspectives, compared with small molecule inhibitors of chemically synthesized origin, γ-secretase inhibitors of natural origin are mostly herbs or edible plants, which are less toxic, resourceful, easily available and safer. Therefore, the scientific development and rational application of natural-derived GSIs to reverse tumor chemotherapy resistance is highly promising in the future.

The findings are summarized schematically in Fig. 6. Elevated γ-secretase activity and the consequent intranuclear accumulation of NICD synergistically exacerbate PXR activation, forming the γ-secretase/NICD-PXR axis that mediates the development of Taxol resistance. Mechanistically, PSEN-1 serves as the catalytic subunit for γ-secretase activation. Increased γ-secretase activity enhances the intranuclear accumulation of NICD, facilitating the interaction between NICD and PXR, thereby promoting PXR activation and fostering drug resistance. Inhibition of γ-secretase activity serves to mitigate the accumulation of NICD in the nucleus, reducing the binding of NICD to PXR. This inhibits PXR activation, resulting in the reversal of Taxol resistance. Both DAPT and Nirogacestat, as γ-secretase inhibitors, effectively enhance the efficacy of Taxol in TNBC/Taxol cells and xenograft tumours, providing a successful strategy for overcoming resistance in TNBC/Taxol. This study uncovers a novel target and mechanism underlying TNBC/Taxol resistance, potentially offering a breakthrough in addressing Taxol resistance in TNBC.

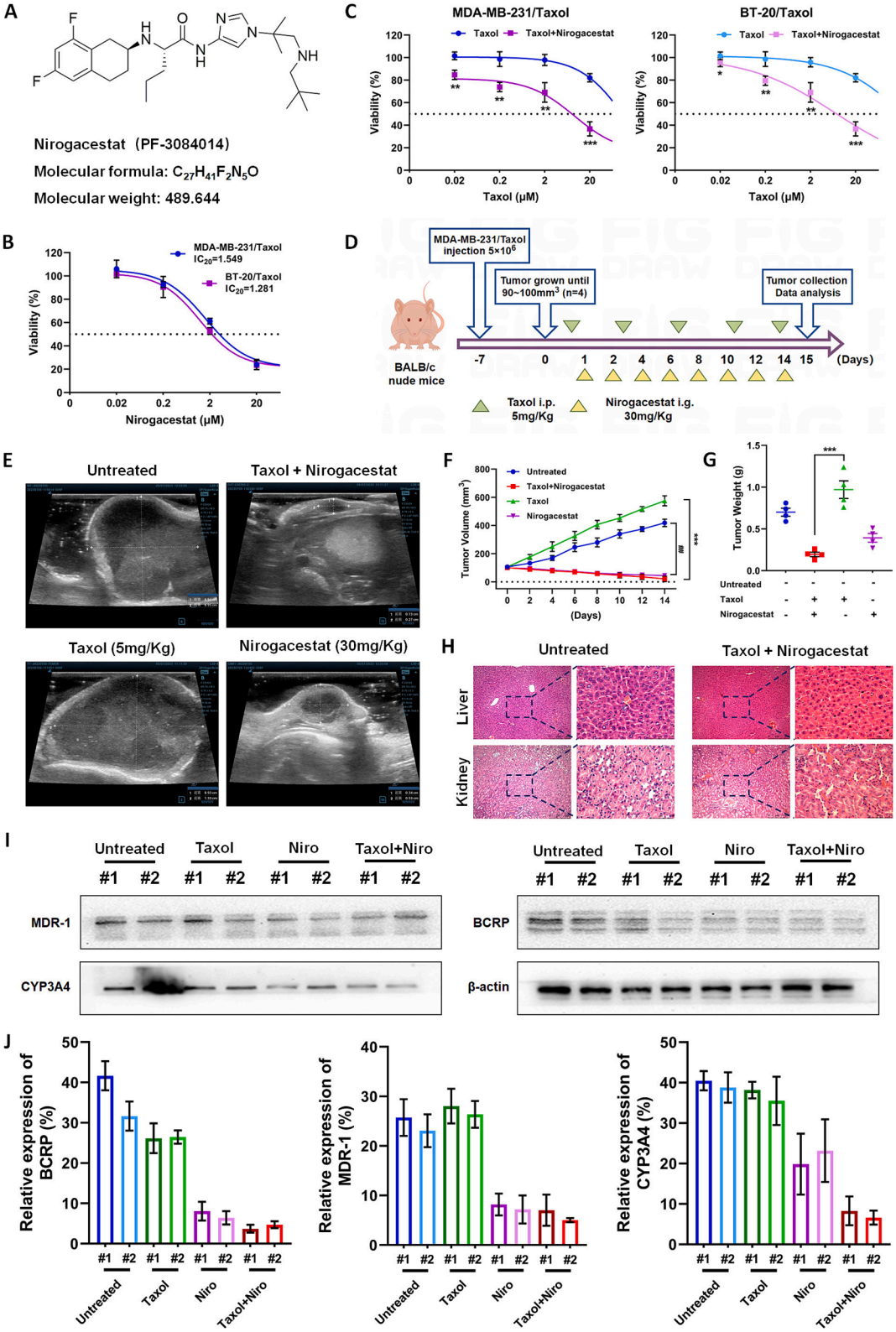

Fig. 5. Nirogacestat significantly decrease Taxol resistance in TNBC/Taxol cells *in vivo*. **(A)** Chemical structure of Nirogacestat (PF-3084014), a selective γ-secretase inhibitor. **(B)** SRB results showing the efficacy of Nirogacestat in TNBC/Taxol cells. **(C)** SRB results showing the efficacy of Taxol in MDA-MB-231/Taxol and BT-20/Taxol cells treated with Nirogacestat (15 μM) or vehicle for 48 h. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *vs.* Taxol group. **(D)** Schematic diagram of the establishment and treatment of MDA-MB-231/Taxol nude mice xenograft model. MDA-MB-231/Taxol cells were subcutaneously injected into the right upper limb axilla of BALB/c nude mice. Treatments were administered as indicated by the colored triangles (i.p., intraperitoneal; i.g., intragastric). **(E)** Tumor ultrasound images from MDA-MB-231/Taxol xenograft nude mice. **(F)** The tumor growth curve shows the inhibitory effect of different treatments on tumor volume. **(G)** The tumor weight (mg) in the different groups. **(H)** The liver and kidney of xenograft mice were histological analyzed by HE staining (scale bar = 100 μm). **(I&J)** The expression level and statistical graphs of PXR downstream related resistance proteins in MDA-MB-231/Taxol xenografts. Nirogacestat (Niro). $^{***}p < 0.001$, *vs.* Taxol group. $^{\#\#}p < 0.01$, *vs.* untreated group.

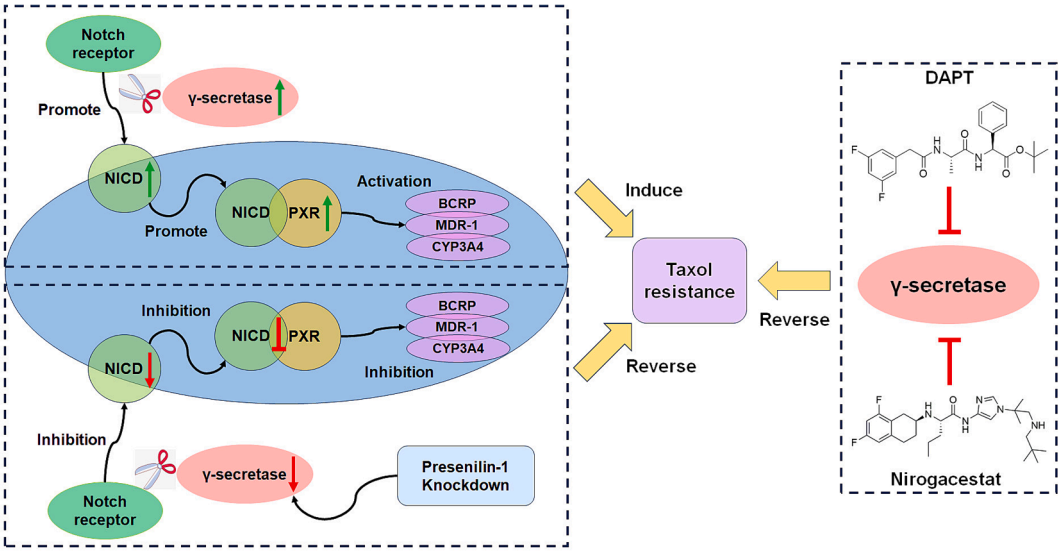

Fig. 6. Schematic diagram showing the mechanism of γ-secretase mediated Taxol resistance and potential treatment strategies. Increased γ-secretase activity promoted the release and intranuclear accumulation of NICD, which interacted with PXR, exacerbated PXR activation, and switched on the Notch pathway and the transcription of resistance-related target genes downstream of PXR, such as BCRP, MDR-1, and CYP3A4. Activation of the γ-secretase/NICD-PXR axis mediates Taxol resistance.

CRediT authorship contribution statement

Zuo-Jun Wang: Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Xiang-Yi Zhan:** Investigation, Formal analysis, Data curation, Conceptualization. **Liang-Yu Ma:** Methodology, Investigation. **Kuo Yao:** Investigation, Data curation. **Han-Yu Dai:** Investigation, Data curation. **Ramesh Kumar Santhanam:** Writing – review & editing. **Ming-Sheng Zhou:** Writing – review & editing, Supervision, Funding acquisition. **Hui Jia:** Writing – review & editing, Writing – original draft, Validation, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

Zuo-Jun Wang and Hui Jia conceived the study. Zuo-Jun Wang, Liang-Yu Ma, Xiangyi Zhan, Kuo Yao and Han-Yu Dai acquired samples and performed the experiments and data analyses. Hui Jia and Zuo-Jun Wang analyzed and interpreted the data. Hui Jia, Ming-Sheng Zhou, and Ramesh Kumar Santhanam wrote and revised the manuscript. All authors read and approved the final manuscript.

Data availability

Data will be made available on request.

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