Depletion of death-associated protein-3 induces chemoresistance in gastric cancer cells through the β-catenin/LGR5/Bcl-2 axis

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ABSTRACT

Previously, we demonstrated that death-associated protein-3 (DAP3) loss drives chemoresistance in gastric cancer cells. In the present study, we aimed to determine the underlying molecular mechanism. The effect of DAP3 silencing on β-catenin signaling was assessed. The direct mediator of DAP3 silencing-induced chemoresistance was identified. Depletion of DAP3 stimulates nuclear accumulation of β-catenin and enhances β-catenin-dependent transcriptional activity in gastric cancer cells. However, the protein kinase B , , extracellular regulated protein kinase and signal transducer and activator of transcription 3 signaling pathways remain unaffected by DAP3 loss. We found that the downstream target gene LGR5 (leucine-rich G-protein coupled receptor 5) is upregulated in DAP3-depleted gastric cancer cells. Moreover, knockdown of LGR5 resensitizes DAP3-depleted gastric cancer cells to 5-fluorouracil (5-FU) and oxaliplatin. We also observed that ectopic expression of LGR5 reduces apoptosis in gastric cancer cells on treatment with 5-FU and oxaliplatin, which is accompanied by prevention of caspase-3 cleavage. The antiapoptotic protein Bcl-2 is identified as a key mediator of LGR5-induced apoptosis resistance in gastric cancer cells. The present findings indicate that DAP3 deficiency-induced chemoresistance in gastric cancer is at least partially mediated through the β-catenin/LGR5/Bcl-2 axis. Targeting LGR5 may provide a novel strategy to overcome chemoresistance in DAP3-deficient gastric cancer cells.

Significance of this study

What is already known about this subject?

- The Wnt/β-catenin signaling pathway is involved in the development of chemoresistance in tumor cells.
- Increased expression of leucine-rich G-protein coupled receptor 5 (LGR5) confers chemoresistance to gastric cancer cells.
- Knockdown of death-associated protein-3 (DAP3) promotes chemoresistance in gastric cancer cells.

What are the new findings?

- Depletion of DAP3 stimulates nuclear accumulation of β-catenin and enhances β-catenin-dependent transcriptional activity.
- Knockdown of LGR5 resensitizes DAP3-depleted gastric cancer cells to chemotherapeutic drugs.
- Bcl-2 is identified as a key mediator of LGR5-induced apoptosis resistance in gastric cancer cells.

How might these results change the focus of research or clinical practice?

- Targeting LGR5 may provide a novel strategy to overcome chemoresistance in DAP3-deficient gastric cancer cells.

INTRODUCTION

Gastric cancer is one of the most lethal malignancies worldwide.[1] Many patients with gastric cancer, in particular in developing countries, are diagnosed at a late stage, which leads to a low rate of complete resection. Adjuvant chemotherapy after curative gastrectomy is considered the standard treatment for advanced gastric cancer.[2] Although patients gain survival benefits from adjuvant chemotherapy,[3] the long-term outcome is limited by acquired or intrinsic chemoresistance of tumor cells.[4 5] Thus, understanding of the molecular mechanisms that induce drug resistance will improve the efficacy of chemotherapy in patients with advanced gastric cancer.

The Wnt/β-catenin signaling pathway is involved in the development of chemoresistance in tumor cells.[6 7] Ng *et al* reported that activation of the β-catenin pathway is responsible for Slit3 loss-induced chemoresistance in hepatocellular carcinoma.[6] Inhibition of the Wnt/β-catenin pathway was found to prevent chemoresistance in cancer cells,[8] suggesting that the β-catenin pathway represents a promising therapeutic target to overcome chemoresistance. Leucine-rich G-protein coupled receptor 5 (LGR5) serves as an important downstream target of β-catenin signaling.[9 10] LGR5 is frequently upregulated in many cancer types

such as colorectal cancer and breast cancer.[11 12] Cao *et al* showed that LGR5 has the capacity to enhance stemness and chemoresistance in cervical cancer cells.[10] Similarly, increased expression of LGR5 confers chemoresistance to gastric cancer cells.[13]

Death-associated protein-3 (DAP3) as a member of the death-associated protein family is known to modulate apoptosis.[14] Mariani *et al* reported that DAP3 expression is elevated in invasive glioblastoma cells.[15] Ectopic expression of DAP3 leads to increased apoptosis in cells after detachment.[16] Our previous work has reported that knockdown of DAP3 promotes chemoresistance in gastric cancer cells.[17] However, the detailed mechanism involved has not been clarified.

In the present study, we aimed to identify the key signaling pathways involved in DAP3 depletion-induced chemoresistance and to search for direct mediators of DPA3 action in gastric cancer.

MATERIALS AND METHODS

Cell culture and treatment

HGC27 and MGC803 cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and grown in ATCC-recommended media supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, California, USA). Cells were treated with 5-fluorouracil (5-FU) and oxaliplatin as described previously.[17]

Plasmids, siRNAs, and transfections

The plasmid expressing a short hairpin RNA targeting DAP3 was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). LGR5-targeting small interfering RNA (siRNA), Bcl-2-targeting siRNA, and negative control siRNA were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). LGR5-expressing plasmid was obtained from OriGene Technologies (Rockville, Maryland, USA). Cell transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen), according to the manufacturer's protocol.

Luciferase reporter assay

For measurement of β-catenin transcriptional activity, TOPFlash/FOPFlash luciferase reporter assay was conducted as described previously.[18] In brief, cells were transfected with the firefly luciferase reporter TOPFlash or TOP-FOP plasmid, together with the *Renilla* luciferase reporter pRL-TK (Promega, Fitchburg, Wisconsin, USA). Twenty-four hours after transfection, the cells were lysed. The luciferase activity of lysate was determined using the Dual Luciferase Assay System (Promega). The firefly luciferase activity was normalized to the activity of the *Renilla* luciferase.

Subcellular fractionation and western blot analysis

Nuclear and cytoplasmic fractionation was prepared using the BioVision Nuclear/Cytosolic Fractionation Kit (Mountain View, California, USA) following the manufacturer's instruction. Protein samples were separated on sodium dodecyl sulfate acrylamide gels and transferred on polyvinylidene difluorid (PVDF) membranes. The membranes were incubated overnight at 4°C with primary antibodies recognizing total β-catenin, non-phosphorylated active β-catenin, phospho-glycogen synthase kinase 3 beta (GSK-3β), LGR5, cleaved caspase-3, Bcl-2, Bcl-xL, Mcl-1, histone h3, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology). Chemiluminescent signals were developed using chemiluminescence (Thermo-Scientific) and quantified by densitometry.

Quantitative real-time PCR analysis

Total RNA was isolated using Trizol reagent, and reverse transcription was performed using SuperScript II (Invitrogen). Quantitative real-time PCR was conducted using the SYBR Green PCR Master Mix (Thermo Fisher Scientific). The PCR primers were as follows: *Lgr5* forward, 5′-TTTGGACAAGGGAGACCTGGAGAAT-3′ and *Lgr5* reverse, 5′-GAAAGCCACAGGGCAGTTTAGGAT-3′.[19] GAPDH served as the internal control. Relative levels of *Lgr5* transcripts were calculated using the $2^{-\Delta\Delta CT}$ method.[20]

Cell viability assay

Cells were seeded onto 96-well plates at a density of 5×10^3 cells/well and treated with 5-FU or oxaliplatin for 48 hours. Cells were added with 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/mL; Sigma-Aldrich, St Louis, Missouri, USA) and incubated for 4 hours at 37°C. Absorbance was measured at 570 nm.

Apoptosis

After treatment with 5-FU or oxaliplatin for 48 hours, cells were fixed and stained with Annexin V-FITC and propidium iodide (Beyotime, Haimen, China) according to the manufacturer's protocol. Stained cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, California, USA).

Statistics

All values are reported as mean±SD and analyzed by Student's t-test or one-way analysis of variance. $P<0.05$ was considered statistically significant.

RESULTS

Depletion of DAP3 enhances β-catenin activation

Depletion of DAP3 markedly raised the levels of total β-catenin and non-phosphorylated active β-catenin in both MGC803 and HGC27 cells (figure 1A). Moreover, DAP3 knockdown led to accumulation of β-catenin in the nucleus (figure 1B). However, DAP3 deficiency had no significant impact on the activation of protein kinase B (Akt), extracellular regulated protein kinase (ERK), and signal transducer and activator of transcription 3 (STAT3) signaling (figure 1A). To validate the regulation of β-catenin signaling, we transiently transfected a β-catenin/TCF4 reporter construct into DAP3-depleted gastric cancer cells. Luciferase reporter assay demonstrated that DAP3 depletion enhanced β-catenin-dependent transcriptional activity (figure 1C). It has been well accepted that phosphorylation and degradation of GSK-3β are associated with β-catenin activation.[9] We found that DAP3 knockdown resulted in increased phosphorylation of GSK-3β (figure 1D). Taken

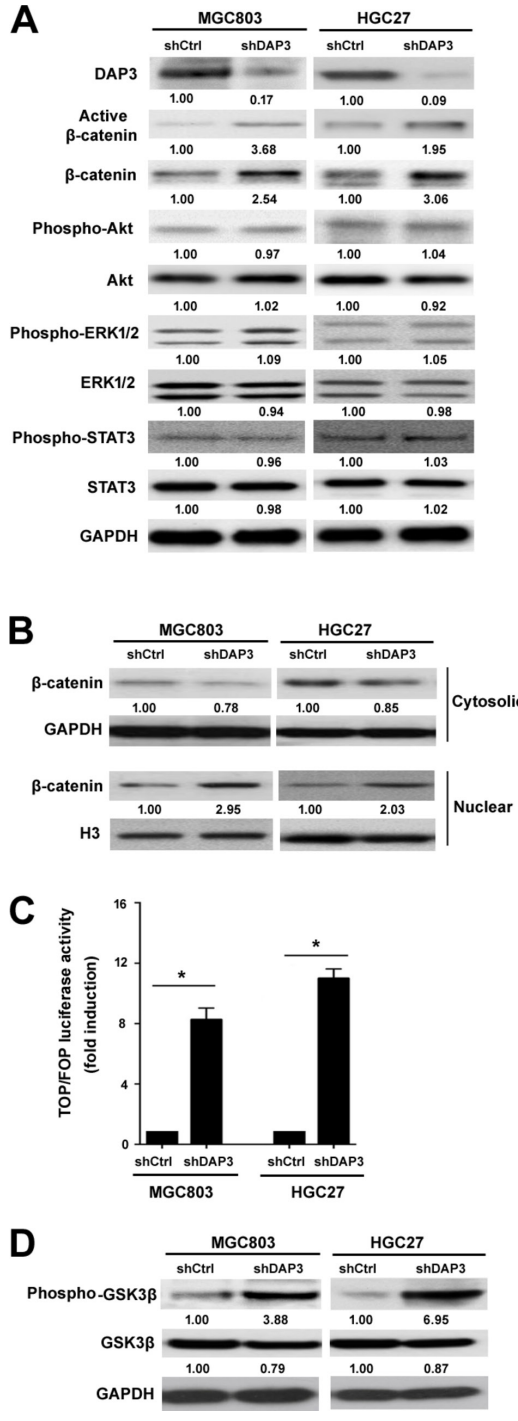

Figure 1 Depletion of DAP3 enhances β-catenin activation. (A) Western blot analysis of indicated proteins in gastric cancer cells transfected with control shRNA (shCtrl) or DAP3-targeting shRNA (shDAP3). (B) Western blot analysis of β-catenin protein in subcellular fractions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and histone H3 were used as the control for the cytoplasmic and nuclear fraction, respectively. (C) Luciferase reporter assay. DAP3 depletion leads to an increase in β-catenin-dependent transcriptional activity. *P<0.05. (D) Western blot analysis of phosphorylation of glycogen synthase kinase 3 beta (GSK-3β). DAP3, death-associated protein-3; shRNA, short hairpin RNA; Akt, protein kinase B; ERK, extracellular regulated protein kinase; STAT3, signal transducer and activator of transcription 3.

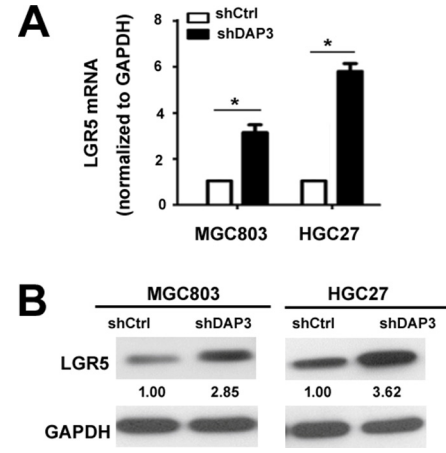

Figure 2 LGR5 is induced in DAP3-depleted gastric cancer cells. (A) Real-time PCR analysis of LGR5 mRNA levels in gastric cancer cells transfected with control shRNA (shCtrl) or DAP3-targeting shRNA (shDAP3). *P<0.05. (B) Western blot analysis of LGR5 protein levels. DAP3, death-associated protein-3; LGR5, leucine-rich G-protein coupled receptor 5; shRNA, short hairpin RNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

together, DAP3 acts as a negative regulator of β-catenin signaling in gastric cancer.

LGR5 is induced in DAP3-depleted gastric cancer cells

LGR5 is known as an important target of β-catenin and can modulate chemosensitivity of cancer cells.[9 10] Therefore, we asked whether DAP3 depletion has an impact on the expression of LGR5. To address this, we examined the expression of LGR5 in DAP3-depleted gastric cancer cells. It was found that DAP3 depletion resulted in a significant increase in the level of LGR5 transcript in MGC803 and HGC27 cells, compared with corresponding control cells (figure 2A). Similar change was observed at the protein level, as assessed by western blot analysis (figure 2B). These results indicate the upregulation of LGR5 after DAP3 depletion.

LGR5 silencing reverses DAP3 depletion-driven chemoresistance in gastric cancer cells

Next, we checked whether the induction of LGR5 accounts for DAP3 depletion-driven chemoresistance in gastric cancer cells. To resolve this, we knocked down LGR5 expression in DAP3-depleted gastric cancer cells. Transfection with specific LGR5-targeting siRNAs effectively downregulated the expression of LGR5 in DAP3-deficient MGC803 and HGC27 cells (figure 3A,B). When LGR5 expression was inhibited, DAP3 silencing-mediated chemoresistance to 5-FU (figure 3C) and oxaliplatin (figure 3D) was abrogated. Together, LGR5 plays a pivotal role in inducing chemoresistance owing to DAP3 deficiency.

LGR5 induces apoptosis resistance in gastric cancer cells

Next, we tested whether LGR5 overexpression can stimulate apoptosis resistance to chemotherapeutic drugs. To this end, we assessed apoptotic responses in LGR5-overexpressing cells after exposure to 5-FU and oxaliplatin. It was found that 5-FU and oxaliplatin treatment significantly induced apoptosis in vector-transfected MGC803 and HGC27 cells

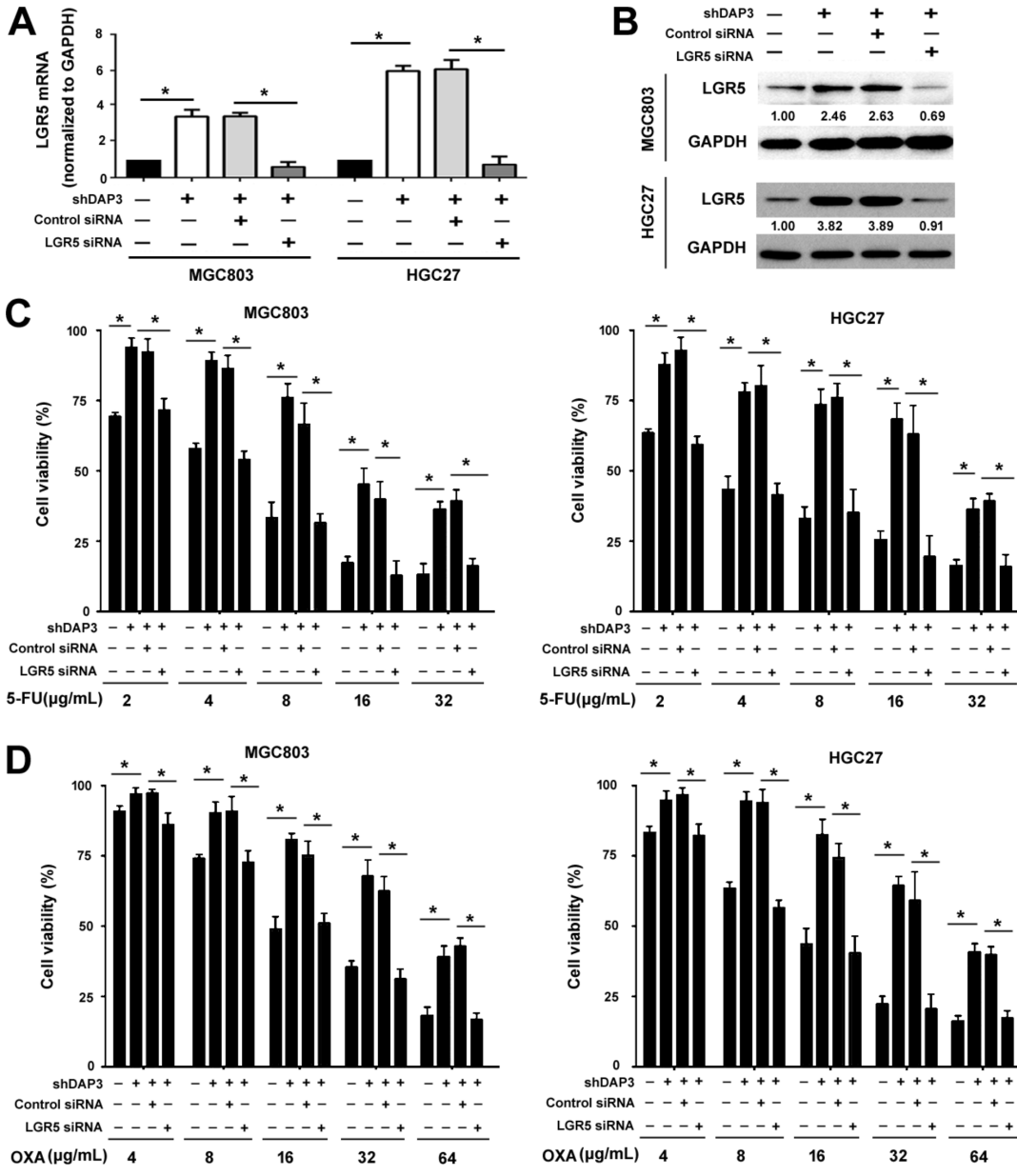

Figure 3 LGR5 silencing reverses DAP3 depletion-driven chemoresistance in gastric cancer cells. (A and B) Transfection with specific LGR5-targeting siRNAs effectively downregulated the expression of LGR5, as determined by real-time PCR (A) and western blot (B) analyses. (C and D) Gastric cancer cells transfected with indicated constructs were treated with different concentrations of 5-FU (C) and oxaliplatin (OXA) (D) and tested for viability. *P<0.05. 5-FU, 5-fluorouracil; DAP3, death-associated protein-3; LGR5, leucine-rich G-protein coupled receptor 5; shDAP3, DAP3-targeting shRNA; shRNA, short hairpin RNA; siRNA, small interfering RNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

(figure 4A,B). LGR5 overexpression prevented the apoptosis induced by these chemotherapeutic drugs. Moreover, 5-FU and oxaliplatin promoted the cleavage of caspase-3 in gastric cancer cells, and such effect was abolished by LGR5 overexpression (figure 4C). The data indicate that LGR5 confers resistance to 5-FU and oxaliplatin in gastric cancer cells.

Upregulation of Bcl-2 accounts for LGR5-mediated apoptosis resistance

To get insight into the mechanism by which LGR5 promotes chemoresistance, we measured the expression of multiple antiapoptotic proteins by western blot analysis. We found that ectopic expression of LGR5 elevated the protein level of Bcl-2, but not Bcl-xL or Mcl-1, in gastric cancer cells (figure 5A). Most importantly, knockdown of Bcl-2 reversed LGR5-mediated apoptosis resistance in gastric cancer cells after treatment with 5-FU and oxaliplatin (figure 5B,C). Taken together, LGR5-induced chemoresistance is ascribed to upregulation of the antiapoptotic protein Bcl-2.

DISCUSSION

In this study, we show that DAP3 depletion leads to increased levels of active β-catenin and nuclear accumulation of

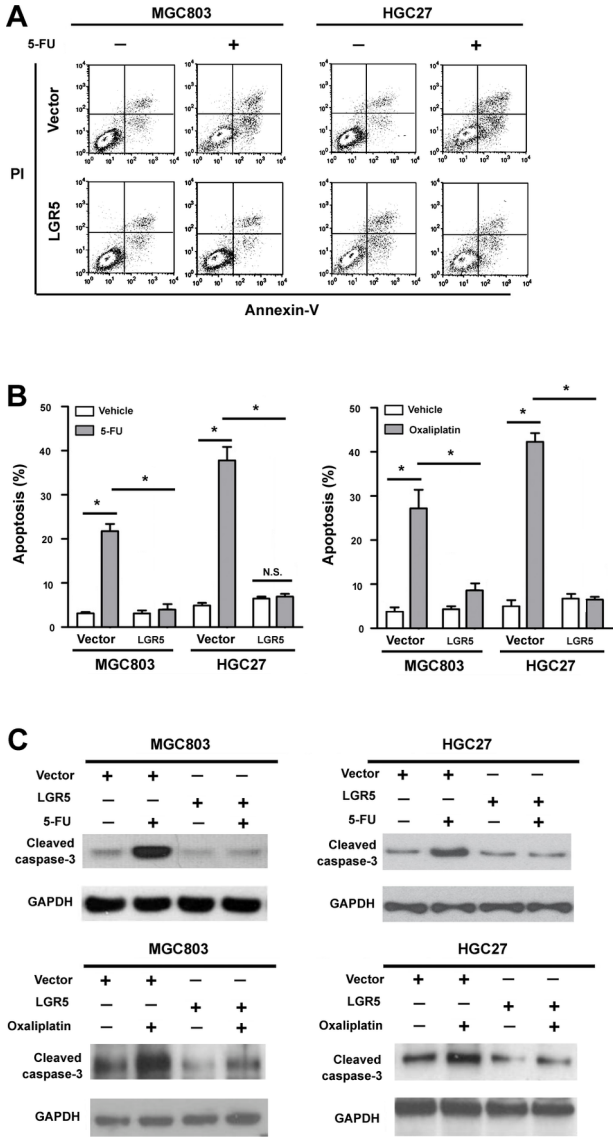

Figure 4 LGR5 induces apoptosis resistance in gastric cancer cells. (A) Apoptosis analysis by flow cytometry. LGR5-overexpressing cells and control cells were treated with 8 µg/mL 5-FU for 48 hours and analyzed for apoptosis. Representative dot plots of flow cytometry are shown. (B) Quantification of apoptosis in gastric cancer cells after treatment with 8 µg/mL 5-FU or 32 µg/mL oxaliplatin for 48 hours. *P<0.05. (C) Western blot analysis of cleaved caspase-3 levels. 5-FU, 5-fluorouracil; LGR5, leucine-rich G-protein coupled receptor 5; PI, propidium iodide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

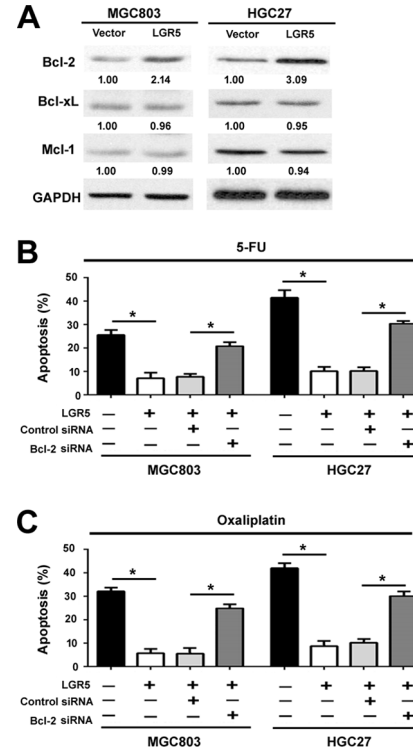

Figure 5 Upregulation of Bcl-2 accounts for LGR5-mediated apoptosis resistance. (A) Western blot analysis of indicated proteins in gastric cancer cells transfected with LGR5-expressing plasmid or empty vector. (B) Gastric cancer cells transfected with indicated constructs were treated with 8 µg/mL 5-FU for 48 hours and analyzed for apoptosis. (C) Gastric cancer cells transfected with indicated constructs were treated with 32 µg/mL oxaliplatin for 48 hours and analyzed for apoptosis. *P<0.05. 5-FU, 5-fluorouracil; LGR5, leucine-rich G-protein coupled receptor 5; siRNA, small interfering RNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

β-catenin in gastric cancer cells, which is accompanied by enhanced phosphorylation of GSK-3β. Luciferase reporter assays indicate the induction of β-catenin-dependent transcriptional activity in DAP3-depleted cells, suggesting DAP3 as an endogenous inhibitor of β-catenin signaling. Activation of β-catenin signaling is considered as an important cause of chemoresistance in tumor cells.[6 7] Santos *et al* reported that SOX9 upregulation promotes stemness and chemoresistance in gastric cancer cells through activation of Wnt/β-catenin signaling.[21] Similarly, the Wnt/β-catenin signaling pathway is involved in tissue transglutaminase-1-induced chemoresistance in gastric cancer cells.[22] These studies, combined with our findings, suggest that DAP3 silencing-induced chemoresistance is likely mediated through activation of β-catenin signaling.

Our data also demonstrate that LGR5, a downstream target gene of β-catenin, is upregulated in DAP3-depleted gastric cancer cells. To validate the involvement of LGR5 in the action of DAP3, we knocked down the expression of LGR5. Of note, LGR5 knockdown resensitizes DAP3-deficient MGC803 and HGC27 cells to 5-FU and oxaliplatin, indicating that LGR5 is required for DAP3 deficiency-induced chemoresistance in gastric cancer cells. In line with our findings, LGR5 upregulation contributes to chemoresistance in multiple cancer types, including gastric cancer, cervical cancer, and colorectal cancer.[10 13 23] LGR5 is known to promote the activation of β-catenin signaling in cancer cells,[24] which provides an explanation for the ability of LGR5 to induce chemoresistance. Although we provide evidence for the involvement of LGR5 in DAP3 loss-induced chemoresistance, we cannot exclude the possibility that other β-catenin target genes may also mediate the activity of DAP3.

LGR5 is known to act as a prosurvival factor in several types of cancer cells.[25 26] Knockdown of LGR5 was observed to induce apoptosis in breast cancer cells.[25] Hsu *et al* reported that LGR5 silencing stimulates mitochondria-mediated apoptosis in colorectal cancer cells.[27] In conjunction with these observations, our data demonstrate that LGR5 overexpression attenuates the apoptotic response to 5-FU and oxaliplatin in gastric cancer cells. At the molecular level, caspase-3 cleavage was abolished after LGR5 overexpression, confirming that LGR5 contributes to the survival of gastric cancer cells on treatment with chemotherapeutic drugs. Our data further reveal that LGR5 overexpression selectively induces the expression of the antiapoptotic protein Bcl-2. Downregulation of Bcl-2 impairs LGR5-mediated apoptosis resistance to chemotherapeutic drugs. Taken together, LGR5 induces chemoresistance in gastric cancer cells at least partially through upregulation of Bcl-2. Future work will be needed to identify the key mediators in the induction of Bcl-2 by LGR5.

In summary, our results show that the β-catenin/LGR5 axis is involved in DAP3 loss-induced chemoresistance in gastric cancer cells. LGR5 overexpression confers survival advantage to gastric cancer cells on exposure to chemotherapeutic drugs, which is largely attributed to induction of Bcl-2 and prevention of apoptotic response. Our data suggest that targeting LGR5 may be beneficial in overcoming chemoresistance in DAP3-deficient gastric cancer cells.

Contributors YJ, LZ, YH, and JJ participated in study design, data collection, and drafting of the manuscript. ZL, XC, XW, FP, JS, SL, and XL conducted the experiments.

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Patient consent for publication Not required.

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