Upregulation of FAM3B Promotes Cisplatin Resistance in Gastric Cancer by Inducing Epithelial-Mesenchymal Transition

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Cisplatin (CDDP) remains one of the primary chemotherapeutic agents for gastric cancer patients. However, relapse and metastasis are common because of innate and acquired chemo-resistance. Family with sequence similarity 3 (FAM3) is a novel cytokine-like protein that has an important role in tumor progression, but little is known about the role of FAM3B in human gastric cancer CDDP resistance. In this study, we investigated the role of FAM3B in gastric cancer CDDP resistance and reveal the possible underlying mechanism.

Material/Methods: We firstly developed a CDDP-resistant gastric cell line AGS/CDDP by treating AGS cells to a continuous exposure of CDDP. The FAM3B levels were compared in these 2 cell lines by quantitative real time polymerase chain reaction (qRT-PCR) and western blotting. Cell viability, apoptosis and epithelial-mesenchymal transition (EMT) related changes were detected after ectopic expression or interfering of FAM3B.

Results: We found increased FAM3B expression in AGS/CDDP cells. FAM3B overexpression induced CDDP resistance in AGS cells. Conversely, FAM3B knockdown enhanced CDDP sensitivity of AGS/CDDP cells. Moreover, FAM3B induced EMT in gastric cancer cells by upregulating snail. Inhibition of snail reversed FAM3B-triggered EMT and CDDP resistance.

Conclusions: Upregulation of FAM3B triggered CDDP resistance in gastric cancer cells by inducing EMT in a snail-dependent manner, making FAM3B a promising therapeutic target to reverse gastric cancer chemo-resistance.

MeSH Keywords: Cisplatin • Epithelial-Mesenchymal Transition • Stomach Neoplasms

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Background

Gastric cancer is one of the most commonly diagnosed malignancy in men and women, with more than 1 million new cases happening every year. It is also one of the leading causes of cancer-related death in males and females, resulting in over 700 000 deaths in 2012 worldwide [1]. Though incidence and mortality rates of gastric cancer have declined steadily in the majority of more developed countries since the middle of the 20th century, they are still higher in China. Gastric cancer ranks the second most commonly diagnosed and the second most deadly cancer in China [2].

The comprehensive therapy of gastric cancer is based on surgery accompanied with radiation and/or chemotherapy. The combined pre-operative and post-operative chemotherapy has been reported to show promising effects by increasing patient survival, among which cisplatin (CDDP) remains one of the primary chemotherapeutic agents for gastric cancer. The anti-cancer effect of CDDP lies in causing DNA damage which leads to inhibition of DNA replication and transcription, thus results in cell cycle arrest and cell death [3]. Despite that it has a consistent rate of initial responses, innate and acquired chemo-resistance remains the most significant obstacles to effective chemotherapy and better prognosis of gastric cancer patients. Thus, the investigation into the mechanisms underlying CDDP resistance of gastric cancer has clinical significance in preventing and reversing chemo-resistance.

FAM3 is a cytokine-like gene family containing 4 genes: FAM3A, FAM3B, FAM3C, and FAM3D. The 4 members all encode a protein (224–235 amino acids) with a hydrophobic leader sequence [4]. FAM3 has been reported to have important functions in amount of major diseases, such as diabetes, Alzheimer disease, and cancer [5–7]. FAM3B, also named pancreatic-derived factor (PANDER), is the most studied FAM3 family member. FAM3B was originally found in pancreatic α and β cells in endocrine pancreas and was involved in glucose metabolism and lipogenesis [4,8,9]. Besides its function in glycolipid metabolism, FAM3B has also been reported to have an important role in tumor progression. In colon cancer, esophageal squamous cell carcinoma (ESCC) and prostate cancer, FAM3B was reported to be upregulated and was correlated with bad prognosis of the patients. FAM3B inhibits cell death through different mechanisms [10–12]. However, the role of FAM3B in human gastric cancer CDDP resistance has not been reported.

In this research, we aimed to investigate the role of FAM3B in gastric cancer CDDP resistance and reveal the possible underlying mechanism. Our study found that FAM3B was upregulated in CDDP-resistant AGS/CDDP cells. FAM3B overexpression induced CDDP resistance in AGS cells, nevertheless, FAM3B knockdown sensitized AGS/CDDP cells to CDDP. Mechanically, FAM3B triggered CDDP resistance by inducing epithelial-mesenchymal transition (EMT) in a snail-dependent manner. Our study demonstrated that the role FAM3B/EMT regulatory axis in mediating gastric cancer CDDP resistance.

Material and Methods

Cell lines and reagents

The human gastric cancer cell line AGS was purchased from ATCC (Manassas, VA, USA). The CDDP-resistant AGS/CDDP cell line was developed by continuous administration of CDDP starting from 0.1 μg/mL to 5 μg/mL and maintained in 5 μg/mL of CDDP. To perform experiments, AGS/CDDP cells were resuspended in CDDP-free RPMI-1640 medium (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA) overnight for attachment onto the culture plate. AGS cells were maintained in the RPMI-1640 medium with 10% FBS and incubated at 37°C with 5% CO_2.

CDDP was obtained from Selleck Chemicals (Houston, TX, USA). The rabbit monoclonal antibodies against human E-cadherin (3195), vimentin (5741), snail (3879), P65 (8242), P-P65 (3033), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5174) were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-human FAM3B polyclonal antibody (ab103154) was obtained from Abcam (Cambridge, UK).

Cell viability

The cell viability was determined by using Cell Counting Kit-8 (CCK-8) assay. In brief, gastric cancer cells were seeded in 96-well plates and cultured overnight. Then the cells were incubated with CDDP of various concentrations for 48 hours. Thereafter, CCK-8 (Dojindo Laboratories, Japan) was added to the plates and incubated for 2 hours at 37°C. The absorbance at 490 nm was detected by a spectrophotometer (Thermo Fisher Scientific, USA).

Flow cytometry

Gastric cancer cells which were used to determine the apoptotic rates were collected by centrifugation at 1000×g for 5 minutes. After resuspension, the cells were incubated with 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) (MultiSciences, Hangzhou, China) and maintained in the dark for 10 minutes, and then cells were subjected to flow cytometric analysis. The apoptotic cell rates were compared.

Western blotting

Whole-cell protein lysates were prepared, and western blotting analysis were performed as previously described [13].

Table 1. The sequences of the primers used in qRT-PCR.

Gene		Primer sequence (5'-3')
SNAIL	Forward	ACTGCAACAAGGAATACCTCAG
	Reverse	GCACTGGTACTTCTTGACATCTG
SLUG	Forward	TGTGACAAGGAATATGTGAGCC
	Reverse	TGAGCCCTCAGATTTGACCTG
ZEB1	Forward	CAGCTTGATACCTGTGAATGGG
	Reverse	TATCTGTGGTCGTGTGGGACT
ZEB2	Forward	GCGATGGTCATGCAGTCAG
	Reverse	CAGGTGGCAGGTCATTTCTT
TWIST1	Forward	GTCCGCAGTCTTACGAGGAG
	Reverse	CTTGAGGGTCTGAATCTTGCT
GAPDH	Forward	ACAACTTTGGTATCGTGGAAGG
	Reverse	GCCATCACGCCACAGTTTC

The signals were developed by using enhanced chemiluminescence (ECL) (Millipore, Switzerland) and captured by a Tanon-5200 Chemi-luminescent Imaging System (Tanon, China). The density of western blotting bands was quantified by ImageJ.

Quantitative real time polymerase chain reaction (qRT-PCR)

The quantitative real time polymerase chain reaction (qRT-PCR) was conducted as previously described [13]. The relative mRNA expression levels were quantified by the $2^{-\Delta\Delta Cq}$ method [14]. The sequences of the primers used in our research were listed in Table 1.

Ectopic expression of FAM3B

Lentivirus carrying pcDNA3.1 plasmid containing FAM3B cDNA was synthesized by GeneChem Company Ltd. (Shanghai, China). The transfection was conducted following the manufacturer's instructions.

Transfection of siRNA

The human FAM3B siRNA (sc-91522) and control siRNA (sc-37007) was obtained from Santa Cruz Biotechnology, Inc. (CA, USA) and transfected using Lipofectamine RNAiMAX (Thermo Fisher Scientific, MA, USA). For transfection, 100 pM of siRNA and 5 μM of Lipofectamine RNAiMAX was mixed with 250 μM Opti-MEM (Thermo Fisher Scientific, USA), respectively. Thereafter, they were mixed and incubated at room temperature for 20 minutes, and then added to the 6-well plate to make the final volume of culture medium to be 2 mL.

Statistical analysis

The data were presented as means±standard deviation (SD). Student's *t*-test and one-way ANOVA were used to analyze the differences by using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). *P* value <0.05 was considered significantly different.

Results

FAM3B was upregulated in AGS/CDDP cells

We firstly established a CDDP-resistant gastric cancer cell line by treating AGS cells with continuous exposure to CDDP at the concentration which started at 0.1 μg/mL and increased to 5 μg/mL in a stepwise manner and we named the cells AGS/CDDP. Then we confirmed the CDDP resistance by CCK-8 assay. The results showed that AGS/CDDP cells had higher cell viability than that in AGS after the incubation with CDDP for 48 hours (Figure 1A). The flow cytometry results detected less cell apoptosis in AGS/CDDP cells (Figure 1B, 1C). To reveal whether FAM3B had a role in CDDP resistance of gastric cancer, FAM3B level was detected in AGS/CDDP cells. The mRNA and protein levels of FAM3B were upregulated in AGS/CDDP cells compared to AGS cells (Figure 1D, 1E).

FAM3B overexpression induced CDDP resistance in AGS cells

To investigate the effect of upregulated FAM3B on gastric cancer CDDP resistance, FAM3B overexpression vector (FAM3B) or empty vector (Vector) were transfected into AGS cells. We firstly confirmed the overexpression efficiency (Figure 2A, 2B). FAM3B overexpression impaired the sensitivity of AGS cells to CDDP (Figure 2C). The AGS cells with or without FAM3B overexpression were treated with 2.5 μg/mL CDDP for 48 hours and subjected to flow cytometry analysis. As expected, FAM3B overexpression significantly decreased CDDP-induced apoptosis in AGS cells (Figure 2D, 2E). The aforementioned results demonstrated that FAM3B overexpression induced CDDP resistance in AGS cells.

FAM3B knockdown overcame CDDP resistance in AGS/CDDP cells

To further reveal whether the upregulated FAM3B was responsible for CDDP resistance in AGS/CDDP cells, we adopted FAM3B siRNA to inhibit the expression of FAM3B in AGS/CDDP cells. The knockdown efficiency was confirmed by qRT-PCR and western blotting (Figure 3A, 3B). The CCK-8 assay showed that FAM3B inhibition reduced the cell viability after CDDP incubation in AGS/CDDP (Figure 3C). To further determine the effect of FAM3B downregulation on CDDP-induced apoptosis, flow
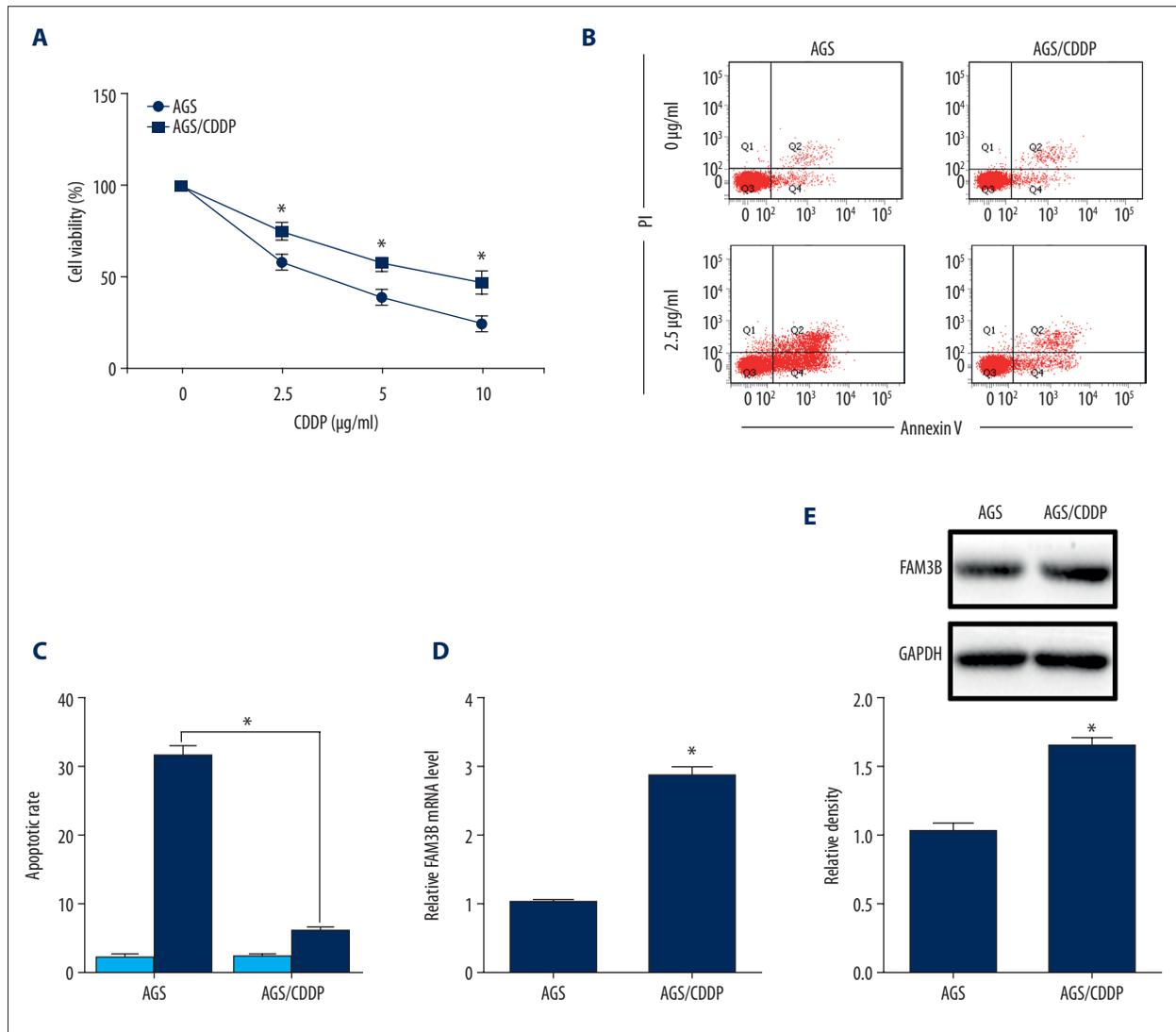
Figure 1. FAM3B was upregulated in CDDP-resistant AGS cells. (**A**) AGS and AGS/CDDP cells were incubated with CDDP (0, 2.5, 5, and 10 μg/mL) for 48 hours. The cell viability was measured by CCK-8 assay. (**B**) AGS and AGS/CDDP cells treated with 0 μg/mL or 2.5 μg/mL CDDP for 48 hours were used in flow cytometry analysis. (**C**) Comparison of apoptotic cell rates. (**D**) qRT-PCR results of the FAM3B mRNA level in AGS and AGS/CDDP cells. (**E**) Western blotting results of the FAM3B protein level in AGS and AGS/CDDP cells. Quantification of the relative band density was in the **lower panel** (* *P*<0.05). CCDP – cisplatin; AGS – human gastric cancer cell line; CCK-8 – Cell Counting Kit-8; qRT-PCR – quantitative real time polymerase chain reaction.

cytometry analysis was conducted in AGS/CDDP cells with or without FAM3B knockdown after the exposure to 2.5 μg/mL CDDP for 48 hours. FAM3B siRNA transfection significantly increased CDDP-induced apoptosis (Figure 3D, 3E). Collectively, FAM3B knockdown reversed CDDP resistance in AGS/CDDP cells.

Cell migration and invasion was enhanced in AGS/CDDP cells

To reveal the mechanism underlying FAM3B-mediated CDDP resistance in gastric cancer, we focused on EMT which was

reported to have an important function in therapy resistance of cancers [15]. We first examined the protein level the epithelial marker E-cadherin and the mesenchymal marker vimentin in AGS/CDDP cells. We found decreased E-cadherin and increased vimentin in AGS/CDDP cells (Figure 4A). Then we compared the cell migration in AGS and AGS/CDDP cells by wound healing assay which showed enhanced migration capacity in AGS/CDDP cells (Figure 4B). The Transwell assay also demonstrated enhanced cell invasion in AGS/CDDP cells (Figure 4C). Together, AGS/CDDP cells might undergo EMT while acquiring CDDP resistance.
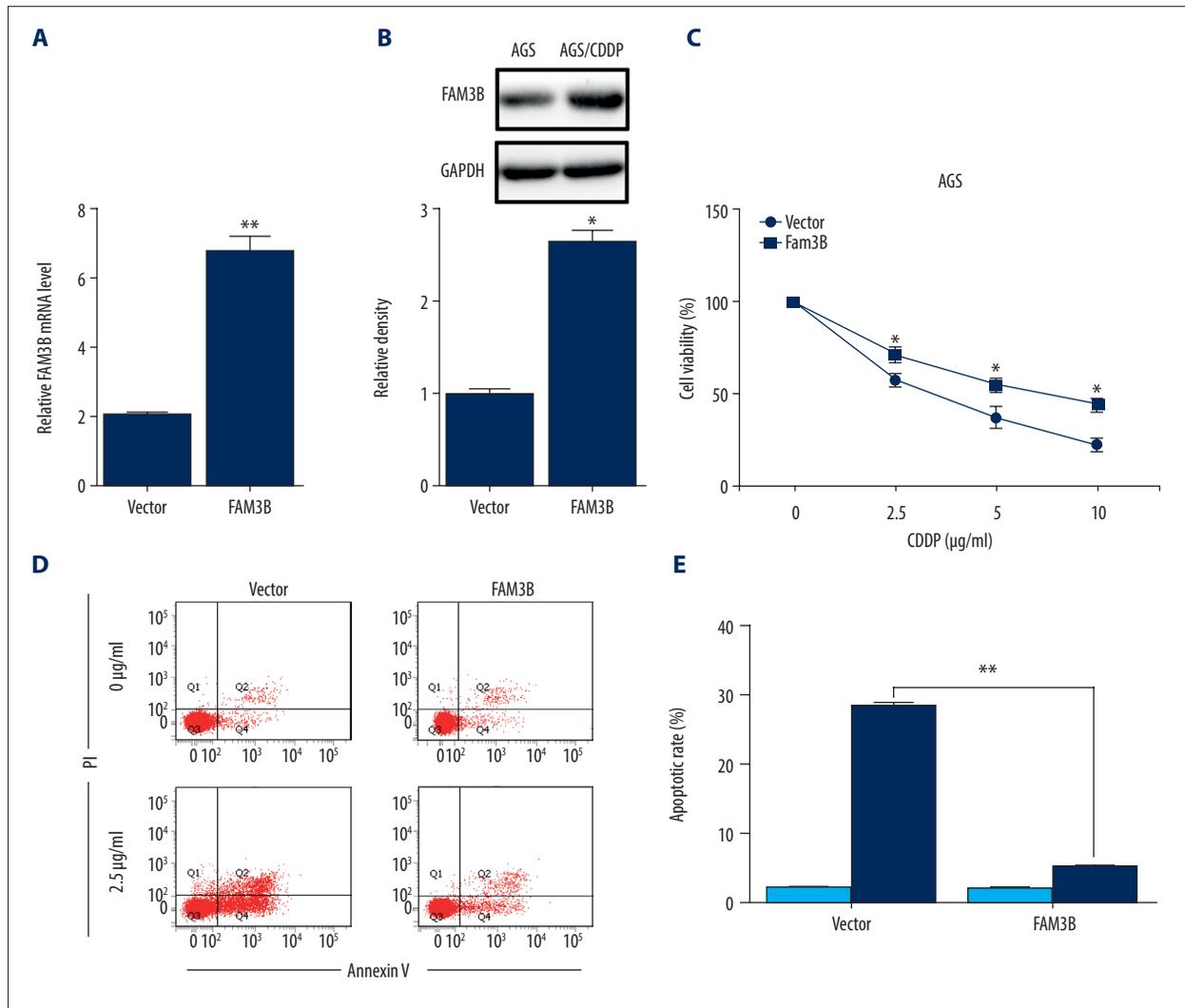
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Figure 2. FAM3B overexpression induced CDDP resistance in AGS cells. (**A**) FAM3B transfection was confirmed by qRT-PCR in AGS cells. (**B**) FAM3B transfection was confirmed by western blotting in AGS cells. Quantification of the relative band density was in the **lower panel**. (**C**) AGS cells with FAM3B overexpression were treated with CDDP (0, 2.5, 5, and 10 μg/mL) for 48 hours, and the cell viability was measured by CCK-8 assay. (**D**) AGS cells with FAM3B overexpression treated with 0 μg/mL or 2.5 μg/mL CDDP for 48 hours were used in flow cytometry analysis. (**E**) Comparison of apoptotic cell rates (* $P<0.05$; ** $P<0.01$). CCDP – cisplatin; AGS – human gastric cancer cell line; CCK-8 – Cell Counting Kit-8; qRT-PCR – quantitative real time polymerase chain reaction.

FAM3B overexpression induced EMT

To find out whether FAM3B overexpression induced EMT in AGS cells, we detected E-cadherin and vimentin protein levels in AGS cells with FAM3B overexpression. The results showed that E-cadherin decreased while vimentin increased after FAM3B overexpression (Figure 5A). The cell migration and invasion were also evaluated. We found enhanced cell migration and invasion after ectopic overexpression of FAM3B (Figure 5B, 5C). Then we detected the mRNA changes of EMT transcription factors *SNAIL*, *SLUG*, *ZEB1*, *ZEB2*, and *TWIST1* by qRT-PCR which showed that *SNAIL* was upregulated in AGS cells with FAM3B

overexpression (Figure 5D). Increased snail protein level was also detected (Figure 5E). As it has been reported that loss of E-cadherin can be mediated through NF-κB-induced snail up-regulation in gastric cancer [16], we also detected P-P65 in AGS cells after FAM3B overexpression and found increased P65 activation (Figure 5F). These results demonstrated that FAM3B induced EMT in AGS cells.
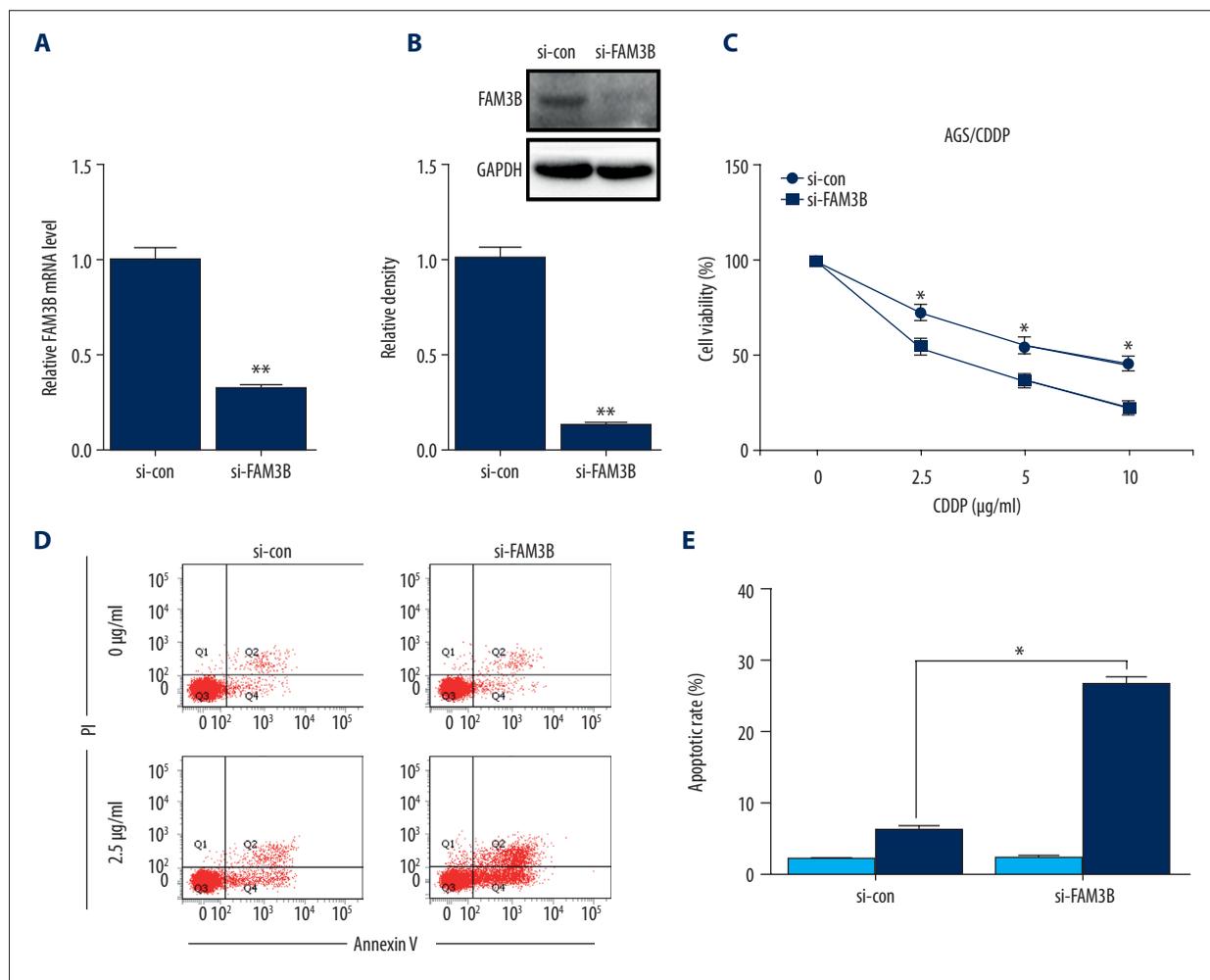
Figure 3. FAM3B knockdown overcame CDDP resistance in AGS/CDDP cells. (**A**) The inhibiting efficiency of FAM3B siRNA was confirmed by qRT-PCR in AGS/CDDP cells. (**B**) The knockdown efficiency of FAM3B siRNA was confirmed by western blotting in AGS/CDDP cells. Quantification of the relative band density was in the **lower panel**. (**C**) AGS/CDDP cells with FAM3B inhibition and control cells were treated with CDDP for 48 hours, and the cell viability was measured by CCK-8 assay. (**D**) AGS/CDDP cells with FAM3B inhibition treated with 0 μg/mL or 2.5 μg/mL CDDP for 48 hours were used in flow cytometry analysis. (**E**) Comparison of apoptotic cell rates (* $P<0.05$). CCDP – cisplatin; AGS – human gastric cancer cell line; CCK-8 – Cell Counting Kit-8; qRT-PCR – quantitative real time polymerase chain reaction.

Inhibition of snail reversed CDDP resistance in AGS/CDDP cells

To further confirm whether FAM3B induced gastric cancer cell CDDP resistance through inducing EMT, we also evaluated the snail protein level in AGS/CDDP cells and found increased snail by western blotting (Figure 6A). Then we used *SNAIL* siRNA to inhibit its expression in AGS/CDDP cells. The inhibition efficiency was firstly confirmed (Figure 6B, 6C). Inhibition of *SNAIL* reversed the E-cadherin decrease and vimentin increase in AGS/CDDP cells (Figure 6D). After the inhibition of *SNAIL*, CDDP resistance was reversed as it was evident by the decrease of cell viability in AGS/CDDP cells after CDDP incubation (Figure 6E). Taken together, the inhibition of EMT reversed CDDP resistance in AGS/CDDP cells.

Discussion

CDDP-based chemotherapy is the primary strategy for adjuvant chemotherapy in early-stage gastric cancer patients and systemic chemotherapy in late-stage gastric cancer patients. Unfortunately, CDDP resistance is a major problem, strongly limiting the efficiency [17]. Thus, elucidating the molecular mechanism underlying CDDP resistance will help develop reasonable and effective therapeutic strategies to overcome CDDP resistance. To date, many researches have revealed different mechanisms in gastric cancer CDDP resistance. Duan et al. found that taxol resistance gene 1 (TXR1) triggered CDDP resistance *in vivo* and *in vitro* [18]. Zou et al. found that inhibition of glutathione S-transferase pi 1 (GSTP1) sensitized CDDP-resistant
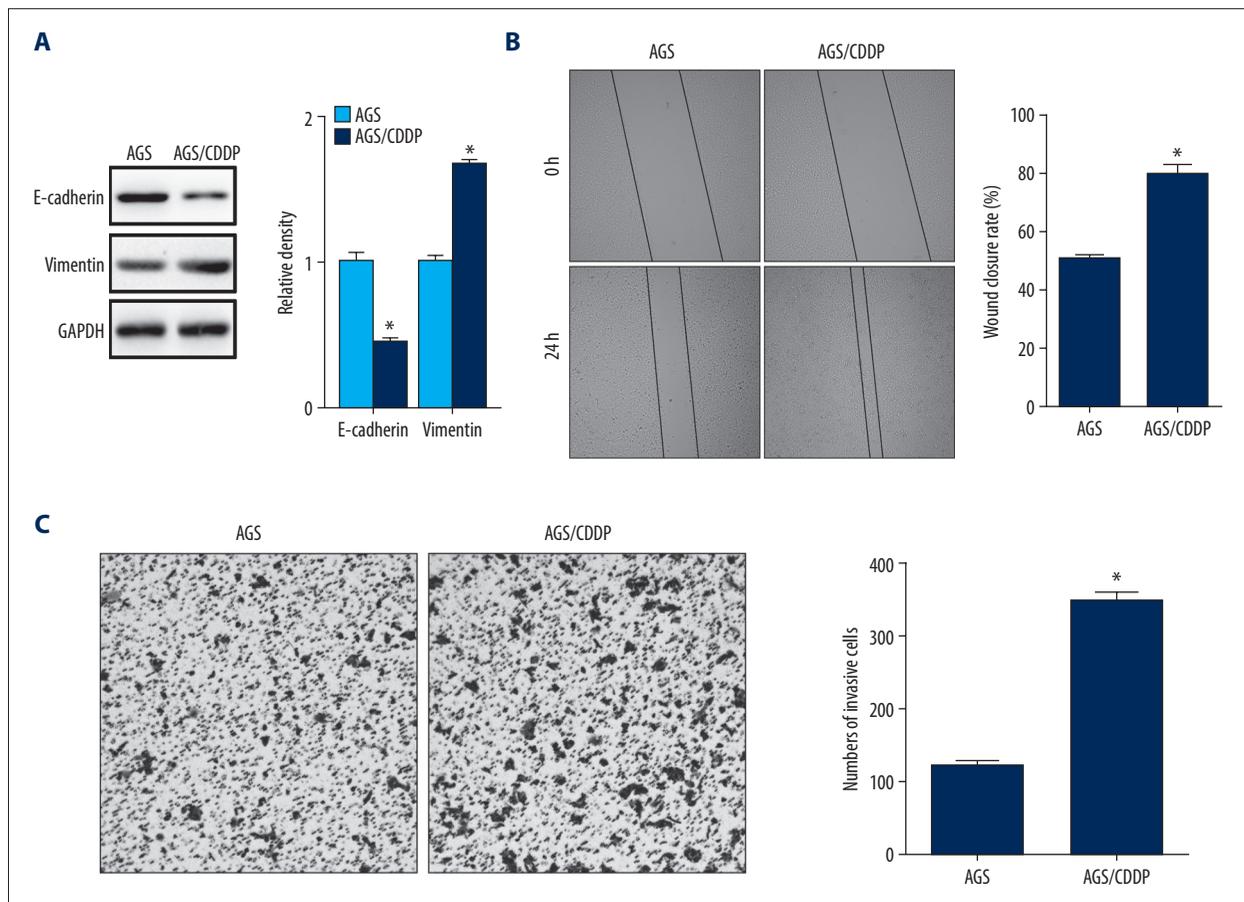
Figure 4. Cell migration and invasion was enhanced in AGS/CDDP cells. (**A**) Western blotting results of E-cadherin and vimentin in AGS and AGS/CDDP cells. Quantification of the relative band density was in the **right panel**. (**B**) The cell migration of AGS and AGS/CDDP cells was evaluated by wound healing assay. Comparison of wound closure rate was in the **right panel**. (**C**) The cell invasion of AGS and AGS/CDDP cells was evaluated by Transwell assay. Comparison of invasive cell counts was in the **right panel** (* $P<0.05$). CCDP – cisplatin; AGS – human gastric cancer cell line.

gastric cancer cells to CDDP treatment [19]. Jiang et al. found that phosphoprotein enriched in astrocytes 15 (PEA-15) was involved in AKT-regulated cisplatin resistance [20]. The research by Li et al. found that psoriasin decreased the sensitivity of gastric cancer cells to CDDP by activating the extracellular signal-regulated kinase (ERK) signaling pathway [21]. Besides, some long non-coding RNA (like PVT1) and microRNAs (like miR-876-3p) have also been demonstrated to function in CDDP resistance of gastric cancer [22,23]. In this study, we found that FAM3B was increased in CDDP-resistant gastric cancer cells. Additionally, FAM3B overexpression could induce CDDP resistance of gastric cancer cells by reducing CDPP-induced apoptosis, while FAM3B knockdown sensitized gastric cancer cells to CDDP treatment. More importantly, FAM3B induced EMT by upregulating snail. Inhibition of EMT by silencing *SNAIL* reversed FAM3B induced CDDP resistance.

FAM3B is also named PANDER due to its robust expression in the endocrine pancreas [5]. It has been shown to regulate

glucose homeostasis, α and β cell function [24]. In recent years, FAM3B has been found to be involved in tumor progression. In colon cancer, a non-secretory form of FAM3B (FAM3B-258) was found abundant in cancer cells and tissues. FAM3B-258 promotes colon cancer cell invasion and metastasis through slug upregulation [10]. In ESCC, FAM3B was also detected higher than adjacent normal tissues. High FAM3B expression was correlated with high TNM stage and predicted poor prognosis of ESCC patients. Mechanically FAM3B could regulate the AKT-MDM2.p53 pathway and EMT which inhibited cell death and promoted tumor growth [11]. In prostate cancer, FAM3B expression was found increased compared to normal tissues. Overexpression of FAM3B contributed to increased resistance to cell death and tumor growth through upregulating gene expression of anti-apoptotic Bcl-2 and Bcl-XL and downregulating expression of pro-apoptotic Bax [12]. We also found increased FAM3B in CDDP-resistant gastric cancer cells, while inhibition of FAM3B reversed CDDP resistance.
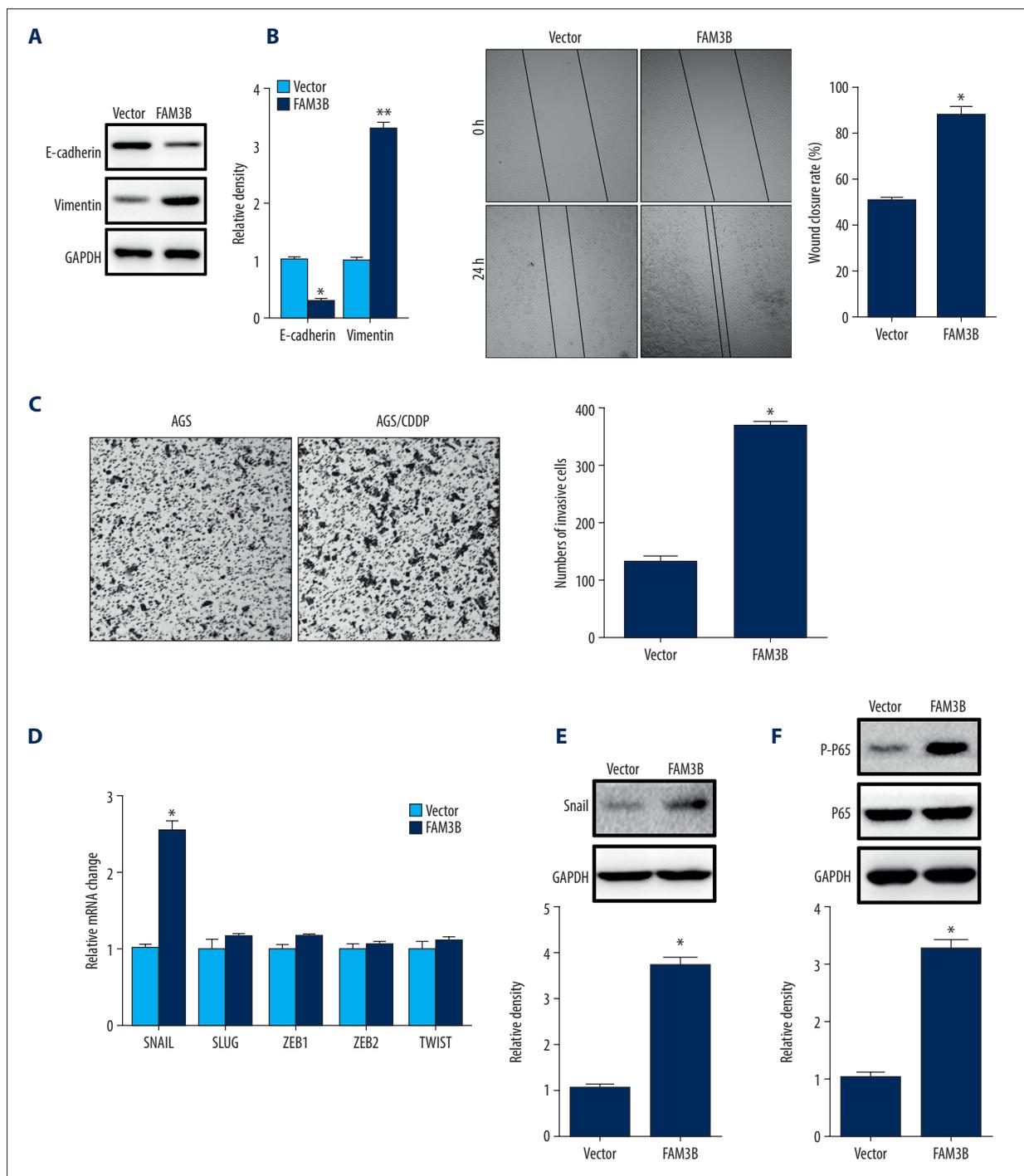
Figure 5. FAM3B overexpression induced EMT. (**A**) Western blotting results of E-cadherin and vimentin in AGS cells with FAM3B overexpression. Quantification of the relative band density was in the **right panel**. (**B**) The cell migration of AGS cells with or without FAM3B overexpression was evaluated by wound healing assay. Comparison of wound closure rate was in the **right panel**. (**C**) The cell invasion of AGS cells with or without FAM3B overexpression was evaluated by Transwell assay. Comparison of invasive cell counts was in the **right panel**. (**D**) qRT-PCR results of the *SNAIL*, *SLUG*, *ZEB1*, *ZEB2*, and *TWIST1* mRNA levels in AGS cells with ectopic FAM3B overexpression. (**E**) Western blotting results of snail in AGS cells with ectopic FAM3B overexpression. Quantification of the relative band density was in the **lower panel**. (**F**) Western blotting results of P-P65 in AGS cells with ectopic FAM3B overexpression. Quantification of the relative band density was in the **lower panel** (* *P*<0.05). EMT – epithelial-mesenchymal transition; AGS – human gastric cancer cell line; qRT-PCR – quantitative real time polymerase chain reaction.
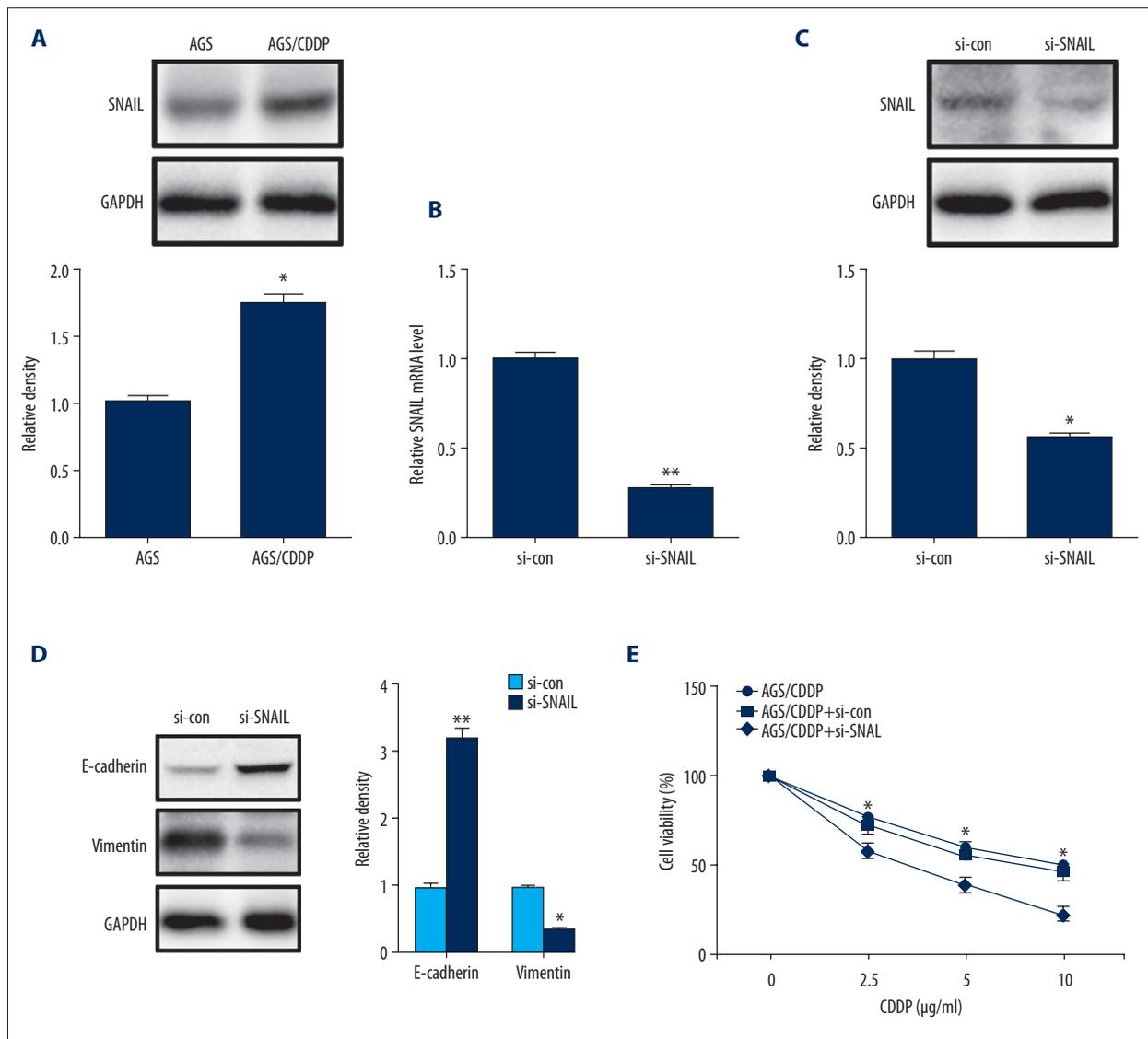
Figure 6. Inhibition of snail reversed CDDP resistance in AGS/CDDP cells. (**A**) Western blotting results of snail in AGS and AGS/CDDP cells. Quantification of the relative band density was in the **lower panel**. (**B**) The knockdown efficiency of *SNAIL* siRNA was accessed by qRT-PCR in AGS/CDDP cells. (**C**) The knockdown efficiency of *SNAIL* siRNA was accessed by western blotting in AGS/CDDP cells. Quantification of the relative band density was in the **lower panel**. (**D**) Western blotting results of E-cadherin and vimentin in AGS/CDDP cells with *SNAIL* inhibition and control cells. Quantification of the relative band density was in the **right panel**. (**E**) AGS/CDDP cells with *SNAIL* inhibition and control cells were treated with CDDP (0, 2.5, 5, and 10 µg/mL) for 48 hours, and the cell viability was measured by CCK-8 assay (* *P*<0.05; ** *P*<0.01). CCDP – cisplatin; AGS – human gastric cancer cell line; CCK-8 – Cell Counting Kit-8; qRT-PCR – quantitative real time polymerase chain reaction.

EMT is reported to have a critical role in mediating cancer progression, recurrence, metastasis and drug resistance [25]. The role of EMT in cancer drug resistance has been increasingly recognized [15,26]. EMT accompanies the development of chemo-resistance in a number of cancer types such as ovarian cancer, hepatocellular carcinoma, neuroblastoma, and gastric cancer [13,15,27,28]. EMT also plays a significant role in resistance to CDDP. CDDP-resistant cells were significantly enriched for a mesenchymal gene expression signature, while highly proliferative

non-EMT cancer cells were sensitive to CDDP [29]. In urothelial cancer, CDDP-resistant cells acquired morphological changes characteristic of EMT with increased invasiveness [30]. Furthermore, EMT transcription factors are involved in CDDP resistance through different molecular mechanisms. Inhibition of TWIST1 sensitized human lung cancer cells to CDDP by activation of JNK/mitochondrial pathway [31]. TWIST2 is associated with the cervical malignant conversion, cervical cancer metastasis, and CDDP resistance [32]. ZEB1 was found important in CDDP sensitivity in

pancreatic cancer cell lines [33]. Hsu et al. found that SNAIL induced EMT and CDDP resistance by direct regulation of excision repair cross-complementation croup 1 (ERCC1) in human head and neck squamous cell carcinoma [34]. Our study revealed that CDDP-resistant gastric cancer cells acquired EMT phenotype with decreased E-cadherin and increased vimentin. Notably, AGS/CDDP cells exhibited enhanced activities of migration and invasion. The phenotypic changes were attributed to snail upregulation induced by P-P65 activation. These findings confirmed that gastric cancer cell CDDP resistance is associated with EMT.

Unlike FAM3C which is revealed to be involved in EMT by extensive researches [35], the relationship between FAM3B and EMT is seldom reported. Li et al. found that FAM3B-258 promoted human colon cancer cell invasion and metastasis by upregulating slug. He et al. demonstrated that FAM3B induced EMT in ESCC cells through increasing snail. In our research, we also found increased snail after FAM3B overexpression. FAM3B promoted EMT via snail upregulation in AGS cells.

Conclusions

Upregulation of FAM3B induced CDDP resistance in gastric cancer cells by regulating EMT in a snail-dependent manner, making FAM3B a novel therapeutic target for gastric cancer chemo-resistance.

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