Received: 2019.07.04
Accepted: 2019.08.07
Published: 2019.11.16

Targeting Frizzled-7 Decreases Stemness and Chemotherapeutic Resistance in Gastric Cancer Cells by Suppressing Myc Expression

Authors' Contribution:

Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BC 1 **Yongzhong Cheng**

BD 2 **Li Li**

D 3 **Sirong Pan**

EF 4 **Huilin Jiang**

AG 1 **Hongyan Jin**

1 Department of Oncology, Wuhan Puren Hospital, Wuhan, Hubei, P.R. China

2 Department of Science and Education, Wuhan Puren Hospital, Wuhan, Hubei, P.R. China

3 Department of Medicine, Wuhan Puren Hospital, Wuhan, Hubei, P.R. China

4 Department of Oncology, Xiehe Jiangbei Hospital, Wuhan, Hubei, P.R. China

Corresponding Author: Hongyan Jin, e-mail: hongyan_jin@sina.cn

Source of support: Departmental sources

Background: Although the promoting roles of Frizzled-7 (Fzd7) have been shown before, its effects in gastric cancer (GC) cell stemness are still unclear. The present study assessed the effects of Fzd7 on GC cell stemness and chemoresistance.

Material/Methods: Clinical samples were used to detect Fzd7 expression and online datasets were used to analyze the correlation between Fzd7 expression and GC patient prognosis. Quantitative real-time PCR (qPCR), Western blot, and spheroid formation were used to detect the stemness of cells and Fzd7-mediated effects on GC cell stemness. Cell viability was assessed to evaluate the role of Fzd7 in chemoresistance of GC cells.

Results: We found that the expression of Frizzled-7 (Fzd7), a Wnt receptor, was increased in gastric cancer (GC) cells and tissues. Additionally, Fzd7 expression was correlated with shorter overall survival of GC patients. Knockdown of Fzd7 or using inhibitors of Wnt/Fzd (OMP-18R5/Vantictumad) decreased GC cell stemness, characterized as a decrease of spheroid formation ability and expression of stemness regulators. Notably, Fzd7 knockdown or inhibitors of Wnt/Fzd attenuated the chemoresistance of GC cells. Furthermore, elevation of Myc expression rescued the effects of Fzd7 inhibition on GC cell stemness and chemoresistance.

Conclusions: Our results suggest that inhibition of Fzd7 decreases the stemness and chemotherapeutic resistance of GC cells.

MeSH Keywords: **Neoplastic Stem Cells • Stomach Neoplasms • Wnt Signaling Pathway**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/918504>

1799

—

5

19

Background

In recent years, although the quality of life of patients with advanced gastric cancer (GC) has improved and their survival time has been prolonged, the 5-year survival rate of patients with advanced GC is still low, and the efficacy of chemotherapy is limited; this may be attributed to an incomplete understanding of the mechanism [1]. Therefore, it is urgent to elucidate the mechanism of GC progression.

Gastric cancer stem cells (CSCs) play an important role in the occurrence and development of GC [2]. They are the main causes of drug resistance, metastasis, and recurrence of GC. After routine surgical treatment or adjuvant radiotherapy and chemotherapy, the number of CSCs in GC did not decrease, but the phenomenon of accumulation and dormancy appeared, which is also the root of metastasis and recurrence of GC [3]. However, the specific mechanism contributing to gastric CSC action is unclear. The important regulatory function of Wnt signaling pathway in animal development has been studied in detail. The Wnt signaling pathway is composed of a series of evolutionarily conserved proteins, such as Frizzled (Fzd) protein, a seven-time transmembrane receptor, which is a sub-family of G protein-coupled receptors [4]. It was found that Fzd protein is a very important key protein connecting Wnt signaling and the downstream pathway, such as the β -catenin pathway, Rho-Rock pathway, and cell plane polarity pathway [5]. Fzd protein is responsible for Wnt protein recognition and fixation, and carries information to the cell membrane. It has been found that the Fzd protein family also plays an important role in cancer [6]. The expression of Fzd is increased in various cancers, such as colon cancer, ovarian cancer, and GC [7]. Previous studies have shown that Frizzled-7 (Fzd7) regulates stem cell function in the gastric and intestinal epithelium [8]. Additionally, Fzd7 expression is upregulated in GC tissues and is negatively correlated with patient prognosis [9]. However, the roles of Fzd7 in GC cell stemness have never been revealed.

In the current work, we found that Fzd7 level is upregulated in GC tissues and cells compared to that in normal tissues and cells, among which Fzd7 exhibited the most change. Additionally, it was found that Fzd proteins, especially Fzd7, contribute to GC cell stemness, partially dependent on Myc. These results suggest that targeting Fzd7 could be used to treat GC.

Material and Methods

Clinical samples, cell culture, and reagents

Thirty-five pairs of GC and normal adjacent paraffin-embedded tissues were obtained from Wuhan Puren Hospital between May 2016 and March 2019. The experiments were performed

with the written consent of each subject and approval of ethics review committees in Wuhan Puren Hospital. Human GC cell lines (SCG7901, MKN-45, MGC-803, BGC-823, and AGS) and the normal gastric epithelial cell line GES-1 were purchased from the Chinese Academy of Sciences Cell Bank. SCG7901-CR (SCG7901 cisplatin-resistant SCG7901) cells were purchased from Shanghai Gegan Biotechnology Co. (Shanghai, China). All cell lines were maintained in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (fetal bovine serum, Biological Industries, Kibbutz Beit Haemek, Israel) and 1% penicillin/streptomycin (Sangon Biotech, Shanghai, China) in a humidified atmosphere with 5% CO₂ at 37°C. OMP-18R5 (OR, 10 μ g/ml) was obtained from Oncomed (Redwood, USA).

Quantitative real-time PCR (qPCR)

Paraffin-embedded tissues were used for extracting RNA using an EASYspin Fast RNA Extraction Kit for Fixed Embedded Tissues (Haoxin BioTech, Hangzhou, China), and total RNA from cells was extracted using an EASYspin Tissue/Cell RNA Rapid Extraction Kit (Haoxin BioTech). Then, cDNA was reverse-synthesized using the SuperScript® III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific) and subjected to qPCR assay with 2 $\times$ SYBR Green qPCR Mix (Haoxin Bio.Tech) on the StepOne Plus PCR system. Gene expression levels were calculated relative to the gene 18S using 2^{- $\Delta\Delta$ CT} method.

Western blot

The detailed procedure was described in a previous study [10]. The detailed information about the primary antibodies were listed as follows: CD44 (Cat. No. 15675-1-AP, 1: 1000, Proteintech, Wuhan, China), ALDH1 (Cat. No. 15910-1-AP, 1: 1000, ProteinTech), β -actin (Cat. No. 60008-1-Ig, 1: 3000, Proteintech), Myc (Cat. No. 10828-1-AP, 1: 1000, Proteintech) and Fzd7 (Cat. No. 16974-1-AP, 1: 1000, ProteinTech).

Spheroid formation assessment

The detailed procedure was reported in a previous study [11].

Plasmid construction, siRNA synthesis and transfection

Myc overexpression (Myc-OE) plasmid (MSCV-Myc) was purchased from Addgene. siRNAs against Fzd7 were purchased from GenePharma (Shanghai, China) and 50 nM was used for transfection. The transfection process was performed using Lipofectamine 3000 (Thermo Fisher Scientific).

Figure 1. Fzd7 expression level is upregulated in GC tissues and cells. **(A)** Fzd7 mRNA level was examined in GC and normal adjacent tissues. **(B)** The correlation between Fzd7 expression and the overall survival of GC patients was analyzed via the KM-plotter online datasets. **(C, D)** Fzd7 expression was measured in GC cells and normal gastric epithelial cells. Data are presented as the mean±s.d., ** P<0.01 vs. Normal adjacent tissues or GES-1.

Cell viability

Cells with different transfection or treatment were maintained in 96-well plates and treated with cisplatin (10 μM) 12 h later [12]. At 24 h, 48 h and 72 h later, cell viability was evaluated via Cell Counting Kit-8 (CCK8) assay (YEASEN, Shanghai, China).

Statistical analysis

All data are expressed as the mean±SEM, where the mean represents the number of independent experiments (n≥3). Statistical analysis was performed using Prism7 GraphPad software). The *t* test was used for analyzing the datasets, with only 2 groups. The differences between the groups were analyzed using one-way ANOVA with the Tukey-Kramer post-test. P value less than 0.05 was considered significant.

Results

Fzd7 expression is significantly increased in GC tissues and cells

We first examined Fzd7 expression in GC and normal adjacent tissues through qPCR assay, showing that Fzd7 expression was significantly increased in GC tissues (Figure 1A). KM-Plotter analysis (<http://kmplot.com/analysis>) through online datasets showed that Fzd7 expression was correlated with shorter overall survival of GC patients (Figure 1B). We also assessed Fzd7 expression in normal gastric epithelial cells and GC cells. As shown in Figure 1C and 1D, Fzd7 expression exhibited a significantly higher level in GC cells compared to GES-1, especially in MKN45 and SCG7901 cells, which were used for subsequent experiments.

Figure 2. Knockdown or inhibition of Fzd7 decreased the expression of stemness regulators in GC cells. **(A, B)** The mRNA levels of GC stemness regulators and Fzd7 were examined in GC cells with Fzd7 knockdown or OR treatment. **(C, D)** The protein levels of GC stemness markers and Fzd7 were detected in GC cells with Fzd7 knockdown or OR treatment. Data are presented as the mean $\pm$ s.d., ** $P < 0.01$ vs. control.

Knockdown or inhibition of Fzd7 downregulated the levels of stemness regulators in GC cells

Since gastric CSCs are involved in the occurrence of GC, we speculated that Fzd7 plays a critical role in GC cell stemness. As expected, knockdown or inhibition of Fzd7 by transfection of siRNA against Fzd7 or OR treatment significantly decreased the mRNA levels of GC stemness markers (ALDH1 and CD44) in MKN45 and SCG7901 cells (Figure 2A, 2B). The knockdown efficiency of Fzd7 siRNA was also confirmed (Figure 2A, 2B). Furthermore, a consistent result was obtained at the protein levels of GC stemness markers (Figure 2C, 2D).

Knockdown or inhibition of Fzd7 attenuated the spheroid formation ability of GC cells

Spheroids formed by tumor cells at non-adherent condition have been regarded as CSCs; thus, we further explored the spheroid formation capacity of GC cells with Fzd7 knockdown

or inhibition. Indeed, the spheroid formation ability was reduced by Fzd7 knockdown or inhibition, showing as the decrease of spheroid number and size (Figure 3A, 3B).

Knockdown or inhibition of Fzd7 reduced the chemoresistance of GC cells

As gastric CSCs are regarded to the critical factor contributing to the chemoresistance of GC cells, we compared the stemness of cisplatin-sensitive and -resistant GC cells. As expected, SCG7901-CR displayed a stronger stemness than the parental SCG7901 cells, characterized by the increase of stemness marker and Fzd7 expression, and spheroid formation ability (Figure 4A–4D). Then, we evaluated the effects of Fzd7 inhibition on the chemoresistance of SCG7901-CR cells. As shown in Figure 4E, knockdown of Fzd7 and inhibition of Fzd7 partially reversed the chemoresistance of SCG7901-CR cells. Indeed, SCG7901-CR cell stemness was reduced by Fzd7 knockdown and inhibition (Figure 4F–4I). Thus, our results suggest that

Figure 3. Knockdown or inhibition of Fzd7 attenuated the spheroid formation ability of GC cells. **(A)** The spheroid size was measured in GC cells with Fzd7 knockdown or OR treatment. **(B)** The spheroid number was assessed in the cells described in **(A)**. Data are presented as the mean $\pm$ s.d., ** $P < 0.01$ vs. control.

targeting Fzd7 decreases the stemness and thus the chemotherapeutic resistance of GC cells.

Fzd7 exerts its effects on GC cell stemness in a Myc-dependent manner

Finally, we explored the underlying mechanisms contributing to the roles of Fzd in GC cell stemness and chemoresistance. Since the transcription factor Myc had been identified to be the downstream effector of Fzd7 and is necessary for Fzd7-mediated effects on the growth of gastric adenomas, we speculated this Fzd7/Myc axis is responsible for GC cell stemness. Indeed, Myc exhibited a similar expression pattern

as found with Fzd7 in SCG7901 and SCG7901-CR cells, with a higher level in SCG7901-CR than in SCG7901 cells (Figure 5A). Knockdown of Fzd7 or inhibition of Fzd7 decreased the expression of Myc in GC cells (Figure 5B, 5C). As expected, overexpression of Myc rescued the decreased expression of stemness markers, which was led by Fzd7 knockdown in GC cells (Figure 5D–5F). Additionally, overexpression of Myc attenuated the effects of Fzd7 knockdown on the spheroid formation ability (Figure 5G, 5H). Notably, Fzd7 and Myc expression exhibited a positive correlation in GC tissues. Thus, these effects demonstrate that Fzd7-dependent Myc expression is essential for GC cell stemness.

Figure 4. Knockdown of or inhibition of Fzd7 reduced the chemoresistance of GC cells. (**A**, **B**) The mRNA and protein levels of GC stemness regulators and Fzd7 were examined in SCG7901-CR and SCG7901 cells. (**C**, **D**) The spheroid size and number were determined in SCG7901-CR and SCG7901 cells. (**E**) SCG7901-CR with Fzd7 knockdown or OR treatment, and SCG7901 cells were treated with or without cisplatin, and cell viability was evaluated. (**F**, **G**) The mRNA and protein levels of GC stemness regulators and Fzd7 were detected in SCG7901-CR cells with Fzd7 knockdown or OR treatment. (**H**, **I**) The spheroid size and number were determined in the cells depicted in (**F**). Data are presented as the mean $\pm$ s.d., ** $P < 0.01$ vs. control.

Figure 5. Fzd7 exerts its effects on GC cell stemness in a Myc-dependent manner. **(A)** Myc expression was examined in SCG7901 and SCG7901-CR cells. **(B, C)** Myc expression was examined in GC cells with Fzd7 knockdown or OR treatment. **(D, E)** ALDH1 and CD44 mRNA levels were determined in GC cells with Fzd7 knockdown plus Myc-OE or not. **(F)** The protein levels of ALDH1 and CD44 were detected in the cells depicted in **(D)**. **(G, H)** The spheroid number and size were measured in the cells described in **(D)**. Data are presented as the mean $\pm$ s.d., ** $P < 0.01$ vs. control.

Discussion

Fzd receptor family includes Fzd1–10 and is deregulated in various tumors, including GC [9,13], among which the high levels of Fzd1 is correlated with poor prognosis and promotes GC progression [14], and knockdown of Fzd2 has been shown to suppress GC proliferation [15]. Although the promoting roles of Fzd7 have been indicated in GC progression [16], the effects of Fzd7 on GC cell stemness and chemoresistance are still unclear. The present study is the first to show that Fzd7 promotes GC cell stemness and chemoresistance.

Previous studies have shown that Fzd7 is enriched in Lgr5(+) stem cells and plays a critical role in intestinal epithelium and organoid formation [17], and Fzd7 is necessary for the formation of mouse gastric epithelium [8]. Since normal stem cells and CSCs have the similar traits [18], we speculated that Fzd7 contributes to gastric CSC progression. Firstly, we showed that knockdown or inhibition of Fzd7 reduced the expression GC cell stemness markers. Secondly, the spheroid formation ability was attenuated by Fzd7 knockdown or inhibition. Thirdly, we found that cisplatin-resistant GC cells exhibit a stronger stemness than the parental GC cells, and their stemness and chemoresistance were reduced by Fzd7 knockdown or inhibition. Finally, we confirmed that Fzd7-dependent Myc expression

is responsible for Fzd7-mediated effects. Notably, OR can target several Fzds (Fzd1, 2, 5, 7, and 8), all of which are responsible for Wnt signaling transmitting; however, as Fzd1, Fzd5, and Fzd8 are not detected in MKN-45 and SCG7901 cells [19], and Fzd2 cannot compensate Fzd7 loss in the intestinal epithelium [17], these effects may suggest that Fzd7 plays a predominant role in GC cell stemness. Additionally, OR, the Fzd inhibitor, is currently being tested in several solid tumor types (<http://www.oncomed.com/Pipeline>), and we expect that OR could be used in combination with chemotherapeutics in treatment for GC patients. It must be noted that more clinical samples are needed to confirm Fzd7 expression in GC and normal adjacent tissues, and further *in vivo* experiments are needed to determine the roles of Fzd7 in GC cell stemness.

Conclusions

Collectively, combined with the analysis of clinical samples, our results demonstrate that targeting Fzd7 might be an attractive therapeutic strategy for GC treatment.

Conflicts of interest

None.

References:

- Yu J, Huang C, Sun Y et al: Effect of laparoscopic vs. open distal gastrectomy on 3-year disease-free survival in patients with locally advanced gastric cancer: The CLASS-01 Randomized Clinical Trial. *JAMA*, 2019; 321(20): 1983–92
- Yoon C, Till J, Cho SJ et al: KRAS activation in gastric adenocarcinoma stimulates epithelial-to-mesenchymal transition to cancer stem-like cells and promotes metastasis. *Mol Cancer Res*, 2019 [Epub ahead of print]
- Courtois S, Duran RV, Giraud J et al: Metformin targets gastric cancer stem cells. *Eur J Cancer*, 2017; 84: 193–201
- Koval A, Purvanov V, Egger-Adam D, Katanaev VL: Yellow submarine of the Wnt/Frizzled signaling: Submerging from the G protein harbor to the targets. *Biochem Pharmacol*, 2011; 82(10): 1311–19
- Tauriello DV, Jordens I, Kirchner K et al: Wnt/beta-catenin signaling requires interaction of the Dishevelled DEP domain and C terminus with a discontinuous motif in Frizzled. *Proc Natl Acad Sci USA*, 2012; 109(14): E812–20
- Strutt D, Madder D, Chaudhary V, Artymiuk PJ: Structure-function dissection of the frizzled receptor in *Drosophila melanogaster* suggests different mechanisms of action in planar polarity and canonical Wnt signaling. *Genetics*, 2012; 192(4): 1295–313
- Siddique A, Yu B, Khan K et al: Expression of the Frizzled receptors and their co-receptors in calcified human aortic valves. *Can J Physiol Pharmacol*, 2018; 96(2): 208–14
- Flanagan DJ, Barker N, Nowell C et al: Loss of the Wnt receptor frizzled 7 in the mouse gastric epithelium is deleterious and triggers rapid repopulation *in vivo*. *Dis Model Mech*, 2017; 10(8): 971–80
- Kirikoshi H, Sekihara H, Katoh M: Up-regulation of Frizzled-7 (FZD7) in human gastric cancer. *Int J Oncol*, 2001; 19(1): 111–15
- Zheng L, Li X, Gu Y et al: The 3'UTR of the pseudogene CYP4Z2P promotes tumor angiogenesis in breast cancer by acting as a ceRNA for CYP4Z1. *Breast Cancer Res Treat*, 2015; 150(1): 105–18
- Zheng L, Xiang C, Li X et al: STARD13-correlated ceRNA network-directed inhibition on YAP/TAZ activity suppresses stemness of breast cancer via co-regulating Hippo and Rho-GTPase/F-actin signaling. *J Hematol Oncol*, 2018; 11(1): 72
- Yu HE, Wang F, Yu F et al: Suppression of fumarate hydratase activity increases the efficacy of cisplatin-mediated chemotherapy in gastric cancer. *Cell Death Dis*, 2019; 10(6): 413
- Ueno K, Hirata H, Hinoda Y, Dahiya R: Frizzled homolog proteins, microRNAs and Wnt signaling in cancer. *Int J Cancer*, 2013; 132(8): 1731–40
- Qu Y, Ray PS, Li J et al: High levels of secreted frizzled-related protein 1 correlate with poor prognosis and promote tumourigenesis in gastric cancer. *Eur J Cancer*, 2013; 49(17): 3718–28
- Tomizawa M, Shinozaki F, Motoyoshi Y et al: Gastric cancer cell proliferation is suppressed by frizzled-2 short hairpin RNA. *Int J Oncol*, 2015; 46(3): 1018–24
- Li G, Su Q, Liu H et al: Frizzled7 promotes epithelial-to-mesenchymal transition and stemness via activating canonical Wnt/beta-catenin pathway in gastric cancer. *Int J Biol Sci*, 2018; 14(3): 280–93
- Flanagan DJ, Phesse TJ, Barker N et al: Frizzled7 functions as a Wnt receptor in intestinal epithelial Lgr5(+) stem cells. *Stem Cell Rep*, 2015; 4(5): 759–67
- Zheng L, Guo Q, Xiang C et al: Transcriptional factor six2 promotes the competitive endogenous RNA network between CYP4Z1 and pseudogene CYP4Z2P responsible for maintaining the stemness of breast cancer cells. *J Hematol Oncol*, 2019; 12(1): 23
- Flanagan DJ, Barker N, Costanzo NSD et al: Frizzled-7 is required for Wnt signaling in gastric tumors with and without Apc mutations. *Cancer Res*, 2019; 79(5): 970–81