



Original Articles

Major vault protein is a direct target of Notch1 signaling and contributes to chemoresistance in triple-negative breast cancer cells



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ABSTRACT

Resistance to chemotherapy remains a significant problem in the treatment of breast cancer, especially for triple-negative breast cancer (TNBC), in which standard systemic therapy is currently limited to chemotherapeutic agents. Our study aimed to better understand the molecular mechanisms that lead to failure of chemotherapy in TNBC. Herein, we observed elevated expression of Notch1 and major vault protein (MVP) in MDA-MB-231DDPR cells compared to their parental counterparts. We demonstrated that Notch1 could positively regulate the expression of MVP. Also, Notch1 intracellular domain (ICD) was capable of binding to CBF-1 on the promoter of MVP to drive its transcription, resulting in activation of AKT pathway and promoting the progress of epithelial to mesenchymal transition (EMT). Conversely, silencing of Notch1 and MVP suppressed AKT pathway, reduced EMT and enhanced the sensitivity of TNBC cells to cisplatin and doxorubicin. Survival analysis indicated that the MVP was closely related to shorter recurrence-free survival (RFS) in patients with TNBC. Collectively, this study provides evidence that Notch1 activates AKT pathway and promotes EMT partly through direct activation of MVP. Targeting Notch1/MVP pathway appears to have potential in overcoming chemoresistance in TNBC.

1. Introduction

Triple negative breast cancers (TNBCs) refers to a particular subtype of breast cancers. IHC results are negative for the protein expression of the estrogen receptor (ER), the progesterone receptor (PR) and lack overexpression/gene amplification of HER2 in TNBCs [1]. Around 12–17% of breast cancers belong to TNBCs [2]. When it comes to the treatment for women with TNBC, standard chemotherapy such as doxorubicin, taxanes [3], or platinum compounds as salvage treatment [4] are commonly used due to the lack of a therapeutic target in these patients. Nevertheless, resistance to chemotherapeutic agents has significantly compromised the efficacy of chemotherapy against TNBC, leading to recurrent or metastatic disease, and ultimately death.

The mechanism of chemoresistance is complex and has been recognized to be involved in ATP transporters, mutations in DNA repair

enzymes and activation of signaling pathways, such as Notch, PI3K/AKT, and NF- κ B signaling, etc. Notch receptors are a set of transmembrane proteins, including Notch1–Notch4. Notch1 experiences a series of proteolytic cleavages when it is activated by an extracellular ligand (for example Jagged or Delta). Mediated by ADAM proteins and the γ -secretase complex, Notch1 then release an intracellular domain named N1ICD which can translocate to the nucleus and serve as a transcriptional co-regulator for extensive target genes [5]. Emerging evidence has shown that Notch1 signaling was closely related to chemoresistance in a variety of malignancies. Previous studies indicated that Notch1 was capable of regulating ABC transporters [6,7], apoptotic proteins [8,9], and the p53 pathway [10,11], thereby affecting the sensitivity of tumor cells to chemotherapy agents. Zhang and colleagues reported that MiR-139–5p modulated chemosensitivity to docetaxel by targeting Notch1 in breast cancer cells [12]. However, the down-stream

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targets of Notch1 signaling remain not fully understood.

MVP is the primary component of the vault complex which is a ribonucleoprotein particle with a hollow barrel-like structure [13]. It was initially reported to be involved in chemotherapy resistance of various tumor types [14,15], which is involved in exporting drugs from the nucleus for sequestration in cytosolic vesicles [16]. It is also connected to chemotherapy resistance-related pathways, such as phosphoinositide-3-kinase/AKT signaling pathways and EGFR-induced MAPK pathway [17]. Furthermore, MVP was associated with the expression of ABCG1 [18] and ABCB1 in MDR-selected tumor cells [19], but the function of this protein in breast cancer and its specific role in the drug resistance mechanism and clinical settings remain yet to be determined.

In the present study, we demonstrated that Notch1 and MVP were pivotal regulators contributing to chemoresistance in TNBC cells. Notch1 could transcriptionally promote the expression of MVP and activate the AKT pathway as well as EMT process, and silencing of this pathway enhanced the sensitivity of TNBC cells to chemotherapy. These results suggested a viable strategy for tackling chemoresistance in TNBC.

2. Materials and methods

2.1. Cell culture and establishment of the cisplatin-resistant MDA-MB-231DDPR cell line

MDA-MB-231, BT549, MCF-7, T47D, SKBR-3, HCC1937, and ZR751 were purchased from American Type Culture Collection (ATCC). The MDA-MB-231DDPR cells were obtained by exposure to CDDP for 6 months, from 0.1 µg/L to 1 µg/L. Finally, the MDA-MB-231DDPR cells were maintained in 1 µg/L DDP concentration. All the cells were maintained in DMEM containing 10% foetal bovine serum (FBS) and were cultured in a humidified 5% CO₂ incubator at 37 °C.

2.2. Plasmids, small interfering RNAs (siRNAs), and transfection

The LV201-SH, LV201-N1ICD, pCMV3 and pCMV3-MVP plasmids were developed by our laboratory. siRNAs (Table 1) were synthesized by GenePharma Company (Suzhou, China). For the transfection, Lipofectamine 3000 (Life Technology, NY, USA) is used according to the manufacturer's protocol.

2.3. Western blotting analysis

Extracted protein was resolved by 10% SDS-PAGE and transferred to PVDF membrane. Next, the membrane was probed with primary antibodies (Table 2), followed by incubations with appropriate secondary antibodies and subjected to ECL detection (Appligen, Beijing, China).

Table 1

Primers and used Small interference RNAs in this study.

Gene/ChIP/luciferase assay	Forward primer	Reverse primer
Notch1	5'-CGGGTCCACCAGTTTGAATG -3'	5'-GTTGTATTGGTTCGGCACCAT-3'
MVP	5'- ACAACTACTGCGTGATTCTC-3'	5'-TCAGCATGTAGGTGCTTC-3'
GAPDH	5'-TGGACTCCAGACGTACTCAG-3'	5'-ACATGTTCCAATATGATTCCA-3'
Luciferase assay: wild-type CSL-binding site	5'-GCCAGACATTGAGAACTGAAGAGAGAAACCTC-3'	5'-TCCCCGGGTCCCGCAACGTCGTGTTT-3'
Luciferase assay: mutated CSL-binding site	5'-GCCAGACATTGAGAACTGAAGAGAGAAACCTC-3'	5'-GAGGTTTCTCTCTCAGTTTCTCA-3'
ChIP primers NC	5'-CCTCAGTTAGCACCTGTCCC -3'	5'-CAGCTGGTTCTCATTTTATCCT-3'
ChIP primers contain CSL-binding site	5'-CCCACTGCTCTGCCTCTA-3'	5'-TCCCGCAACGTCGTGTTT-3'
Small interference RNAs	Sense(5'-3')	
siNotch1a	5'-CACCAGUUUGAAUGGUCAATT-3'	
siNotch1b	5'-AAGTGTCTGAGGCCAGCAAGA-3'	
siMVPa	5'-CCUACAUGCUGACCCAGGATT-3'	
siMVPb	5'-ATCATTGCGACTGCTGTCTT-3'	

Table 2

Antibodies used in this study.

Antibody	Cat.#	Company	Con	Species
Anti-Notch1	ab178948	Abcam (Danvers,MA,USA)	1:1000	Rabbit
Anti-MVP	EPR13227(B)	Abcam (Danvers,MA,USA)	1:2000	Rabbit
Anti-MVP	SC-390134	Santa Cruz (Santa Cruz,CA,USA)	1:1000	Mouse
Anti-AKT	4685	Cell Signaling Technology (Danvers,MA,USA)	1:2000	Rabbit
Phospho-AKT (Ser473)	4073	Cell Signaling Technology (Danvers,MA,USA)	1:1000	Rabbit
Anti-mTOR	2972	Cell Signaling Technology (Danvers,MA,USA)	1:1000	Rabbit
Phospho-mTOR (Ser2448)	5536	Cell Signaling Technology (Danvers,MA,USA)	1:1000	Rabbit
Vimentin	5741	Cell Signaling Technology (Danvers,MA,USA)	1:1000	Rabbit
N-cadherin	13116	Cell Signaling Technology (Danvers,MA,USA)	1:1000	Rabbit
GAPDH	TA-08	ZhongshanJinqiao (Beijing,China)	1:3000	Mouse

2.4. Quantitative real-time PCR

The RNA was extracted with the Trizol reagent (Life Technology, NY, USA). Total RNA (1 µg) was reverse-transcribed into cDNA using the PrimeScript™ RT reagent kit (Takara Bio Inc., Dalian, China). The real-time PCR assay was performed in a CFX96 Real-time PCR Detection System (Bio-Rad, CA, USA). The primer sequences are shown in Table 1.

2.5. Anti-cancer drug sensitivity assays

Cells were seeded into 96-well plates at a density of 5×10^3 cells/well. Each plates contain 1–1000 µM/L cisplatin and 0.001–10 µM/L doxorubicin. After incubation for 48 h, viability of the cells was tested using Cell Counting Kit-8 (CCK-8, Dojindo, Japan). The absorbance of each well was measured at 450 nm using a spectrophotometer (Thermo). The IC₅₀ was calculated with GraphPad Prism5.

2.6. Immunofluorescence assay

The cells were treated with 4% paraformaldehyde and 0.5% Triton X-100. Subsequently, cells were incubated with the primary antibodies (Notch1 1:100, MVP 1:100) overnight at 4 °C. Next, cells were incubated with secondary antibodies for 1 h in the dark. Finally, the samples were stained with DAPI and were observed with immunofluorescence microscope (Carl Zeiss, Jena, Germany).

2.7. Immunohistochemistry

Tissue samples collected from 52 primary breast cancer patients at the Cancer Hospital of Shantou University Medical College from June 2014 to August 2016 were used for immuno-histochemical analysis. MVP (1:100) and Notch1 (1:100) were used as primary antibodies. The staining score of each section was calculated by staining intensity and number of positive cells. Immunohistochemistry (IHC) staining score was determined through assessment of staining intensity and number of positive cells. Staining intensity was categorized and recorded as 0, 1, 2, and 3, which representing no staining, weak staining, moderate staining and strong staining, respectively. Score of tumor cells positivity were recorded as 0, 1, 2, 3 and 4, with percentage of positive cells calculated within the ranges of 0–5%, 5–24%, 25–49%, 50–74% and > 75%, respectively. Multiplication of positive cell score and staining intensity were used to produce a weighted score for each case. Finally, grading was calculated based on the sum of scores: 0 (–); 1–4 (+); 5–8 (+ +); > 9 (+ + +). Weighted score of – and + were defined as negative case, and Weighted score of + + and + + + was defined as positive case.

2.8. Luciferase reporter assay

MDA-MB-231 cells (1×10^5) were seeded in 24-well plates, and co-transfected with LV201-SH, LV201-N1ICD, pGL3MVP-Wt promoter reporter, pGL3MVP-Mt promoter reporter and pRL-SV40 using Lipofectamine 3000. The luciferase activity was measured by using the Dual-Luciferase Reporter Assay System 48 h after transfection.

2.9. Chromatin immunoprecipitation (ChIP)

Cells were fixed with 1% formaldehyde for 15 min, and then used to perform ChIP experiments using the ChIP assay kit (Beyotime, Shanghai, China). PCR was performed using primers (Table 1) for the promoter region of MVP.

2.10. Transwell assay

Cell culture inserts (8 mM pore size; BD, CA, USA) and Matrigel invasion chambers (BD, CA, USA) were used. Approximately 2×10^5 cells were seeded into the upper chamber in serum-free DMEM. The lower chamber contained DMEM with 10% FBS. After incubation for 36 h, the non-migrated cells on the upper side of the membranes were removed and the migrated cells were stained with 0.1% crystal violet, and counted in five randomized fields.

2.11. GEO dataset analysis

GEO accession number: GSE77515 was used to analyze Notch family protein and some drug resistance proteins, following which the MeV4.9 software was used to draw the heat map. GSE25065 and GSE25055 were used to analyze the MVP level in residual tumors and pathological complete response (pCR) tumors after chemotherapy.

2.12. Clinical database and statistical analysis

The connection between MVP mRNA expression and survival was accessed by online database (<http://kmplot.com>) [20]. The ONCOMINE database (www.oncomine.org) was used to analyze the levels of MVP in breast cancer tissues (BC) and normal breast tissues. The relation between the mRNA levels of MVP and Vimentin in breast cancers was determined through analysis in the cBioportal database (<http://www.cbioportal.org/index.do>) [21]. We selected TCGA, cell 2015 database for the analysis and the correlation coefficients between mRNA levels were obtained by Pearson correlation analysis. SPSS version 23.0 was used for all the statistical analysis in the study. Each experiment was

performed in triplicate with data showing as the mean \pm SEM. Student's t-test and unpaired two-tailed Student's t-test were used for between-group and paired comparison. Two-sided p value of less than 0.05 was considered statistically significant.

3. Results

3.1. MDA-MB-231 cells with acquired cisplatin resistance expressed high levels of Notch1 and MVP

MDA-MB-231 cells were treated with cisplatin for 12 h and 24 h and then used for high-throughput RNA-seq analysis (GEO accession number: GSE77515). The results of the heat map showed the expression of the notch family as well as of some drug resistance proteins (Fig. 1A). The expression of Notch1 was increased while the expression of other Notch family members, including Notch2, 3, and 4 was unchanged. Furthermore, MVP expression was significantly higher in the cells treated with cisplatin than in the control group.

We next established a cisplatin resistant MDA-MB-231DDPR cell line by cultivating the cells in cisplatin to perform further mechanistic studies. We observed that the morphology of MDA-MB-231DDPR cells differed from the parental cells with long protrusions (Fig. 1B). To validate cisplatin resistance in the MDA-MB-231DDPR cells, we performed CCK8 assay to determine the IC50 value. Our data showed that the sensitivity to cisplatin of MDA-MB-231DDPR cells was much less than that of parental cells with an IC50 value of $78 \pm 4.04 \mu\text{M/L}$ which was about 7 times higher than $11 \pm 1.15 \mu\text{M/L}$ for the parental cells (Fig. 1C). These data suggested that MDA-MB-231DDPR cells are resistant to cisplatin. We then tested the expression of Notch1, MVP, and the key molecules in the AKT pathway in MDA-MB-231DDPR cells. As shown in Fig. 1D and E, both Notch1 and MVP were significantly higher at both mRNA and protein levels in the MDA-MB-231 DDPR cells than in the MDA-MB-231 cells, and the AKT pathway was also activated. Moreover, we treated MDA-MB-231 cells with 0.04, 0.08, and $0.16 \mu\text{mol/L}$ of doxorubicin or treated it with $0.08 \mu\text{mol/L}$ in 3, 6, 9, 12 h, and measured Notch1 and MVP proteins by immunoblotting. As expected, the Notch1 and MVP protein levels increased with the concentration gradient of doxorubicin and a time-dependent manner (Supplementary Fig S1A and S1B).

We also examined the expression and localization of Notch1 and MVP in both cell types by immunofluorescence. The expression of Notch1 and MVP were up-regulated in MDA-MB-231DDPR cells and Notch1 was localized mainly in the nucleus in both cells, while MVP was present predominantly in the cytoplasm in the parental MDA-MB-231 cells but mostly accumulated in the nucleus in MDA-MB-231DDPR cells (Fig. 1F). Because of the simultaneous up-regulation in cisplatin-resistant cells, we explored a functional linkage among Notch1, MVP and AKT pathway.

3.2. Inhibition of Notch1 down-regulated MVP expression and suppressed AKT pathway in MDA-MB-231DDPR cells

To further investigate the function of Notch1 and MVP, we knocked down their expression in MDA-MB-231DDPR cells via transient transfection of Notch1 and MVP siRNAs. As shown in Fig. 2A and B, both Notch1 and MVP siRNAs significantly knocked-down their protein expression, respectively. Because siNotch1-A inhibited Notch1 more efficiently than siNotch1-B (Fig. 2A), we chose siNotch1-A as main sequence for the following experiments. As shown in Fig. 2C and D, knocking down Notch1 could reduce expression of MVP both at mRNA and protein level, and also suppress the AKT pathway.

We next determined whether Notch1 or MVP expression correlated with cisplatin resistance. First, we knocked down Notch1 or MVP in MDA-MB-231DDPR cells and measured resistance to cisplatin. The IC50 value of cisplatin in MDA-MB-231DDPR cells transfected with siNotch1 and siMVP was $54 \pm 1.16 \mu\text{M/L}$ and $43 \pm 1.4 \mu\text{M/L}$,

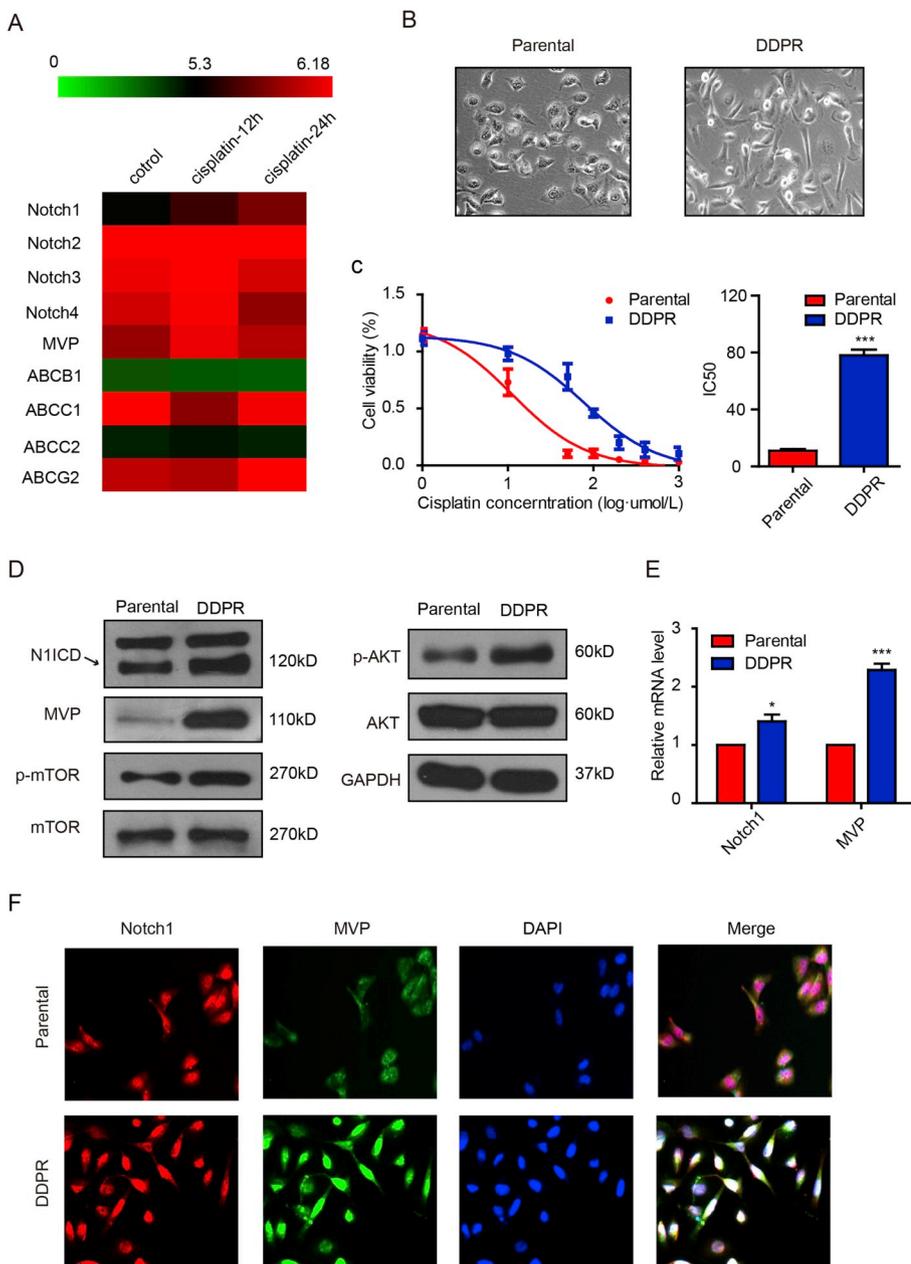


Fig. 1. MDA-MB-231DDPR cells expressed a high level of Notch1 and MVP. (A). Heatmap of differentially expressed genes in the NOTCH family and some drug resistance proteins. (B). Morphology of MDA-MB-231 and MDA-MB-231DDPR cells. (C). Cell viability analysis of MDA-MB-231 and MDA-MB-231DDPR cells after treatment with cisplatin. (D). Relative protein levels of Notch1, MVP, p-AKT, AKT, p-mTOR, and mTOR were detected by Western blot analysis in MDA-MB-231 and MDA-MB-231DDPR cells. (E). The mRNA levels of Notch1 and MVP in MDA-MB-231 and MDA-MB-231DDPR cells were determined by real-time RT-PCR. (F). Immunofluorescence of MDA-MB-231 and MDA-MB-231DDPR cells stained with anti-Notch1 (red signal) and anti-MVP (green signal) antibody, as well as DAPI for the nuclei (blue). Data are presented as the mean \pm SEM. of three experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (Student's t-test) as compared with control cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

respectively, and that of the control group was $69 \pm 2.6 \mu\text{M/L}$. The data showed that siNotch1 and siMVP could both increase the cisplatin sensitivity. We then knocked down Notch1 and MVP simultaneously and observed that the combined depletion of Notch1 and MVP increased the sensitivity to cisplatin with an IC50 value of $26 \pm 1.73 \mu\text{M/L}$. Finally, when we co-transfected MDA-MB-231DDPR cells with siNotch1 and pCMV3-MVP, the effect of siNotch1 could be partially restored (IC50 = $65 \pm 2.31 \mu\text{M/L}$) (Fig. 2E and F). These findings indicated that Notch1 was a pivotal mediator of cisplatin resistance by modulating MVP expression and the AKT pathway in MDA-MB-231DDPR cells.

3.3. MDA-MB-231DDPR cells exhibited an EMT-like phenotypic change

As drug-resistant cells often develop EMT phenotype [22,23], we examined whether the acquisition of the cisplatin resistance was accompanied by morphological changes. As shown in Fig. 1B, since MDA-MB-231 DDPR cells exhibited elongated and more aggressive

morphology, we speculated that they would exhibit enhanced migration and invasion ability. Utilizing the Transwell assay, we observed that invasive and migratory capacity of MDA-MB-231DDPR cells was significantly enhanced compared with parental cells (Fig. 3A and B). Next, we examined whether MDA-MB-231DDPR cells carried EMT molecular changes. We found that compared with parental cells, expression of both Vimentin and N-cadherin increased in MDA-MB-231DDPR cells by Western blotting (Fig. 3C). Increased expression of Vimentin in MDA-MB-231DDPR cells was also detected by immunofluorescence (Fig. 3D). These data indicated that MDA-MB-231DDPR cells acquired EMT-like properties, enhanced motility and an invasive behavior compared with parental cells.

3.4. Depletion of Notch1 or MVP reversed EMT in the MDA-MB-231 DDPR cells

It is well-established that EMT plays a vital role in drug resistance in several types of cancers [24–26]. Reversing EMT may be an important

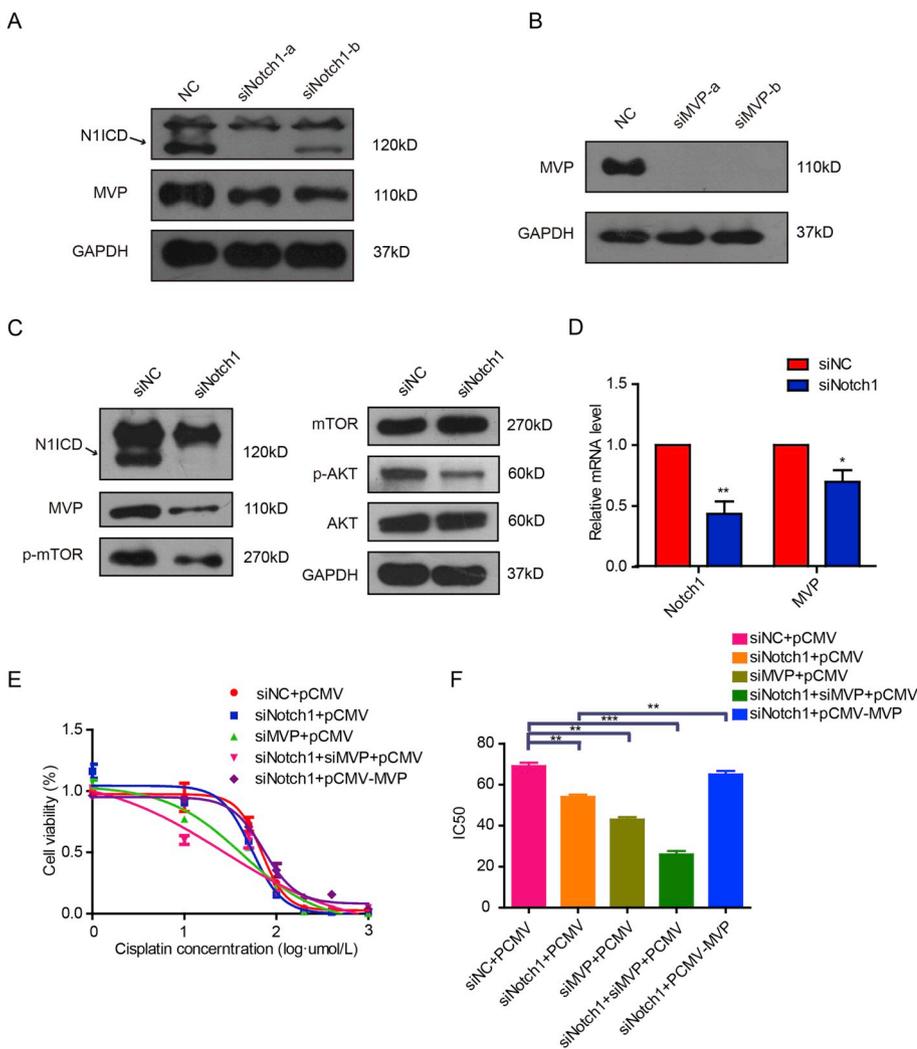


Fig. 2. Notch1 knockdown in MDA-MB-231DDPR cells decreased MVP level and suppressed the activity of AKT pathway. (A) and (B). MDA-MB-231 cells were transfected with two different sequences of Notch1 or MVP siRNAs. (C). Western blotting confirmed that Notch1 knockdown in MDA-MB-231DDPR cells could decrease the expression of MVP and inhibit the AKT pathway. (D). The Notch1 and MVP mRNA levels were measured by RT-PCR. (E) and (F). Cell viability was examined using the CCK-8 assay after treatment with different concentrations of cisplatin in MDA-MB-231DDPR cells transfected with siNC, siNotch1, siMVP, siNotch1 + siMVP or siNotch1 + pCMV3-MVP. Data are presented as the mean \pm SEM. of three experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (Student's t-test) as compared with control cells.

strategy for overcoming chemotherapy resistance. Based on the above results, we hypothesized that the role of Notch1 and MVP in drug resistance phenotype is mediated by EMT. To determine the possible regulation of EMT by Notch1 and MVP, we analyzed the expression of EMT markers in MDA-MB-231DDPR cells by Western blotting. The results show that Notch1 or MVP knockdown in MDA-MB-231DDPR cells led to a significant decrease in the expression of N-cadherin and Vimentin (Fig. 3E and F), while their overexpression resulted in the opposite molecular changes (Supplementary Fig S2A and S2C).

We next examined the effect of Notch1 and MVP on cell motility and invasion in MDA-MB-231DDPR cells. Transwell assay showed that depletion of Notch1 or MVP significantly inhibited the migration and invasion of MDA-MB-231DDPR cells. and simultaneous knockdown of Notch1 and MVP resulted in further decline of migratory and invasive abilities of MDA-MB-231DDPR cells. On the other hand, upon co-transfection of MDA-MB-231DDPR cells with siNotch1 and pCMV3-MVP, the effect of siNotch1 could be partially restored (Fig. 3G and H). Finally, ectopic Notch1 or MVP expression in MDA-MB-231DDPR cells significantly increased their migratory and invasive potential (Supplementary Fig S2B and S2D).

We also utilized an online Database (TCGA CELL 2015) to determine the relationship between the expression of MVP and Vimentin in 825 breast cancer patients and found a positive correlation in all patients (Pearson = 0.178, $p < 0.001$). In patients with subtypes of basal-like breast cancer, the relationship was more prominent (Pearson = 0.404, $p < 0.001$) (Supplementary Fig S3A and S3B). These results supported

the hypothesis that Notch1 and MVP regulate cisplatin resistance by promoting EMT.

3.5. Notch1 regulated the expression of MVP in triple-negative breast cancer cells

We determined expression levels of Notch1 and MVP by Western blotting and RT-PCR in seven human breast cancer cell lines, MDA-MB-231, BT549, HCC1937, SKBR3, MCF7, T47D and ZR751. The results showed that Notch1 and MVP were highly expressed in MDA-MB-231, BT-549 and HCC1937 triple-negative breast cancer cells. In contrast, non-triple-negative breast cancer cells, such as SKBR3, MCF7, T47D and ZR751, showed lower expression levels of Notch1 and MVP (Fig. 4A).

Positive regulation between expression of Notch1 and MVP in MDA-MB-231DDPR cells led us to investigate whether Notch1 regulates MVP in other breast cancer cell lines. Western-blotting and RT-PCR analysis showed that inhibition of Notch1 markedly suppressed MVP both at mRNA and protein levels in MDA-MB-231 cells (Fig. 4B). Overexpression of N1ICD, on the other hand, could up-regulate MVP protein and mRNA level (Fig. 4C). To exclude the possibility that these effects may be specific to MDA-MB-231 cells, the mRNA and protein expression of MVP was investigated in another triple-negative breast cancer cell line, BT549. As displayed in Fig. 4D and E, similar results were seen in BT549 cells. To down-regulate N1ICD, we treated MDA-MB-231 cells with concentration gradient of γ -secretase inhibitor (DAPT), which blocks processing of Notch1 into N1ICD. Compared with DMSO

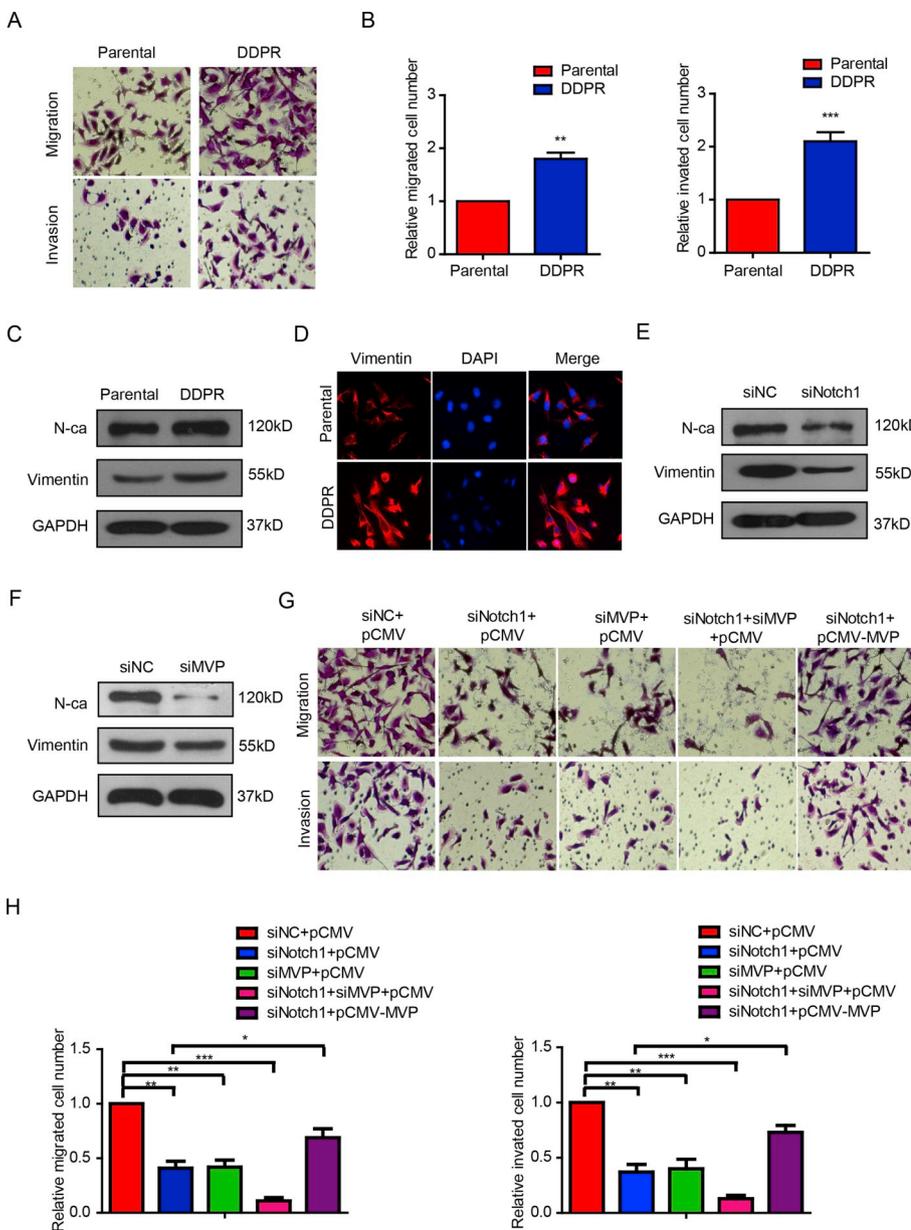


Fig. 3. EMT was induced in MDA-MB-231DDPR cells, and Notch1 and MVP inhibition reversed EMT. (A). The cell migration and invasion ability was evaluated using a transwell assay in MDA-MB-231DDPR cells and its parental cells. (B). N-cadherin and Vimentin were assessed by Western blot analysis. (C). Immunofluorescence of MDA-MB-231DDPR cells and their parental cells stained with anti-Vimentin (green signal) antibody. DAPI (blue signal). (D) and (E). Effect of Notch1 or MVP inhibition on N-cadherin and Vimentin were measured by Western blotting. (F). The cell invasion and migration capacity was examined using the Transwell assay in MDA-MB-231DDPR cells transfected with NC, siNotch1, siMVP, siNotch1 + siMVP or siNotch1 + pCMV3-MVP. Data are presented as the mean \pm SEM. of three experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 (Student's t-test) as compared with control cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

controls, DAPT caused a reduction of N1ICD in a dose-dependent manner and MVP protein level gradually reduced (Supplementary Fig.S4). These results suggested that N1ICD could positively regulate MVP in triple-negative breast cancer cell lines.

3.6. Notch1 enhanced chemoresistance through AKT pathway mediated by MVP

To further confirm that Notch1 could increase chemoresistance via MVP, we performed the following experiments. First, knocking down Notch1 or MVP, proteins that suppressed the AKT pathway, increased the sensitivity of MDA-MB-231 and BT549 cells to doxorubicin and cisplatin (Supplementary Fig.S5 and S6). Next, to reduce the effects of N1ICD, the rescue experiments by co-transfecting MDA-MB-231 cells and BT549 cells with siNotch1 and pCMV-MVP is performed. Western blotting analysis revealed down-regulated expression of MVP and AKT pathway was significantly attenuated by pCMV-MVP in Notch1-silenced MDA-MB-231 and BT549 cells as compared with cells co-transfected with pCMV(Fig. 5A and C). Next, following the rescue

experiments, we treated MDA-MB-231 and BT549 cells with increasing concentrations of cisplatin or doxorubicin followed by CCK assays. As expected, MVP could partially reverse enhanced cisplatin or doxorubicin resistance caused by depleting Notch1 expression (Fig. 5B and D). These results indicated that Notch1 could affect the AKT pathway by regulating the expression of MVP, which conferred resistance to chemotherapy.

3.7. Notch1 enhanced MVP transcription by binding to its promoter

Our results provided strong evidence that Notch1 could regulate MVP both at mRNA and protein levels. We speculated that Notch1 could regulate transcriptionally MVP through the CBF-1 transcription factor. We, therefore analyzed the MVP promoter for putative CBF1 binding sites. The region between bases -617 and -611 revealed one putative CBF1 binding site indicating that Notch1 might act as an upstream inducer of MVP. To investigate whether Notch1/CBF1 could bind to the MVP promoter, we designed sequence primers (- 580 to -716) containing the CBF1-binding sites (TGGGAA) and a negative

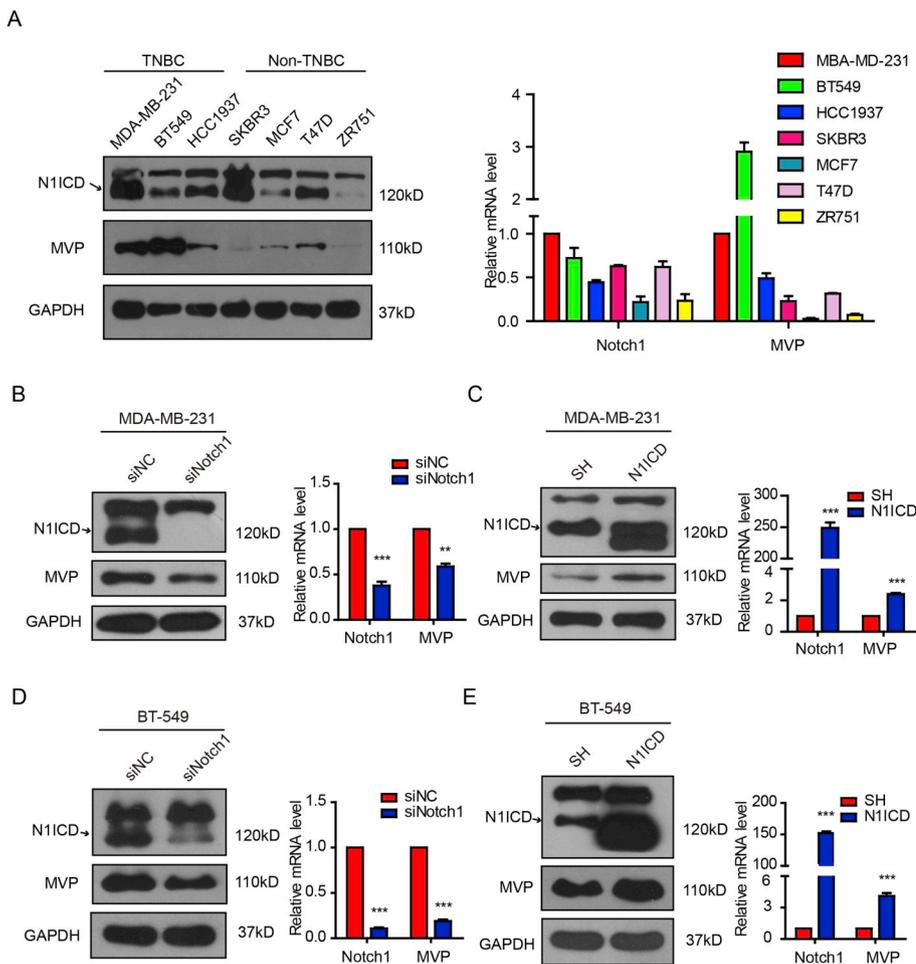


Fig. 4. Notch1 could regulate the expression of MVP in triple-negative breast cancer cells. (A). Notch1 and MVP expression levels in various human breast cancer cell lines, including MDA-MB-231, BT549, HCC1937, SKBR3, MCF-7, T47D, and ZR751 were tested via Western blotting and RT-PCR. (B) and (C). Western blotting and RT-PCR confirmed that, in MDA-MB-231 cells, ectopically over-expressed N11CD or Notch1 knockdown could increase or decrease the expression of MVP, respectively. (D) and (E). Western blotting and RT-PCR analysis confirmed that, in BT549 cells, ectopically over-expressed N11CD or Notch1 knockdown could increase or decrease the expression of MVP, respectively. Data are presented as the mean \pm SEM. of three experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (Student's t-test) as compared with control cells.

control (−108 to −240) that did not contain CBF1-binding sites (Fig. 6A). We performed a ChIP assay to examine whether Notch1 could bind to putative CBF1 binding sites within the promoter region of MVP. The results showed that Notch1 antibody could bind to the MVP promoter region containing putative CBF1 binding sites, whereas the control IgG as well as the negative control did not (Fig. 6B). These results clearly showed binding of Notch1 to the CBF1-binding sites on the MVP promoter.

To further demonstrate Notch1 could regulate MVP promoter activity, we cloned a luciferase reporter vector containing the CBF1-binding sites (pGL3MVP Wt) and a mutant vector in which the CBF1-binding site was deleted (pGL3MVP Mt) (Fig. 6C). When co-transfected with 100 ng, 200 ng and 400 ng LV201-N11CD plasmid, MVP promoter activity increased 2.35-fold to 4.04-fold in the MDA-MB-231 cell line in a dose-dependent manner, while up-regulating Notch1 did not increase the mutant MVP promoter activity (Fig. 6D) We concluded that Notch1 could transcriptionally regulate MVP expression by binding to the MVP promoter.

3.8. Expression of Notch1 and MVP in triple-negative breast cancer patients

Our studies have confirmed that Notch1 could transcriptionally regulate MVP in breast cancer cells. To examine their clinical relation, we collected 52 cases of triple-negative breast cancer from Cancer Hospital of Shantou University Medical College and analyzed the relation between Notch1 and MVP. The results showed that the expression of Notch1 and MVP had a positive correlation (Pearson = 0.309, $p = 0.026$) (Table 3). Next, we analyzed the levels MVP, but not Notch1, in breast cancer (BC) and normal breast (NB) tissues, because the key

measure for Notch is its activity and not necessarily its expression [6]. MVP was 1.311-fold elevated in breast cancer samples as compared with normal breast tissues ($p = 4.96E-9$) in a dataset with 2136 samples derived from Curtis breast database (Fig. 7B). These results suggested MVP play a crucial role in the occurrence of breast cancer (see Fig. 8).

We then analyzed HER2-negative breast cancer patients from MD Anderson Cancer Center in GEO (GSE25065 and GSE25055) for MVP expression. Finding from the study demonstrated that gene expression profiles for breast cancer cases received neoadjuvant treatment with anthracycline-based regimen in two separate cohorts, along with treatment efficacy and survival outcomes. The MVP was present at a relatively higher level in residual tumor than when acquired by PCR after the chemotherapy (GSE25055: $p = 0.0002$; GSE25065: $p < 0.0001$) (Fig. 7C). Finally, we analyzed the relationship between MVP mRNA levels and clinical outcomes in patients with breast cancer from the online database. Firstly, we selected patients with subtypes of basal-like breast cancer who had received chemotherapy ($n = 230$) and observed that high MVP expression was significantly associated with lower recurrence-free survival (RFS, HR = 1.51, $p = 0.067$). When we selected patients with subtypes of basal-like breast cancer who had received adjuvant chemotherapy ($n = 136$), the high MVP (HR = 2.01, $p = 0.028$) expression predicted lower RFS with greater significance (Fig. 7D). In conclusion, our analysis indicated that MVP may play a role in chemoresistance and act as a prognostic factor.

4. Discussion

Standard chemotherapy remains the backbone for the treatment of TNBCs due to the absence of a target. At present, the cure for TNBC

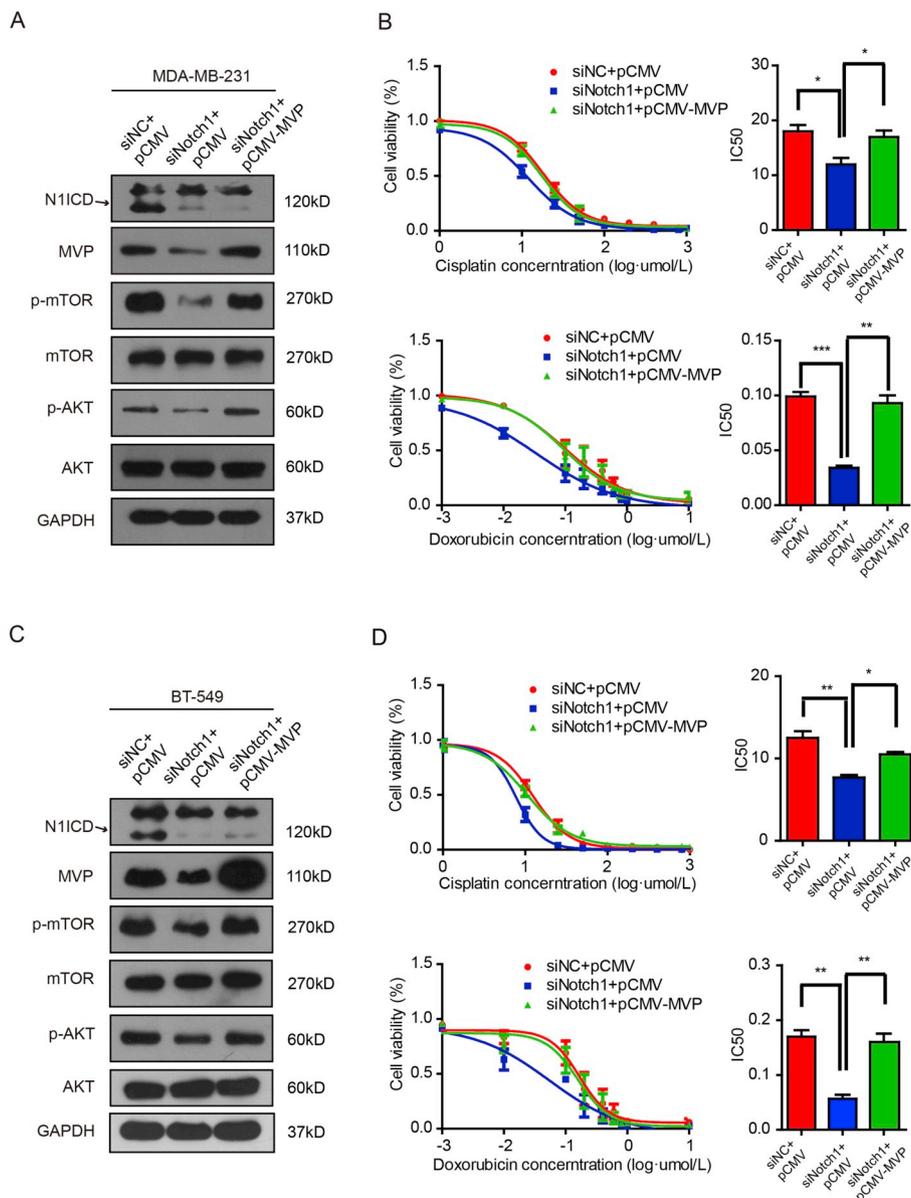


Fig. 5. Notch1 could regulate the AKT pathway via MVP, thus affecting chemoresistance. (A) and (C). MDA-MB-231 cells and BT549 cells were simultaneously transfected with siNotch1 and PCMV-MVP and the levels of Notch1, MVP and the AKT pathway were analyzed by Western blotting. (B) and (D). Cell viability analysis of MDA-MB-231 cells and BT549 cells, co-transfected with siNotch1 and PCMV-MVP, 48 h after treatment with cisplatin and doxorubicin. Data are presented as the mean ± SEM. of three experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 (Student's t-test) as compared with control cells.

remains a conundrum mainly due to either intrinsic or secondary chemoresistance during therapy [27] accounting for 90% of drug failures in the advanced or metastatic disease.

The established mechanisms of chemoresistance in TNBC involve ABC transporters, mutations in DNA repair enzymes, alterations in apoptosis-associated protein, or NF-κB signaling pathways. More recently, Notch signaling has been reported to participate in chemoresistance in a wide range of cancer types [10,28,29]. Many studies have proved that Notch1 could regulate the ATP-binding cassette (ABC) transporters protein, ABCC1/MRP1, but not ABCB1/MDR1 or ABCG2/BCRP, to affect the chemosensitivity [6,30,31].

In our study, we found that, the expression of Notch1 and MVP were both elevated in cisplatin-resistant breast cancer cells compared to the parental cells, and their protein levels increased with the concentration gradient of doxorubicin in a time-dependent manner. Moreover, silencing Notch1 could down-regulate MVP expression resulting in

restoration of their sensitivity to cisplatin. This phenomenon implied that Notch1 and MVP were vital regulators of response to cisplatin in TNBC cells. It is possible that Notch1 could increase the expression of ABCC1/MRP1 or p53, which have been shown to exert pro-survival function, may be partly through increasing the expression of MVP to counteract the killing effect of cisplatin [32]. Furthermore, we also demonstrated that silencing of Notch1 sensitized TNBC cells to doxorubicin, and ectopic MVP expression could partly recover their drug tolerance, indicating that MVP was a mediator of the Notch1 signaling in conferring doxorubicin resistance.

MVP is the major component of the vault complex, which plays a pivotal role in chemoresistance by allowing the intracellular drug to enter the nucleus [16] or regulating MAPK, JAK/STAT and phosphoinositide 3-kinase/Akt signaling pathways [17,33]. MVP is also linked to multidrug resistance (MDR) in cancer, although the exact mechanism is not yet clear [34,35]. Few studies have showed that elevated MVP

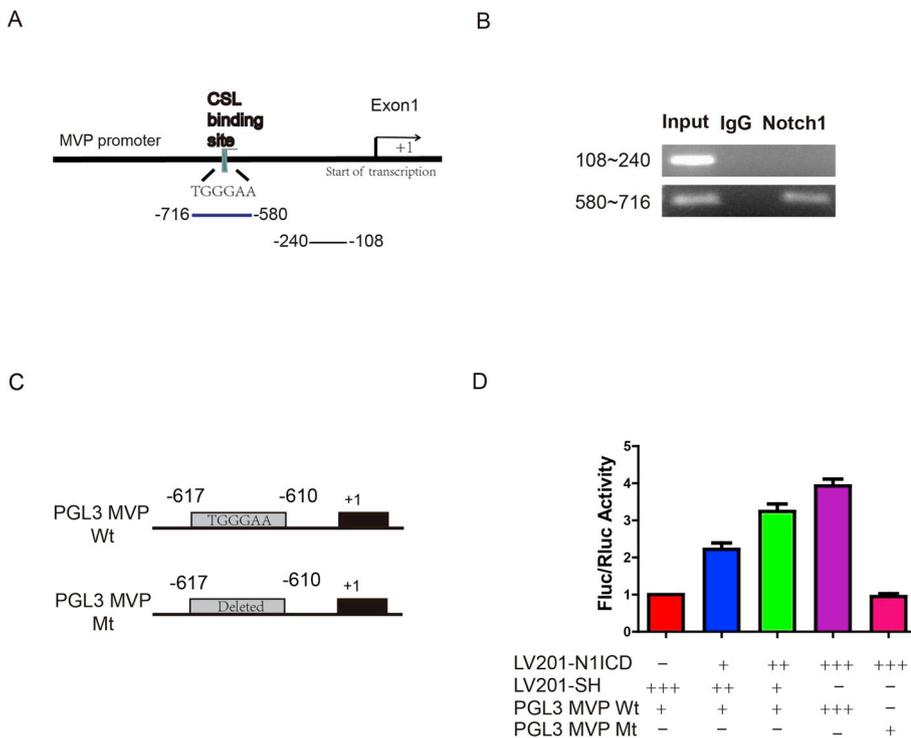


Fig. 6. Notch1 regulated the expression of MVP by binding to the MVP promoter. (A). The human MVP promoter sequence. (B). Notch1 binding on the MVP promoter is shown using the ChIP assay. (C). The schematic diagram of constructs with luciferase reporter plasmid. (D). Luciferase assays were performed in MDA-MB-231 cell by co-transfection with LV201-SH, LV201-N1ICD, PGL3 MVP Wt or PGL3 MVP Mt and luciferase activity was normalized to the Renilla minimal reporter.

expression was significantly associated with chemoresistance, due to its ability to export drugs from the tumor cells thus enhancing their chemoresistance. In the present study, we have shown that MVP was capable of activating AKT pathway independently, which was consistent with observation by Lötsch and colleagues that MVP could activate EGFR/PI3K signaling pathway in glioblastoma [36]. Another significant observation of our study was that MVP could regulate EMT in MDA-MB-231 DDPR cells thereby, at least partially contributing to their tolerance to chemotherapeutic agents. An increasing number of studies have suggested that EMT was associated with drug resistance in cancer, including breast and pancreatic cancers [37–39]. It has been recognized that cancer cells undergoing EMT process, usually presented with phenotype conversion to mesenchymal cells that resemble stem-like cells. This usually quiescent subpopulation was thought to be a mechanism partly responsible for chemoresistance. In this respect, we postulated that one of the avenues that MVP contributed to chemoresistance of TNBC cells was by modulating EMT.

Analysis of tissue samples of pathologically confirmed triple negative breast cancer showed a significant association between the expression of Notch1 and MVP. Furthermore, mechanistic studies demonstrated that MVP was a direct transcriptional target of Notch1 signaling and identified the specific promoter region allowing Notch1-dependent MVP regulation. This discovery provided a possible explanation for the molecular underpinning of Notch1 in the induction of

chemoresistance in TNBC.

So far, the involvement of MVP has not been extensively studied in chemotherapeutic response or survival outcome of breast cancer in clinical settings. Analysis of an original dataset in GEO derived from MD Anderson Cancer Center demonstrated that the expression of MVP was higher in residual tumor than in non-invasive tumor after chemotherapy. Survival analysis using Kaplan-Meier Plotter supported this perspective and indicated that high MVP expression was significantly associated with poor RFS in basal-like breast cancer patients, especially in those who had received chemotherapy. These results consistently supported the notion that MVP is a strong predictor of poor chemotherapy response, as well as a poor prognostic factor in patients with TNBC.

In summary, our study demonstrated that Notch1 signaling significantly contributed to chemoresistance in TNBC cells by partially promoting MVP expression which could induce chemoresistance via activating the AKT pathway and promoting EMT. Furthermore, MVP predicted poor survival outcome in patients with TNBC, and might serve as a unique predictive marker of chemotherapy response for this subgroup of patients. Our results suggest that targeting Notch1/MVP pathways has the potential to enhance the efficacy of the current artillery of drugs for women presenting with TNBC.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Ying-Sheng Xiao, De Zeng and Guo-Jun Zhang conceived and designed the project. Ying-Sheng Xiao, De Zeng, Mei-Fang Li performed the experiments. Min Chen, Xiao-Long Wei, Mei-Fang Li analyzed the data. Wen-He Huang analyzed the data and provided tissue samples. Ying-Sheng Xiao, De Zeng and Guo-Jun Zhang wrote the manuscript. Guo-Jun Zhang approved the final version to be submitted.

Table 3
The relationship between Notch1 and MVP expression.

Notch1	MVP				Spearman	P value
	-	+	++	+++		
-	10	7	3	2	0.309	0.026
+	2	5	0	2		
++	2	4	2	3		
+++	2	3	1	4		

Spearman rank correlation was used to analyze correlation. Two-tailed were statistically tested, p < 0.05 has statistical significance

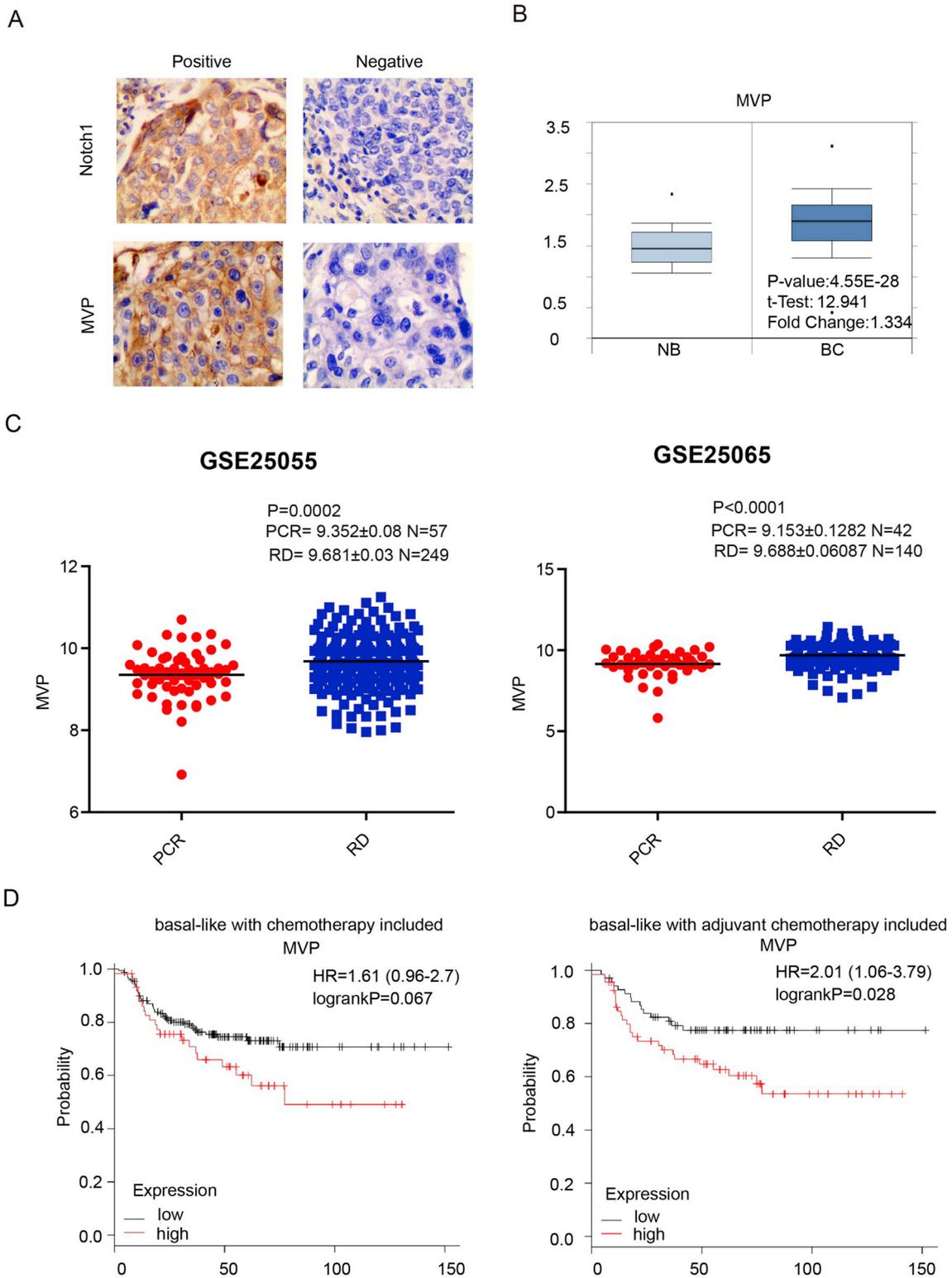


Fig. 7. Expression of Notch1 and MVP in patient tissues. (A). Immunohistochemical analysis of Notch1 and MVP in patients' tissues. **(B).** Expression of MVP in breast cancer tissues and adjacent tissues. **(C).** MVP expression after neoadjuvant chemotherapy in HER-negative breast cancer. **(D).** Prognostic effect of high and low expression of MVP in patients with breast cancer.

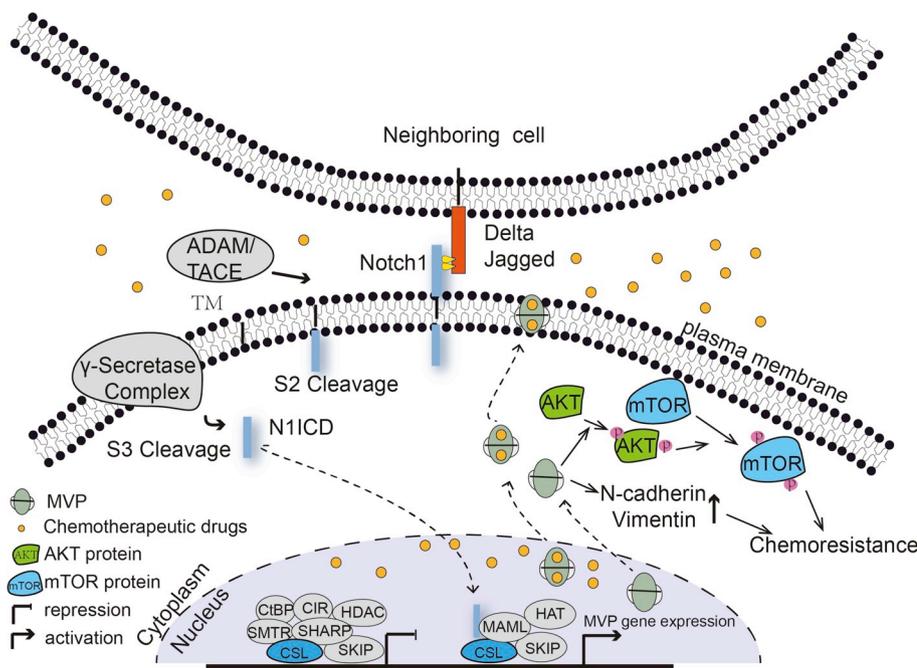


Fig. 8. Schematic diagram showing enhanced notch1-mediated chemoresistance by modulating MVP expression. Notch1 could regulate the MVP expression at both transcriptional and protein levels through the CBF-1 transcription factor. MVP functioned in vesicular transport of drugs and could activate the AKT pathway and induce EMT leading to chemoresistance.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2018.09.031>.

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