

Quantitative proteomics analysis of the role of tetraspanin-8 in the drug resistance of gastric cancer

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Abstract. Gastric cancer, due to its high incidence rate, is the second leading cause of cancer-related mortality worldwide. Chemotherapy is an important component of the multimodal treatment for gastric cancer; however, a significant impediment to successful treatment is multidrug resistance (MDR) in patients with gastric cancer. In the present study, the protein profiles of the MDR cell line, SGC7901/DDP, and its parental cell line, SGC7901, were comparatively analyzed through an iTRAQ-based quantitative proteomics technique. The protein tetraspanin-8 (TSPAN8) was found to be highly expressed in the SGC7901/DDP cells. To examine the role of TSPAN8 in the MDR of SGC7901/DDP cells, we increased cell sensitivity to drugs by increasing apoptosis. Additionally, the silencing of TSPAN8 downregulated Wnt pathway activity, β -catenin expression and β -catenin transfer to the nucleus. TSPAN8 was found to bind to NOTCH2, facilitating its mediation of the Wnt/ β -catenin pathway by regulating β -catenin expression. Overall, the suppression of TSPAN8 expression may prove to be a promising strategy which may aid in the development of novel gastric cancer therapeutic drugs.

Introduction

Globally, gastric cancer is the fourth most common malignancy and the second leading cause of cancer-related mortality, affecting approximately one million individuals each (1,2). Chemotherapy has been applied widely in the treatment of gastric cancer at different stages (3). However, a major issue in the treatment of gastric cancer is the development of resistance to multiple chemotherapeutic agents in tumor cells (4). Multidrug resistance (MDR) in cancer cells is an

acquired resistance to multiple drugs, which may be structurally and functionally different (5). Various mechanisms may lead to the development of MDR in cancer cells, including the altered expression of drug influx/efflux transporters, aberrant DNA repair and impairment, the prevention of apoptosis, the mutation of drug targets in targeted therapy, alterations in the cell cycle and checkpoints and an altered tumor microenvironment (5,6). The signaling pathways involved include, in some cancers, Wnt/ β -catenin, NOTCH and PI3K/AKT, among others, leading to increased resistance to drug treatment with both chemotherapy and targeted therapy (7-10). Interfering with these signaling pathways may be a novel antitumor strategy with which to prevent/inhibit MDR in clinical therapies.

Isobaric tags for the relative and absolute quantification (iTRAQ) analysis is an emerging quantitative proteomics technology that utilizes peptides labeled with isotope-coded covalent tags for the analysis of changes in protein expression in different samples (11). In the present study, the iTRAQ-based proteomic approach was applied to identify differentially expressed proteins in the SGC7901 and SGC7901/DDP cell lines. Among the proteins screened by this approach, tetraspanin-8 (TSPAN8) expression was found to be significantly increased in the SGC7901/DDP cells.

The *TSPAN8* gene encodes a cell surface glycoprotein characterized by 4 transmembrane domains and well-conserved cysteine residues in a large extracellular loop, and is expressed in gastric, colon, rectal and pancreatic carcinomas, but not in the majority of normal tissues (12-15). Within the Tetraspanin-enriched microdomain (TEM), TSPAN8 acts as a molecular facilitator (16), being involved in tissue differentiation (17), tumor-cell metastasis (18), and cell motility and cell fusion (18,19). TSPAN8 has been shown to be overexpressed in gastric cancer and to promote cancer cell proliferation, migration and invasion (20). However, the role of TSPAN8 in MDR gastric cancer cells remains unknown. Thus, in the present study, we identified TSPAN8 as a pro-drug resistance protein using iTRAQ-based quantitative proteomics. The silencing of TSPAN8 enhanced the sensitivity of the SGC7901/DDP cells to chemotherapeutic drugs. Additionally, TSPAN8 mediated the activation of the Wnt/ β -catenin pathway by binding to NOTCH2. These results indicate that TSPAN8 increases the MDR of gastric cancer cells. The inhibition of TSPAN8 may reduce drug resistance and may prove to be a strategy for the clinical treatment of patients with gastric cancer.

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Materials and methods

Cell culture, transfection and drug treatment. The cell lines used in this study were purchased from the China Center for Type Culture Collection (Wuhan, China). SGC7901/DDP is an MDR gastric cancer cell line in which resistance was induced by cisplatin and it is derived from the human gastric cancer cell line, SGC7901. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (both from Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin solution. The biological characteristics of MDR of the SGC7901/DDP cell line were maintained by the addition of 1 μ g/ml cisplatin (Sigma-Aldrich, St. Louis, MO, USA) to the complete medium. The cells were incubated in an atmosphere with 5% carbon dioxide at 37°C. Three small interfering RNA (siRNA) duplexes targeting human TSPAN8 and a control siRNA were synthesized by GenePharma Co., Ltd. (Shanghai, China). The sequences of the siRNA-TSPAN8 were as follows: Sequence 1 forward, 5'-GUAUCUUGAUCCUAGCAUUD TdT-3' and reverse, 5'-AAUGCUAGGAUCAAGAUACdTdT-3'; sequence 2 forward, 5'-GUCUGAUCGCAUUGUGAAUD TdT-3' and reverse, 5'-AUUCACAAUGCGAUCAGACdTdT-3'; sequence 3 forward, 5'-GAGUUUAAAUGCUGCGGUUD TdT-3' and reverse, 5'-AACCGCAGCAUUUAAACUCdTdT-3'; and siRNA-NC forward, 5'-UUCUUCGAAGGUGUCACG UTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'. The SGC7901/DDP cells were transfected with the siRNA using siRNA-Mate (GenePharma Co., Ltd.) following the manufacturer's instructions. The inhibitors of the Wnt pathway (CCT036477 and XAV939) and the inhibitor of NOTCH2 (DAPT) were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA, USA). All these drugs were suspended in dimethyl sulfoxide (DMSO; Sangon Biotech, Shanghai, China) at a stock concentration according to the manufacturer's instructions and stored at -80°C. Following siRNA transfection for 48 h, the cells were exposed to the inhibitors, which were diluted into the culture medium (10 μ M CCT036447, 10 μ M XAV939, 20 μ M DAPT or DMSO alone as a control) for 48 h, respectively.

Determination of half maximal inhibitory concentration (IC_{50}). The cytotoxic effects of the cisplatin, 5-fluorouracil and adriamycin (both from Sangon Biotech) on the SGC7901 and SGC7901/DDP cells were measured by cell counting kit-8 (CCK-8) assay (21). The cells were counted using the Neubauer cell-counting chamber (BRAND GMBH + CO KG, Wertheim, Germany) following the manufacturer's instructions. The cells were then seeded in 96-wells at a density of 5×10^3 cells/well, and cultured in an incubator at 37°C for 24 h before being treated with the chemotherapeutic drugs. Cisplatin, 5-fluorouracil (5-Fu) and adriamycin in graded concentrations were added to the cells. Following treatment for 48 h, the medium was replaced with fresh medium containing 10% CCK-8 reagent (Dojindo, Kumamoto, Japan), and the cells were incubated for an additional 1-4 h. The optical density was then measured by Thermo Scientific Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm. The IC_{50} values obtained following treatment of the SGC7901 and SGC7901/DDP cells with each drug were analyzed using IBM

SPSS Statistics v21 software (SPSS Inc., Chicago, IL, USA) via probit analysis (22).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using a High Purity Total RNA Rapid Extraction kit (RP1201; BioTeke, Beijing, China) according to the manufacturer's instructions. cDNA was synthesized using a iSCRIPT cDNA Synthesis kit (GeneCopoeia Co., Ltd., Guangzhou, China). The primers used for the amplification of TSPAN8, β -catenin, NOTCH2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were synthesized by GeneCopoeia Co., Ltd. GAPDH was used as an internal standard, and the relative expression of each gene was normalized to GAPDH. The real-time PCR kit was purchased from GeneCopoeia Co., Ltd. PCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 10 sec. The relative quantification of gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method (23). Each sample was analyzed in triplicate.

Western blot analysis. Protein was extracted from the cells using RIPA lysis buffer (Beyotime, Shanghai, China) and the concentration was determined using the 2D Quantification kit (Amersham Biosciences, Little Chalfont, UK). The protein samples were separated on a 10% polyacrylamide gel, and electrotransferred onto polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA, USA). The membranes were then blocked with 5% non-fat dried milk for 1 h at room temperature. This was followed by the addition of the primary antibodies: anti-TSPAN8 antibody (ab70007), anti- β -catenin antibody (ab16051) (both from Abcam, Cambridge, MA, USA), anti-cellular retinoic acid-binding protein 2 (CRABP2) antibody (10225-1-AP), anti-voltage-dependent anion-selective channel protein 2 (VDAC2) antibody (11663-1-AP), anti-Bcl-2 antibody (12789-1-AP) (all from Proteintech, Wuhan, China), anti-heat shock protein 90 (HSP90) antibody (bs-0889R), anti-erythrocyte membrane protein band 4.1 (EPB41) antibody (bs-13080R), anti-tumor protein D54 (TPD54) antibody (bs-6743R), anti-mucin 13 (MUC13) antibody (bs-10074R), anti-GAPDH antibody (bs-10900R), anti-caspase-3 antibody (bs-0081R) and anti-Bax antibody (bs-0127R) (all from Bioss, Beijing, China) and overnight incubation at 4°C. All primer antibodies were diluted (1:1,000) by Tris-buffered saline containing 0.1% Tween-20 (TBS-T). After washing 3 times with TBS-T, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG as a secondary antibody (1:5,000, ab6721; Abcam) for 2 h at room temperature. After washing 3 times with TBS-T buffer, the membranes were visualized with an ECL detection system (KeyGen Biotech Inc., Nanjing, China). All western blot analyses were repeated at least 3 times.

Luciferase reporter assay. The cells were seeded in a 6-well plate and transfected with siRNA according to the protocol of siRNA-Mate (GenePharma Co., Ltd.). TOP-flash reporter plasmid was purchased from Shanghai Qcbio Science and Technologies Co., Ltd. (Shanghai, China) and was transfected into the cells by Endofectin™-Plus (GeneCopoeia) according to the manufacturer's instructions 48 h after siRNA transfection. The reporter gene assay was performed 48 h post-plasmid-transfection using the Dual Luciferase Assay

System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized for transfection efficiency using the corresponding *Renilla* luciferase activity. All experiments were performed at least in triplicate.

Immunoprecipitation. The plasmids (HA-TSPAN8, Flag-TSPAN8, HA-NOTCH2 and Flag-NOTCH2) used for exogenous co-immunoprecipitation were synthesized by GeneCopoeia Co., Ltd. The cells were lysed by sonication and centrifugation at 4°C, 16,000 x g, 10 min (TDZ4-WS centrifuge; Thermo Fisher Scientific) in IP lysis buffer (Beyotime, Beijing, China) supplemented with phosphatase/protease inhibitor cocktail and 1 mM PMSF. The supernatant was transferred to a separate microfuge tube, pre-cleared with protein A/G agarose beads (Yanji Biotechnology, Shanghai, China) and centrifuged at 4°C, 16,000 x g, 5 min (TDZ4-WS centrifuge; Thermo Fisher Scientific) to pellet the beads and remove protein impurities. The supernatant was collected and incubated with rabbit IgG (bs-0295P; Bioss) overnight at 4°C. The beads were collected by centrifugation at 4°C, 16,000 x g, 10 min (TDZ4-WS centrifuge; Thermo Fisher Scientific), washed 3 times with IP lysis buffer and resuspended with 2X sodium dodecyl sulfate (SDS) loading buffer. Bound protein was eluted off the beads by boiling and examined by western blot analysis as described above.

Immunofluorescence. The cells were incubated with 4.0% paraformaldehyde for 15 min at room temperature. The cells were then washed 3 times with phosphate-buffered saline (PBS). To increase permeability, 0.1% Triton X-100 was added to the cells for 10 min. The cells were then washed again thrice with PBS. The anti- β -catenin antibody (ab16051; 1:100 diluted by PBS; Abcam) was added to the wells followed by incubation overnight at 4°C. The cells were then washed and incubated in Alexa Fluor-conjugated secondary antibody (1:100 diluted with Bioss antifade mounting medium; Bioss). DAPI (Invitrogen, Carlsbad, CA, USA) was used to dye the nuclei. The cells were incubated with DAPI for 20 min at room temperature. After being washed 3 times with PBS, the cells were imaged under a microscope (Ci-L; Nikon, Tokyo, Japan).

Protein extraction and iTRAQ labeling. Total protein extracts were prepared in lysis buffer [7 M urea, 1 mg/ml DNase I, 1 mM Na₃VO₄ (all from Sangon Biotech), and 1 mM PMSF (Bioss, Beijing, China)] using the Sample Grinding kit from Amersham Biosciences. Following being centrifuged at 17,000 x g for 15 min at 4°C, the supernatant was collected and the protein concentrations were quantified with a 2-D Quantification kit (Amersham Biosciences).

From each sample, 100 μ g of protein was precipitated, denatured, cysteine-blocked and digested with sequencing-grade modified trypsin, according to the manufacturer's instructions (iTRAQ Reagent 8 Plex Multi-plex; Applied Biosystems, Foster City, CA, USA). The samples were then labeled with the iTRAQ tags (SGC7901, 113, 115 tags; SGC7901/DDP 114, 116 tags; Applied Biosystems). The labeled samples were pooled prior to further analysis.

Fractionation of peptides. The iTRAQ-labeled samples were solubilized in 300 μ l of 1% Pharmalyte (Amersham

Biosciences) and 8 M urea solution. The samples were rehydrated on IPG gel strips (pH 3.0-10.0; Amersham Biosciences) at 30 V for 14 h. The peptides were subsequently focused successively at 500 V for 1 h, 1,000 V for 1 h, 3,000 V for 1 h and 8,000 V for 8.5 h. Following electrofocusing, the peptides were extracted from the gel using a solution containing 0.1% formic acid and 2% acetonitrile for 1 h. The fractions were then purified and concentrated on a C18 Discovery DSC-18 SPE column (Sigma-Aldrich), lyophilized and maintained at -20°C.

Mass spectrometry. The samples were analyzed using a QStar Elite hybrid mass spectrometer (Applied Biosystems) coupled with a liquid chromatography system (Amersham Biosciences, Little Chalfont, UK).

The mass spectrometer was set to perform information-dependent acquisition (IDA) in the positive ion mode at a mass range of 300-1800 m/z. Peptides with +2 to +4 charge states were selected for tandem mass spectrometry, and the time of summation of MS/MS events was set to 3 sec. We selected the two most abundantly charged peptides above a 20-count threshold for MS/MS and dynamic exclusion was set to 30 sec with a 50 mDa mass tolerance. Data were processed using ProteinPilot version 2.0 software (Applied Biosystems) and searched against the UniProt (<http://www.uniprot.org/>) human protein database (v3.77). Protein identification was based on selection thresholds of ProtScore >1.3 or ProtScore <0.77, and false discovery rate P-values <0.05.

Bioinformatics analysis. The results obtained by iTRAQ-labeled proteomics were analyzed using protein analysis using the evolutionary relationships (PANTHER) classification system (www.pantherdb.org) following the instructions available online (24). STRING 10.5 (<http://string.embl.de/>) was used to predict the interaction between proteins following the instruction online (25).

Statistical analysis. The *in vitro* experiments were repeated at least 3 times. Data are presented as the means \pm standard deviation (SD). Significance between groups from *in vitro* experiments was determined using the Student's t-test or Dunnett's T3 test. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

iTRAQ-coupled 2D LC-MS/MS analysis of differentially expressed proteins. To identify potential proteins associated with resistance to cisplatin, iTRAQ-based quantification was performed on proteins isolated from cisplatin-sensitive gastric cancer cells (SGC7901) and from DDP-resistant gastric cancer cells (SGC7901/DDP). The specimens were iTRAQ-labeled in duplicate in order to verify the results. Protein samples were labeled as follows: SGC7901, tags 113 and 114; SGC7901/DDP, tags 115 and 116. The relative abundance of protein from the SGC7901/DDP cells with respect to proteins from SGC7901 cells was calculated as the iTRAQ ratios 115:113 and 116:114. These fractions were analyzed by LC/MS/MS. The workflow of the iTRAQ proteomics approach is presented in Fig. 1. ProteinPilot 2.0 software was

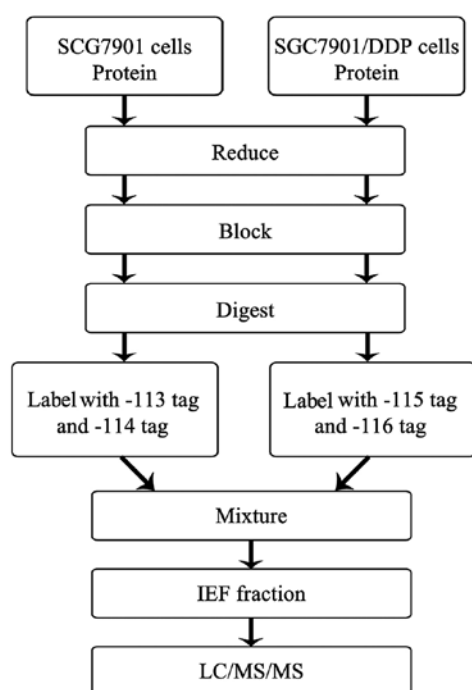


Figure 1. Flowchart of the isobaric tags for the relative and absolute quantification (iTRAQ)-based MS proteomics approach used in this study.

used for protein quantification and identification. Considering the technical variations of the method and statistical analysis in the relative quantification analysis, and in order to reduce false-positives and increase accuracy, a 1.3-fold cut-off for all iTRAQ ratios was used (26,27). Therefore, proteins with iTRAQ ratios <0.77 - or >1.3 -fold cut-off ($P < 0.05$) were considered to be downregulated or upregulated, respectively. A total of 1,324 differentially expressed proteins were identified, regardless of whether or not there was a significant P-value in the iTRAQ ratios. Of these, 112 proteins were differentially expressed in the SCG7901/ddp cells compared to the SGC7901 cells (64 upregulated and 48 downregulated proteins). The top 30 downregulated and upregulated proteins are shown in Table I.

Cellular and molecular functional characteristics of the proteins. The 112 proteins, which were potentially differentially expressed between the SGC7901/DDP cells and SGC7901 cells, were classified into 5 functional categories using the Protein Analysis through Evolutionary Relationships (PANTHER) classification system (Fig. 2). The molecular function categories were binding (23.5%), receptor activity (2.9%), structural molecule activity (14.7%), catalytic activity (44.1%) and transporter activity (14.7%) (Fig. 2).

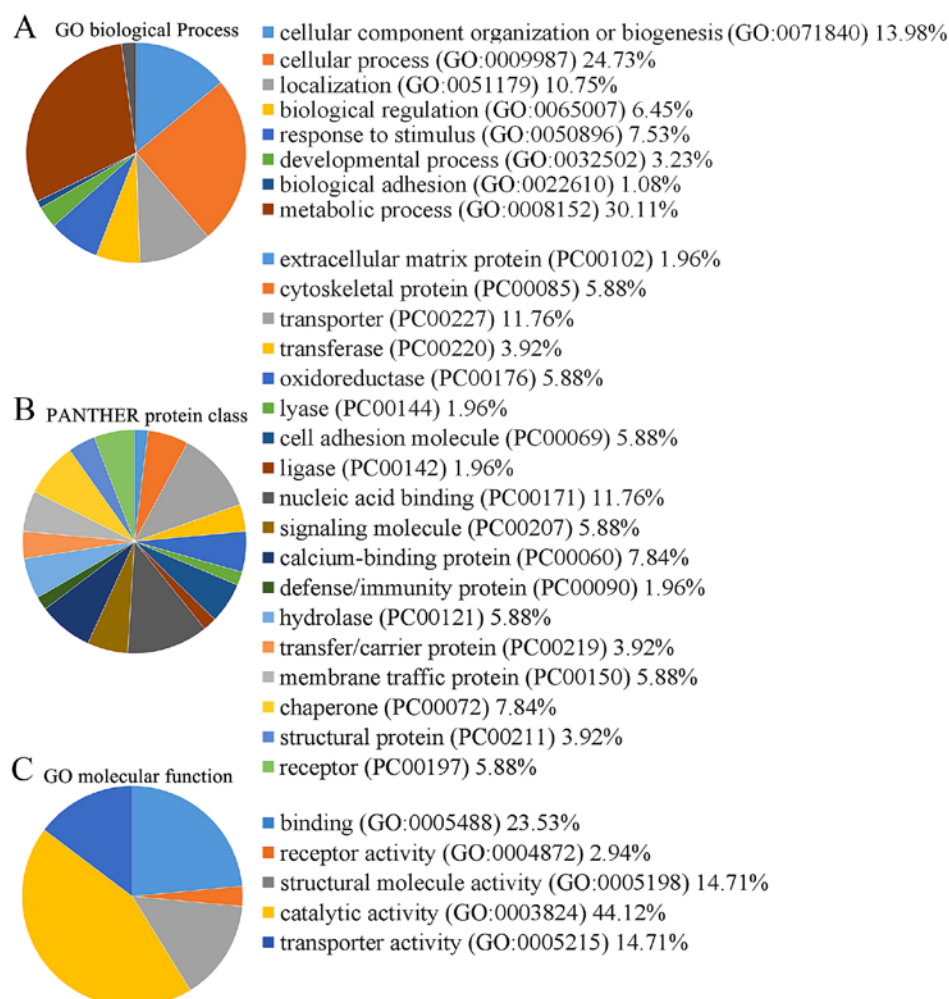


Figure 2. Classification of proteins identified through proteomics into their (A) GO biological process, (B) Protein Analysis through Evolutionary Relationships (PANTHER) protein class, and (C) GO molecular processes. This was carried out using the PANTHER Classification system (www.pantherdb.org/).

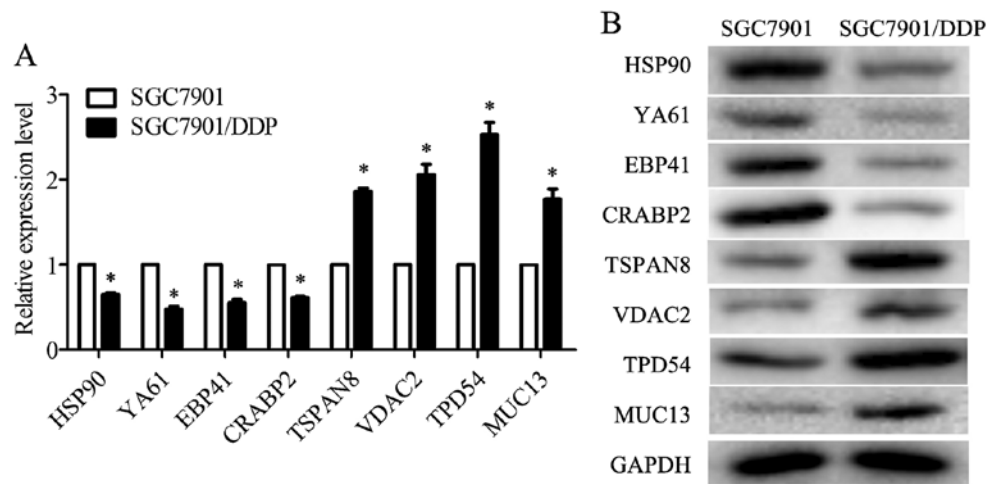


Figure 3. Validation of heat shock protein 90 (HSP90), drug-sensitive protein 1 (YA61), erythrocyte membrane protein band 4.1 (EPB41), cellular retinoic acid-binding protein 2 (CRABP2), tetraspanin-8 (TSPAN8), voltage-dependent anion-selective channel protein 2 (VDAC2), tumor protein D54 (TPD54) and mucin 13 (MUC13) expression in the SGC7901 and SGC7901/DDP cells. (A) RT-qPCR was used to detect the relative mRNA expression levels of HSP90, YA61, EPB41, CRABP2, TSPAN8, VDAC2, TPD54 and MUC13, as normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) ($P < 0.05$). (B) Representative western blot analyses for HSP90, YA61, EPB41, CRABP2, TSPAN8, VDAC2, TPD54 and MUC13 expression in cells. GAPDH was used as the normalization standard. Data are the means \pm SD; * $P < 0.05$ vs. negative control (NC).

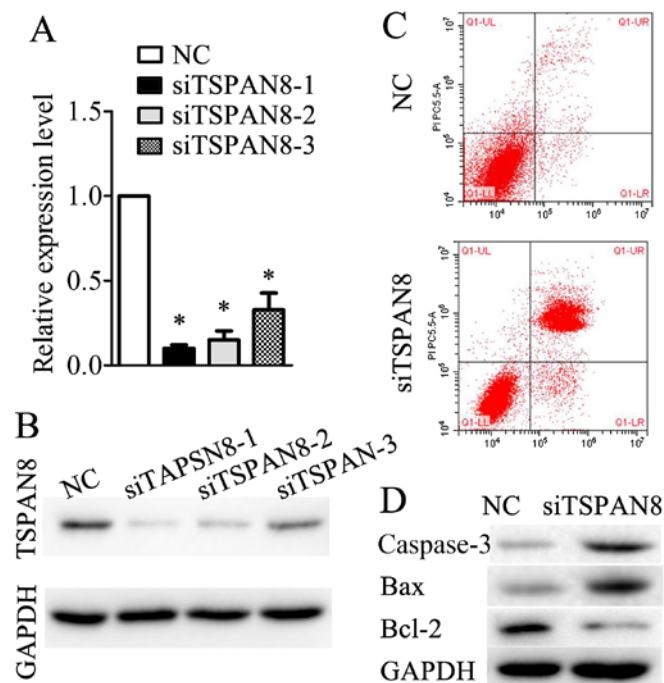


Figure 4. Validation of the silencing of tetraspanin-8 (TSPAN8) using siRNA. (A) The mRNA expression of TSPAN8 was determined by RT-qPCR. TSPAN8 was effectively silenced at the mRNA level by siRNA sequences. (B) The protein expression of TSPAN8 was determined by western blot analysis. TSPAN8 was effectively silenced at the protein level by siRNA sequences. (C) Cell apoptosis was examined by Annexin V FITC-A flow cytometry. Apoptosis was increased in the TSPAN8-silenced SGC7901/DDP cells. (D) The protein expression of caspase-3, Bax and Bcl-2 was examined by western blot analysis. In TSPAN8-silenced SGC7901/DDP cells, the expression of caspase-3 and Bax was increased, while that of Bcl-2 was decreased. Data are the means \pm SD; * $P < 0.05$ vs. negative control (NC).

Validation of differentially expressed proteins. The differentially expressed proteins identified by iTRAQ were validated by RT-qPCR and western blot analysis. The proteins selected for validation were the ones most significantly dysregulated

according to protein classification or the ones closely related to multidrug resistance. TSPAN8 has been reported to promote the proliferation and metastasis of SGC7901 cells. The results from iTRAQ-coupled 2D LC-MS/MS revealed that TSPAN8 was potentially related to drug resistance in the SGC7901/DDP cells. Thus, it was selected as the object of the following analysis. The mRNA levels of HSP90, drug-sensitive protein 1 (YA61), EPB41 and CRABP2 were decreased in the SGC7901/DDP cells when compared with those in the SGC7901 cells, whereas the mRNA levels of TSPAN8, VDAC2, TPD54 and MUC13 were increased (Fig. 3A). The results of western blot analysis revealed that the protein expression levels of HSP90, YA61, EPB41 and CRABP2 were downregulated in the SGC7901/DDP cells when compared to those in the SGC7901 cells, whereas the levels of TSPAN8, VDAC2, TPD54 and MUC13 were upregulated (Fig. 3B). These results were consistent with the trend observed in iTRAQ analysis.

Silencing of TSPAN8 in SGC7901/DDP cells reduces MDR. TSPAN8 has been reported to be an oncoprotein in gastric cancer, enhancing gastric cancer cell proliferation and metastasis (20). However, the role of TSPAN8 in gastric cancer cell drug resistance remains unclear. In the present study, TSPAN8 was knocked down by siRNA. RT-qPCR and western blot analysis confirmed the efficacy of the silencing of TSPAN8. As shown in Fig. 4A, the relative mRNA level of TSPAN8 was significantly decreased following transfection with siRNA against TSPAN8. The results of western blot analysis revealed that TSPAN8 protein expression in specific siRNA-transfected SGC7901/DDP cells was effectively suppressed (Fig. 4B). Of the 3 siRNA sequences, sequence 1 was found to be the most suitable for our purposes (Fig. 4A and B), and was thus used in all subsequent experiments.

MDR is the main cause of chemotherapy failure in gastric cancer treatment. Thus, in this study, to assess the association between TSPAN8 and MDR, the siRNA-transfected

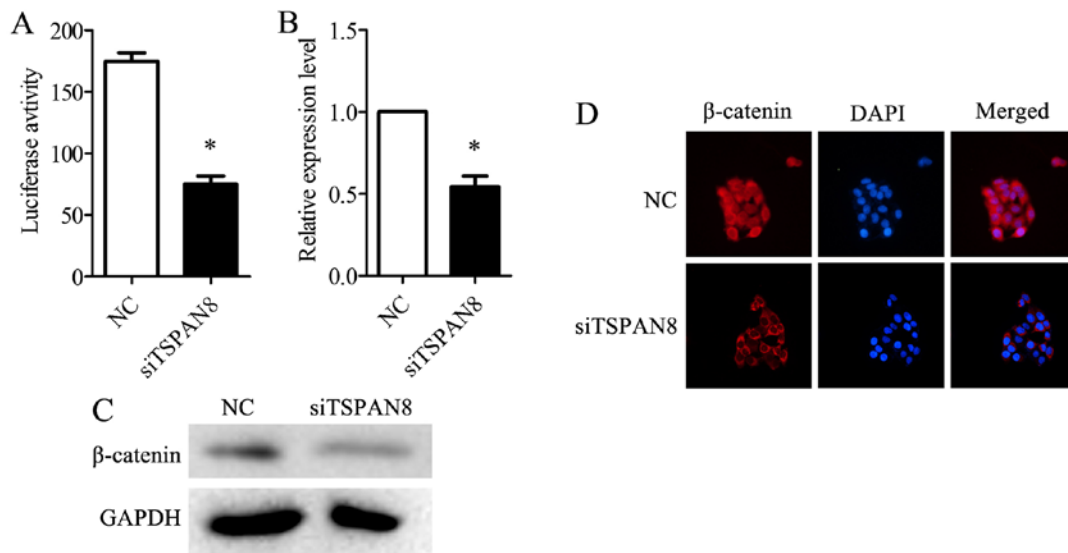


Figure 5. Tetraspanin-8 (TSPAN8) silencing decreases Wnt/β-catenin activity. (A) Wnt pathway activity was examined by luciferase reporter assay. Wnt activity was decreased in the TSPAN8-silenced SGC7901/DDP cells. The expression of β-catenin was examined at (B) the mRNA level using RT-qPCR and at (C) the protein level using western blot analysis. (D) The expression of β-catenin was decreased at both the mRNA and protein level in TSPAN8-silenced SGC7901/DDP cells. The transfer of β-catenin into the nucleus was determined using immunofluorescence. The red fluorescence represents β-catenin, while the blue fluorescence represents the nucleus of SGC7901/DDP cells. The pink color in the merged photos represents β-catenin in the nucleus. When TSPAN8 was silenced, the β-catenin staining in the nucleus was less than that in the negative control (NC) group. Data are the means ± SD; *P<0.05 vs. negative control NC.

SGC7901/DDP cells were treated with cisplatin, 5-Fu and adriamycin (the most commonly used drugs in clinical practice for the chemotherapeutic treatment of gastric cancer), for 2 days and the IC₅₀ values were determined. The IC₅₀ values of cisplatin, 5-Fu and adriamycin were significantly decreased in the TSPAN8-silenced SGC7901/DDP cells compared with the negative controls (Table II). This result suggested that the silencing of TSPAN8 reduced the resistance of the SGC7901/DDP cells to the aforementioned drugs, which, in turn, indicated that TSPAN8 may contribute to the MDR of this cell line. In the following experiments, only cisplatin was used to maintain the drug resistance of the SGC7901/DDP cells.

Furthermore, compared with the negative control SGC7901/DDP cells, apoptosis was increased in the TSPAN8-silenced cells (Fig. 4C). Moreover, the levels of apoptosis-related proteins (caspase-3, Bax and Bcl-2) were examined by western blot analysis. The results (Fig. 4D) revealed that the levels of caspase-3 and Bax were upregulated, while those of Bcl-2, an anti-apoptotic protein, were downregulated in the TSPAN8-silenced SGC7901/DDP cells. These results indicated that the silencing of TSPAN8 promoted SGC7901/DDP cell apoptosis.

Silencing of TSPAN8 sensitizes SGC7901/DDP cells to chemotherapy by mediating Wnt/β-catenin. Thus far, our findings suggested that TSPAN8 plays a critical role in the drug resistance of SGC7901/DDP cells. It is believed that metastasis is the persistence of cancer stem cells (CSCs), which are highly resistant to chemotherapy (28). The Wnt/β-catenin signaling pathway has been reported to increase gastric cancer cell migration and invasion (29). Therefore, in this study, we investigated whether TSPAN8-mediated gastric cancer cell drug resistance is also related to the Wnt/β-catenin pathway. The Wnt/β-catenin pathway activity was detected using a TOP-flash luciferase reporter. The silencing of TSPAN8 in

the SGC7901/DDP cells significantly decreased TOP-flash luciferase activity (Fig. 5A). The TSPAN8-silenced cells displayed a decreased expression of β-catenin at both the mRNA (Fig. 5B) and protein level (Fig. 5C), compared to negative control (NC)-infected SGC7901/DDP cells. Additionally, the accumulation of β-catenin in the nucleus was impaired in the TSPAN8-silenced SGC7901/DDP cells (Fig. 5D). The cells were treated with CCT036477 (CCT) and XAV939 (inhibitors of the Wnt-β-catenin pathway) (30). The reduced IC₅₀ value caused by TSPAN8 silencing was partially reversed when the Wnt-β-catenin pathway inhibitors were added (Table III). These data indicated that TSPAN8 enhanced the resistance of the SGC7901/DDP cells to chemotherapy through the activation of the Wnt/β-catenin pathway and by increasing β-catenin expression and accumulation in the nucleus. However, compared to the NC group, the inhibitors of the Wnt pathway still decreased the IC₅₀ values (Table III).

TSPAN8 mediated Wnt/β-catenin through binding to NOTCH2. To identify which protein or proteins interact with TSPAN8, we utilized STRING 10.5. NOTCH2 was predicted to interact with TSPAN8. Co-immunoprecipitation was used to validate the association between TSPAN8 and NOTCH2. Endogenous co-immunoprecipitation assays revealed that TSPAN8 interacted with NOTCH2 in the SGC7901/DDP cells (Fig. 6A). Consistent with this result, the exogenous interaction between TSPAN8 and NOTCH2 was also observed in the SGC7901/DDP cells that were co-transfected with HA-TSPAN8 and Flag-NOTCH2 (Fig. 6B). These findings revealed that TSPAN8 acts in combination with NOTCH2 in gastric cancer cells. Furthermore, we found that the impairment of β-catenin expression was partially compensated when DAPT (30), a NOTCH2 inhibitor, was used in the TSPAN8-silenced SGC7901/DDP cells (Fig. 6C and D). The results data indicated that TSPAN8 mediated the activation of the Wnt/β-catenin pathway by binding to NOTCH2.

Table I. Partial list of proteins differentially expressed between the SGC7901 and SGC7901/DDP cells.

No.	UniProtKB accession ID	UniProtKB ID	Protein name	Peptides (95%)	115:113	pvAl 115:113	116:114	pvAl 116:114
Top 30 downregulated proteins in the multidrug-resistant SGC7901/DDP gastric cancer cells								
1	B4DQE1	B4DQE1_HUMAN	Annexin	48	0.622325778	0.02227027	0.634360694	7.79E-05
2	P11171	41_HUMAN	Protein 4.1	72	0.624904871	0.03530645	0.710090963	4.57E-06
3	G3V4C1	G3V4C1_HUMAN	Heterogeneous nuclear ribonucleoproteins C1/C2	31	0.628175676	0.000760296	0.725739842	4.02E-05
4	P22626	ROA2_HUMAN	Heterogeneous nuclear ribonucleoproteins A2/B1	10	0.655659914	0.00013268	0.639985561	0.001628224
5	A8K2H4	A8K2H4_HUMAN	cDNA FLJ78235	15	0.660123587	0.005608639	0.673964346	0.00055461
6	Q9NZ23	Q9NZ23_HUMAN	Drug-sensitive protein 1	9	0.663049996	0.01362959	0.715051212	0.007643274
7	P14866	HNRPL_HUMAN	Heterogeneous nuclear ribonucleoprotein L	32	0.663075626	4.51E-05	0.614491193	2.93E-08
8	O43390	HNRPR_HUMAN	Heterogeneous nuclear ribonucleoprotein R	16	0.675710618	7.89E-07	0.617349922	0.01043923
9	P10253	LYAG_HUMAN	Lysosomal α -glucosidase	7	0.677277982	0.004195308	0.622946714	0.001522158
10	B4DVA7	B4DVA7_HUMAN	β -hexosaminidase	25	0.690114915	5.21E-05	0.728313433	0.003678592
11	Q6RFH5	WDR74_HUMAN	WD repeat-containing protein 74	8	0.703277528	0.038117841	0.727350508	0.01727649
12	B3KM89	B3KM89_HUMAN	cDNA FLJ10528 fis, clone NT2R2P2000943, highly similar to protein transport protein Sec24D	36	0.706753314	0.024660509	0.737365516	0.000550256
13	D3DQU2	D3DQU2_HUMAN	Tripeptidyl peptidase I, isoform CRA_a	22	0.707172573	0.003796435	0.658462612	1.84E-05
14	P07900	HS90A_HUMAN	Heat shock protein HSP 90- α	7	0.71462971	8.03E-08	0.703796549	0.002079676
15	Q53GL6	Q53GL6_HUMAN	RNA binding protein (autoantigenic, hnRNP-associated with lethal yellow) long isoform variant (Fragment)	5	0.729635179	0.002883428	0.721853618	0.024822449
16	P17050	NAGAB_HUMAN	α -N-acetylgalactosaminidase	3	0.736388922	0.016721571	0.683589351	0.0137383
17	Q15334	L2GL1_HUMAN	Lethal(2) giant larvae protein homolog 1	23	0.741260827	0.01128356	0.676991196	8.27E-05
18	P08238	HS90B_HUMAN	Heat shock protein HSP 90- β	31	0.745372176	4.53E-05	0.736570076	3.96E-05
19	Q9UHL4	DPP2_HUMAN	Dipeptidyl peptidase 2	15	0.745664179	0.01644465	0.723860763	0.001684257
20	B3KXS5	B3KXS5_HUMAN	Lon protease homolog, mitochondrial	9	0.750456929	1.18E-07	0.623211837	0.02029554
21	P29373	RABP2_HUMAN	Cellular retinoic acid-binding protein 2	6	0.752079427	0.000808526	0.647008409	0.0207015
22	B3KQS9	B3KQS9_HUMAN	cDNA PSEC0141 fis, clone PLACE1005913, highly similar to deoxyribonuclease-2- α (EC 3.1.22.1)	17	0.753284812	0.02952897	0.736696043	0.01036579
23	A8K9X5	A8K9X5_HUMAN	cDNA FLJ76472, highly similar to <i>Homo sapiens</i> Fas (TNFRSF6) associated factor 1 (FAF1), transcript variant 1, mRNA	12	0.754278779	0.000863104	0.61996146	0.009885758
24	Q53FG3	Q53FG3_HUMAN	Interleukin enhancer binding factor 2 variant (Fragment)	12	0.760721684	0.000109113	0.713951109	0.006046766
25	F8W1F5	F8W1F5_HUMAN	Formin-like protein 3	2	0.762245297	0.04186723	0.695746288	0.009152975
26	O60826	CCD22_HUMAN	Coiled-coil domain-containing protein 22	19	0.766084313	0.031637449	0.680582983	7.48E-05
27	D6RD18	D6RD18_HUMAN	Heterogeneous nuclear ribonucleoprotein A/B	11	0.768395722	0.001917408	0.630256551	0.003946933

Table I. Continued.

No.	UniProtKB accession ID	UniProtKB ID	Protein name	Peptides (95%)	115:113	pvAl 115:113	116:114	pvAl 116:114
28	Q9HB71	CYBP_HUMAN	Calcyclin-binding protein	12	0.768992722	0.01458319	0.735216274	0.03016348
29	O43175	SERA_HUMAN	D-3-phosphoglycerate dehydrogenase	8	0.769186914	1.83E-05	0.709687752	0.005728934
30	Q53FB6	Q53FB6_HUMAN	Mitochondrial aldehyde dehydrogenase 2 variant (Fragment)	62	0.7699821	2.35E-06	0.695289527	1.13E-10
Top 30 upregulated proteins in the multidrug-resistant SGC7901/DDP gastric cancer cells								
1	P02768	ALBU_HUMAN	Serum albumin	21	2.153665066	0.004250416	1.802739155	0.003726222
2	P05787	K2C8_HUMAN	Keratin, type II cytoskeletal 8	188	1.95599401	1.16E-14	1.636557607	0.043917108
3	P08727	K1C19_HUMAN	Keratin, type I cytoskeletal 19	105	1.943524003	0.003219714	1.795400163	0.001931372
4	Q9GZL9	Q9GZL9_HUMAN	β -globin (Fragment)	5	1.916771054	0.01608417	1.928079502	0.004268234
5	O75348	VATG1_HUMAN	V-type proton ATPase subunit G1	4	1.765862942	0.02341911	1.686415416	0.021376461
6	B7Z8Q2	B7Z8Q2_HUMAN	cDNA FLJ55606, highly similar to α -2-HS-glycoprotein	5	1.763658047	0.0266499	1.5277603	0.02854233
7	B2RA03	B2RA03_HUMAN	cDNA, FLJ94640, highly similar to <i>Homo sapiens</i> keratin 18 (KRT18), mRNA	139	1.743123055	1.17E-11	1.530218785	0.037320711
8	B2RAU8	B2RAU8_HUMAN	cDNA, FLJ95131, highly similar to <i>Homo sapiens</i> nucleolar and coiled-body phosphoprotein 1 (NOLC1), mRNA	33	1.705428004	0.002081443	1.580844317	0.03559231
9	P45880	VDAC2_HUMAN	Voltage-dependent anion-selective channel protein 2	32	1.693142056	8.20E-08	1.963505651	0.035407521
10	Q9NY12	GAR1_HUMAN	H/ACA ribonucleoprotein complex subunit 1	4	1.674811006	0.033414129	1.769748098	0.003661653
11	I1VZV6	I1VZV6_HUMAN	Hemoglobin α 1	5	1.66385603	0.00999979	1.995862361	0.03718495
12	D3DV26	D3DV26_HUMAN	S100 calcium binding protein A10 [Annexin II ligand, calpactin I, light polypeptide (P11)], isoform CRA_b (Fragment)	5	1.651872993	0.024565291	1.546541111	0.039673839
13	A0A024RD07	A0A024RD07_ HUMAN	Trinucleotide repeat containing 5, isoform CRA_c	4	1.56981504	0.04436332	1.891481629	0.01262225
14	B2R6W1	B2R6W1_HUMAN	cDNA, FLJ93143, highly similar to <i>Homo sapiens</i> complement component 7 (C7), mRNA	6	1.568076968	0.02081763	1.911388803	0.042174641
15	B4DRB6	B4DRB6_HUMAN	cDNA FLJ59394, highly similar to <i>Homo sapiens</i> ubiquitin associated protein 2 (UBAP2), transcript variant 1, mRNA	7	1.550289989	0.00021213	2.001291157	0.016664799
16	B2RAW0	B2RAW0_HUMAN	cDNA, FLJ95154, highly similar to <i>Homo sapiens</i> disabled homolog 2, mitogen-responsive phosphoprotein (<i>Drosophila</i>) (DAB2), mRNA	3	1.548756003	0.002253909	1.844182745	0.04310175
17	Q96AG4	LRC59_HUMAN	Leucine-rich repeat-containing protein 59	28	1.539394975	1.52E-06	2.009917718	0.045030121

Table I. Continued.

No.	UniProtKB accession ID	UniProtKB ID	Protein name	Peptides (95%)	115:113	pvAl 115:113	116:114	pvAl 116:114
18	E9PR17	E9PR17_HUMAN	CD59 glycoprotein	9	1.53618896	0.0128732	1.634750878	0.03102315
19	Q8IVT2	MISP_HUMAN	Mitotic interactor and substrate of PLK1	25	1.531394005	6.58E-11	1.83911107	5.09E-06
20	Q6IBP2	Q6IBP2_HUMAN	HMOX2 protein	17	1.531041026	9.00E-06	1.997268331	0.04397554
21	P19075	TSN8_HUMAN	Tetraspanin-8	4	1.520612955	0.035661951	1.841773812	0.01545809
22	Q15847	ADIRF_HUMAN	Adipogenesis regulatory factor	5	1.518458962	0.003934072	1.812744723	0.01480945
23	Q9H6F5	CCD86_HUMAN	Coiled-coil domain-containing protein 86	5	1.511031032	0.047092739	1.67638497	0.003332002
24	P21796	VDAC1_HUMAN	Voltage-dependent anion-selective channel protein 1	41	1.473088026	4.93E-06	1.983616262	0.00978965
25	Q8NBU5	ATAD1_HUMAN	ATPase family AAA domain-containing protein 1	6	1.471879005	0.01452017	1.612720741	0.002527138
26	Q9H3R2	MUC13_HUMAN	Mucin-13	10	1.469835043	0.016417651	1.633036662	0.000101379
27	O76021	RLID1_HUMAN	Ribosomal L1 domain-containing protein 1	20	1.464844942	0.000926223	1.712203445	0.001586069
28	H0Y7A7	H0Y7A7_HUMAN	Calmodulin (Fragment)	24	1.459040999	0.008054786	1.952625441	7.56E-08
29	Q99808	S29A1_HUMAN	Equilibrative nucleoside transporter 1	2	1.452139974	0.044763569	1.548505666	0.004392849
30	Q14684	RRPIB_HUMAN	Ribosomal RNA processing protein 1 homolog B	21	1.450567961	0.00257107	1.960259671	0.003726222

Table II. IC₅₀ values (mg/l) for selected reagents after siRNA transfection.

Treatment	NC	siTSPAN8
Cisplatin	8.25±0.57	3.89±0.27 ^a
5-Fu	4.43±0.22	2.41±0.16 ^a
Adriamycin	2.48±0.19	1.54±0.10 ^a

Data are the means ± SD. ^aP<0.05 vs. the negative control (NC) in the same treatment group. 5-Fu, 5-fluorouracil.

Table III. TSPAN8-silencing mediated reduction of IC₅₀ could be partially reversed by Wnt/β-catenin inhibitors.

Treatment	NC	siTSPAN8 +		
		siTSPAN8	CCT036477	XAV939
Cisplatin	7.97±0.62	3.77±0.41 ^a	5.92±0.51 ^{a,b}	5.84±0.48 ^{a,b}

Data are the means ± SD. ^aP<0.05 vs. negative control (NC); ^bP<0.05 vs. siTSPAN8 group. TSPAN8, tetraspanin-8.

Discussion

Gastric cancer is one of the most common malignant tumors worldwide (31), and is the leading cause of morbidity and mortality among malignant tumors in East Asia (32). Unfortunately, the majority of patients are diagnosed at the advanced stages of the disease, when chemotherapy is regarded as an important component of multimodal treatment (33). Platinum- or fluorouracil-based chemotherapy is established as the first-line treatment for patients with advanced gastric cancer (34). Cisplatin and other platinum-based cancer drugs destroy tumor cells by binding to DNA strands and interfering with DNA replication (33,34). While cisplatin is often effective when first administered, clinical drug resistance to cisplatin-based chemotherapy is considered a major impediment in the treatment of patients with gastric cancer (3,34). Drug resistance in cancer patients includes the development of intrinsic or acquired drug resistance against chemotherapeutic agents (35). The resistance phenotype is associated with cancer cells gaining a cross-resistance to a large range of drugs that are structurally and functionally different, referred to as MDR (36). The mechanisms of MDR in cancer remain understood on only a limited basis. A wide range of mechanisms contribute to MDR, including drug efflux mediation by ATP-binding cassette (ABC) transporter, the prevention of apoptosis, alterations in drug targets, the aberrant activation of cell signaling pathways, altered cell cycle events, cancer stem cells (CSC), epigenetic regulation, tumor microenvironment and many other causes (8,36). MDR results in treatment failure or even death in patients with gastric cancer (4,37) and, as such, strategies to reverse MDR have been a high priority goal in cancer research.

In the present study, we searched for proteins possibly related to drug resistance in the human gastric cancer cell

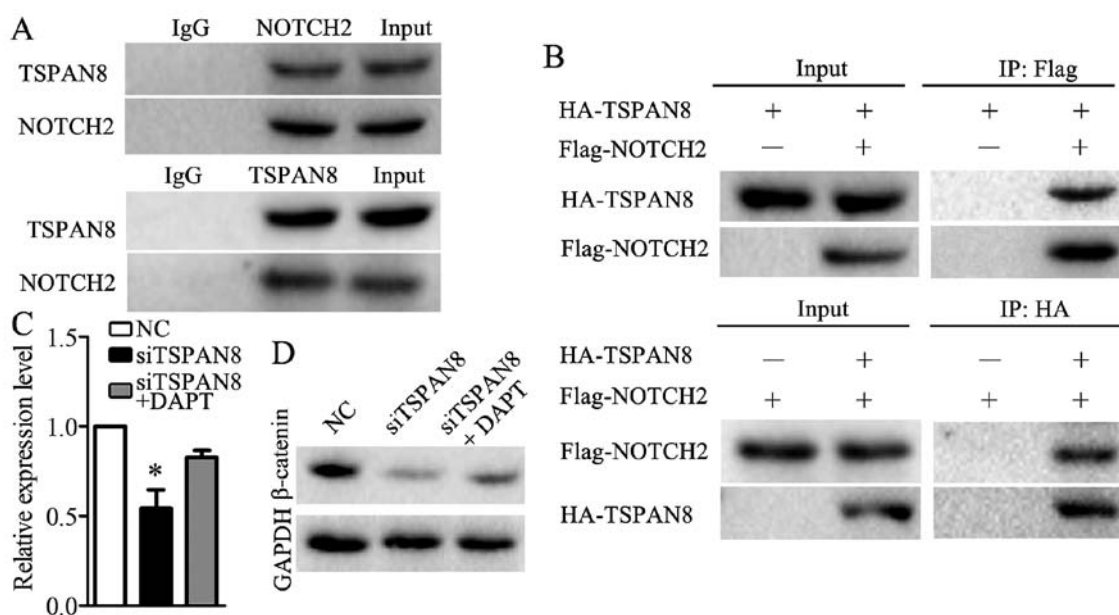


Figure 6. Tetraspanin-8 (TSPAN8) mediates β -catenin expression by binding to NOTCH2. (A) Co-immunoprecipitation of endogenous TSPAN8 and NOTCH2. Non-denatured cell extracts prepared from SGC7901/DDP cells were immunoprecipitated with anti-TSPAN8, anti-NOTCH2 or control IgG antibody. The immune complexes and the input (10% of cells extracts used in the immunoprecipitation step) were analyzed by immunoblotting with antibody specific to TSPAN8 (the first panel) or NOTCH2 (the fourth panel). The same membrane was stripped and reprobed to detect NOTCH2 (the second panel) or TSPAN8 (the third panel). (B) Immunoblot analysis of extracts after immunoprecipitation from SGC7901/DDP cells transfected with HA-TSPAN8 and Flag-NOTCH2. When NOTCH2 was blocked by DAPT, the TSPAN8-mediated downregulation of β -catenin was partially reversed at both (C) the mRNA level and (D) protein level. Data are the means \pm SD; * P <0.05 vs. negative control (NC).

lines, SGC7901 and SGC7901/DDP, using iTRAQ-based quantitative proteomics. In total, 64 proteins were found to be increased, while 48 proteins were found to be decreased, in the SGC7901/DDP cancer cells, compared with the drug-sensitive SGC7901 cells. VDAC2, TPD53, MUC13 and HSP90 (38-41) have been previously reported to be closely associated with MDR. Thus, these proteins and another 4 of the mostly dysregulated proteins were selected for validation. Western blot analysis revealed that the expression levels of TSPAN8, VDAC2, TPD54, MUC13, HSP90, YA61, EPB41 and CRABP2 were validated at the same levels as those obtained from the results of the quantitative proteomic analysis, confirming that the iTRAQ-based quantitative proteomics is an efficient and powerful method for the analysis of MDR-related proteins. TSPAN8 expression was found to be significantly increased in the SGC7901/DDP cells, the drug-resistant gastric cancer cell line. The overexpression of TSPAN8 has been reported in many types of cancer, including hepatocellular carcinoma, pancreatic cancer, colon carcinoma and gastric cancer (14-15,20,42). TSPAN8 has been implicated as increasing the proliferation, migration and invasion of many types of cancer cells, including gastric cancer cells (20). However, the role of TSPAN8 in the MDR of gastric cancer cells remains unknown. In this study, the iTRAQ-based quantitative proteomics data indicated that TSPAN8 contributed to MDR in the SGC7901/DDP cells. To confirm this, we silenced TSPAN8 in the SGC7901/DDP cells via RNA interference. The IC_{50} results revealed that the silencing of TSPAN8 increased the response of the gastric cancer cells to the anticancer drugs. The silencing of TSPAN8 also increased cell apoptosis. These results indicated that TSPAN8 facilitates the MDR of SGC7901/DDP cells by suppressing apoptosis.

The aberrant activation of the Wnt/ β -catenin pathway leads to cancer cell invasion, migration and MDR (27,43). Thus, in this study, Wnt/ β -catenin activity was monitored in the TSPAN8-silenced cells. The results revealed that silencing TSPAN8 significantly decreased Wnt activity and β -catenin expression in the SGC7901/DDP cells. We also found that the IC_{50} of the SGC7901/DDP cells treated with cisplatin was decreased when TSPAN8 was silenced; however, this effect of TSPAN8 silencing was partially reversed when Wnt/ β -catenin pathway inhibitors were used. All these data indicated that TSPAN8 enhanced the resistance of SGC7901/DDP cells to chemotherapy through the activation of the Wnt/ β -catenin pathway and by increasing β -catenin expression and accumulation in the nucleus. When the Wnt/ β -catenin pathway is aberrantly activated, the transcription of downstream genes mediated by Wnt signaling increases. A number of Wnt targeting genes, such as LEF1 and c-MYC, induce drug resistance in cancer cells (44,45). This explains how TSPAN8 increases the MDR of SGC7901/DDP cells by mediating the Wnt/ β -catenin pathway.

To further explore the mechanisms of action of TSPAN8 as regards MDR, we searched the biological database. It was predicted that TSPAN8 may interact with NOTCH2 (46), which has been reported to participate in Wnt/ β -catenin-based MDR in osteosarcoma (31). We hypothesized that TSPAN8 mediated the activation of the Wnt/ β -catenin pathway by binding to NOTCH2 in SGC7901/DDP cells. Co-immunoprecipitation revealed that TSPAN8 bound to NOTCH2. The impairment of β -catenin expression was partially compensated when DAPT, a NOTCH2 inhibitor, was used in TSPAN8-silenced SGC7901/DDP cells. These data indicated that TSPAN8 mediated Wnt/ β -catenin pathway activation by binding to NOTCH2. However, further studies are warranted in order to

elucidate the mechanisms through which TSPAN8 interacts with NOTCH2 in MDR. Taken together, our study indicates that the inhibition of TSPAN8 sensitizes gastric cancer cells to chemotherapeutic drugs. However, to obtain a more complete picture of the molecular mechanisms involved in the regulation of the MDR of SGC7901/DDP by TSPAN8, further studies are required in the future.

In conclusion, the present study demonstrates that TSPAN8 impairs the sensitivity of SGC7901/DDP gastric cancer cells to chemotherapeutic agents by mediating Wnt/ β -catenin activity. TSPAN8 also mediates β -catenin expression and accumulation by binding to NOTCH2. This study provides novel insight for drug designs that overcome cisplatin resistance in gastric cancer cells.

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