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CLINICAL TRIALS STUDY

Inhibition of Girdin enhances chemosensitivity of colorectal cancer cells to oxaliplatin

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Abstract

AIM: To investigate the effect of Girdin knockdown on the chemosensitivity of colorectal cancer cells to oxaliplatin and the possible mechanisms involved.

METHODS: Four siRNAs targeting Girdin were transfected into the chemoresistant colorectal cancer cell line DLD1. Real-time polymerase chain reaction (PCR) was employed to assess Girdin mRNA expression and the most effective siRNA was chosen for conversion into shRNA. Then, DLD1 cells were infected with lentiviruses expressing the Girdin shRNA and a scramble control, respectively, and Girdin mRNA and protein expression levels were assessed by real-time PCR and Western blotting. Furthermore, microarray experiments were used to assess global gene expression profile after Girdin suppression in DLD1 cells. Finally, the cytotoxic effect of simultaneous treatment with oxaliplatin and adriamycin (an inhibitor of a significantly downregulated gene after Girdin suppression in DLD1 cells) was examined by MTT assay.

RESULTS: The most effective siRNA suppressed Girdin expression with an inhibition efficiency of 57%. Compared with the scramble control, DLD1 cells infected with the Girdin shRNA displayed decreased Girdin mRNA and protein levels ($P < 0.05$), and Girdin knockdown significantly enhanced chemosensitivity to oxaliplatin in colorectal cancer cells ($P < 0.05$). Microarray data revealed that 381 and 162 genes were upregulated and downregulated in response to Girdin reduction, respectively, with ratios > 1.2 or < 0.8 ($P < 0.01$). Interestingly, TOP2B (DNA topoisomerase 2-β) was downregulated (ratio = 0.78, $P = 0.0001$) and oxaliplatin/adriamycin combination resulted in increased cell death compared with treatments with individual agents ($P < 0.05$).

CONCLUSION: Girdin knockdown enhances chemosensitivity of colorectal cancer cells to oxaliplatin *via* TOP2B down-regulation. These findings provide a promising approach to overcome the chemoresistance of colorectal cancer cells.

Key words: Oxaliplatin; Chemosensitivity; Girdin; Colorectal cancer; TOP2B

Core tip: The chemoresistance to oxaliplatin is a major problem in the treatment of colorectal cancer (CRC). In this study, a lentivirus-mediated shRNA was constructed to investigate the role of Girdin, a potential regulator of chemotherapy sensitivity. We found that Girdin knockdown enhanced chemosensitivity of colorectal cells to oxaliplatin *via* reduction of TOP2B. This suggested that Girdin is a modulator of CRC chemoresistance and a potential therapeutic target.

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INTRODUCTION

Colorectal cancer (CRC) is currently the third most common cancer worldwide and the fourth cause of cancer-related death[1]. Administration of oxaliplatin-based regimens is considered the first-line chemotherapy in the adjuvant and palliative settings for CRC patients[2-4]. However, treatment efficacy has reached a plateau with a response rate of 40%[5], which highlights the need for novel strategies to enhance the chemosensitivity to oxaliplatin in CRC.

Accumulating studies have demonstrated that the phosphatidylinositol 3-kinase (PI3K)/Akt pathway plays a crucial role in chemotherapy resistance[6]. This pathway affects chemotherapy-induced apoptosis in various cancers, including lung, ovarian, breast, liver and pancreatic tumors[7-11]. In CRC, activation of the PI3K/Akt pathway has been shown to potently affect oxaliplatin resistance[12]. Girdin (also called coiled-coil domain-containing protein 88A), a multidomain molecule, plays important roles in diverse biological processes, *e.g.*, wound healing, tumor cell motility, and angiogenesis[13]. Recent data described Girdin as a novel nonreceptor guanine nucleotide exchange factor (GEF) for Gai proteins that enhances PI3K-Akt signals by activating Gαi1, 2, 3[14]. Moreover, Matsushita proposed that Girdin family proteins play an essential role in apoptosis *via* activation of the IRE1-JNK pathway[15]. Based on these findings, it is conceivable that Girdin may constitute a potential regulator of chemotherapy sensitivity, which prompted us to investigate the effect and possible mechanisms of Girdin knockdown on the chemosensitivity of CRC cells to oxaliplatin.

To date, previous studies have focused on the role of Girdin in the progression and metastasis of CRC cells[16,17]; however, studies assessing the contribution of Girdin to chemoresistance in CRC are inexistent. Herein, we report for the first time that Girdin knockdown enhances chemosensitivity of CRC cells to oxaliplatin *via* reduction of TOP2B.

MATERIALS AND METHODS

Cell lines and cell culture

The 17 cell lines (CACO-2, D2, DLD1, HCT15, HCT116, HUTU80, SW48, SW480, SW620, SW837, CX-1, COLO205, GP2D, GP5D, HCT15, LS174T and LS180) were obtained from American Type Culture Collection (ATCC, United States) and Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. All cell lines were maintained according to ATCC protocols. 293FT cells were purchased from Invitrogen (United States) and maintained according to the manufacturer's specifications in DMEM supplemented with 10% FBS, 0.1 mmol/L minimum essential medium (MEM) nonessential amino acids, 2 mmol L-glutamine, 1% penicillin/streptomycin and 500 μg/mL geneticin (Invitrogen). Sodium butyrate and polyethylene glycol (PEG)-8000 were obtained from Sigma-Aldrich (United States).

Table 1 Four siRNA duplexes targeting Girdin

siRNA	Synthetic siRNA sequence
siRNA1	Sense: CCAGAAUGUACCGAGAUGAUU
	Antisense: UCAUCUCGGUACAUUCUGGUU
siRNA2	Sense: CUUCAUUAGUUCUGCGGGAUU
	Antisense: UCCCGCAGAACUAAUGAAGUU
siRNA3	Sense: GGACCAACCUUGAUGAAUAUU
	Antisense: UAUUCAUCAAGGUUGGUCCUU
siRNA4	Sense: CAAGAGUUGAGGAAUUAAAUU
	Antisense: UUUAAUUCCUCAACUCUUGUU
scramble siRNA	Sense: GAGGCGAAACGCTAAATATCT
	Antisense: AGATATTTAGCGTTTCGCCTC

siRNA design and synthesis

Four target sequences were chosen (Table 1) from the human Girdin gene (GenBank, Gene ID: 55704) to design siRNA online (WI siRNA selection program, http://sirna.wi.mit.edu/), and a scramble siRNA that does not match any known mammalian GenBank sequence was designed using the Invivogen scramble siRNA online program (Invivogen, United States). All oligonucleotides were synthesized by Shanghai GenePharma Co., Ltd (China) (Table 1).

Evaluation of chemosensitivity to oxaliplatin

Oxaliplatin was purchased from Sigma (United States) and stock solutions of 5 mg/ml were prepared in DMSO. Aliquots of oxaliplatin were stored at -20 ℃ until use. Chemosensitivity of 17 CRC cell lines to oxaliplatin was examined using MTT assay. Briefly, cells were harvested in the exponential growth phase, seeded in 96-well plates (3000 cells/well), and incubated overnight. Then, oxaliplatin was added at various concentrations (0, 3, 10, 30 μmol). At the end of treatment period, 10 μL of MTT was added for 4 h and the media gently aspirated. DMSO was used to dissolve the purple crystals and absorbance was determined at 490 nm using a spectrophotometer (Thermo, United States). The inhibition rate (IR) was derived as $1-(A490_{\text{experimental group}}-A490_{\text{blank}})/(A490_{\text{control group}}-A490_{\text{blank}}) \times 100\%$.

siRNA transfection

DLD1 cells were seeded in 24-well plates and incubated for 24 h before transfection. DLD1 cells at 30% confluence were transfected with 50 nmol/L (final concentration) siRNA per well using Lipofectamine 2000 and Opti-MEM (Invitrogen) media according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested for further analysis.

Real-time quantitative polymerase chain reaction

Total RNA was isolated using the TRIzol reagent (Invitrogen), and 1 μg total RNA was reverse transcribed in 50 μL reaction to yield cDNA, which was stored at -20 ℃ until use. Girdin mRNA level was assessed by real-time quantitative polymerase chain reaction (PCR) with β-actin used as an internal control. The primer

sequences are as follows: β-actin (forward: AGCGAGCATCCCCCAAAGTT; reverse: GGGCACGAGGGCTCATCATT), Girdin (forward: CCAGGCATGAAGCGAACA; reverse: CGAGCATCCGAAAGCAAAT) and TOP2B (forward: AAGAAGAGTCAGAAACTGCCAACA; reverse: ACCCACATGAACTGCGTCAA). Quantitative RT-PCR was carried out using the SYBR Green kit (Qiagen, United States) in a final volume of 25 μL. Each PCR cycle included 15 s of denaturation at 95 ℃, 20 s of primer annealing at 58 ℃ and 20 s of extension at 72 ℃.

Lentiviral plasmid construction, lentivirus production and cell transfection

Based on the interference efficiency of the four siRNAs, the most effective sequence in knocking down Girdin was chosen and converted into shRNA with an online tool (http://www.broadinstitute.org/rnai/public/). Both forward oligonucleotide (CCGGAGGCAAGAGTTGAGGAATTAACTCGAGTTAATTCCTCAACTCTTGCCTTTTTTG) and reverse olignucleotide (AATTCAAAAAAGGCAAGAGTTGAGGAATTAACTCGAGTTAATTCCTCAACTCTTGCCT) were synthesized, annealed and cloned between the BamHI and EcoRI sites of the lentiviral shRNA expression vector LvUCTP derived from FugW (Addgene, United States). The U6 promoter was added for shRNA expression and tdTomato gene for indication of transfection and infection. A scramble shRNA was derived from the scramble siRNA (forward oligonucleotide: CCGGGAGGCGAAACGCTAAATATCTCTCGAGAGATATTTAGCGTTTCGCCTCTTTTTG; reverse oligonucleotide: AATTCAAAAAGAGGCGAAACGCTAAATATCTCTCGAGAGATATTTAGCGTTTCGCCTC). Lentivirus production was performed using the ViraPower™ Lentiviral Packaging Mix (Invitrogen) following the manufacturer's instructions. In brief, the lentiviral expression vector was cotransfected with an optimized mixture of three packaging plasmids (pLP1，pLP2 and pLP/VSVG) into 293FT cells. Cell supernatants containing viral particles were harvested 48 h after transfection, filtered through a 0.45 μm filter (Millipore), and stored at -70 ℃. Lentivirus preparations containing only the scramble shRNA were used as negative controls.

Protein extraction and Western blotting

Total protein was extracted from cells using RIPA buffer (Pierce, United States) and quantified using BCA protein assay kit according to the manufacturer's instructions (Pierce, United States). Totally 10 μg protein was loaded onto SDS-PAGE gels for electrophoresis. After transfer, membranes were blocked with TBST containing 5% nonfat milk for 0.5 h at room temperature, and incubated with primary polyclonal antibodies raised in rabbits against Girdin, TOP2B and β-tublin (Santa Cruz, United States). Signals were detected after incubation in HRP-conjugated secondary antibodies and addition of ECL substrate kit (Thermo, United States). β-tublin acted as the reference protein for loading control.

Cytotoxicity assays

DLD1 cells were infected with the recombinant lentivirus for 90 min, incubated for an additional 24 h, and seeded in 96-well plates at 4000 cells/well. Seventy-two hours after infection, cells were treated with oxaliplatin at the indicated concentrations for 72 h and analyzed by MTT assay. Analysis of combinational inhibition of oxaliplatin and adriamycin was conducted at indicated concentrations and cell viability was assessed 72 h after treatment.

cDNA microarrays

The mRNA extracted from lentivirus mediated Girdin shRNA (Lv-Girdin shRNA) infected DLD1 cells was reverse transcribed into cDNA using Cy5-dUTP labeling, while the mRNA from the Lv-scramble shRNA infected DLD1 cells was processed with Cy3-dUTP labeling, following the manufacturer's protocols. The labeled probes were then hybridized to the cDNA microarray chips from Affimetrix (United States). Ratios of Cy5: Cy3 greater than 1.2 or less than 0.8 were chosen for further analysis.

Statistical analysis

Values obtained from real-time PCR and MTT assay and IC_{50} are expressed as mean ± SD. Statistical analysis was carried out by one-way ANOVA using the statistical package SPSS 19.0 (Chicago, IL, United States). Differences were considered statistically significant at $P < 0.05$. For microarray analysis, $P < 0.01$ was considered statistically significant.

RESULTS

Chemosensitivity of CRC cell lines to oxaliplatin

To explore the mechanisms by which CRC cells resist cytotoxicity of oxaliplatin, it is of prime importance to identify at least one cell line resistant to the drug. We examined the chemosensitivity of 17 CRC cell lines to oxaliplatin at various concentrations as previously described[18]. All CRC cells were inhibited by oxaliplatin in a dose-dependent manner (Figure 1). Of the 17 cell lines, DLD1 cells displayed the highest resistance to oxaliplatin at various concentrations. Based on these findings, DLD1 was chosen to further assess oxaliplatin resistance.

Inhibition of Girdin significantly enhanced in vitro chemosensitivity of DLD1 cells

We then examined cell viability of Girdin knockdown DLD1 cells after treatment with oxaliplatin to determine the effect of Girdin on resistance. Four pairs of siRNAs targeting different sites of Girdin mRNA were tested for knockdown efficacy. Real-time PCR was performed to evaluate Girdin mRNA levels after siRNA transfections. The relative Girdin mRNA levels in DLD1 cells were 58% ± 3%, 43% ± 2%, 47% ± 3% and 54% ± 3% for siRNA1, siRNA2, siRNA3 and siRNA4, respectively, compared with the scramble siRNA ($P < 0.05$) (Figure 2A), indicating that siRNA2 was the most effective sequence.

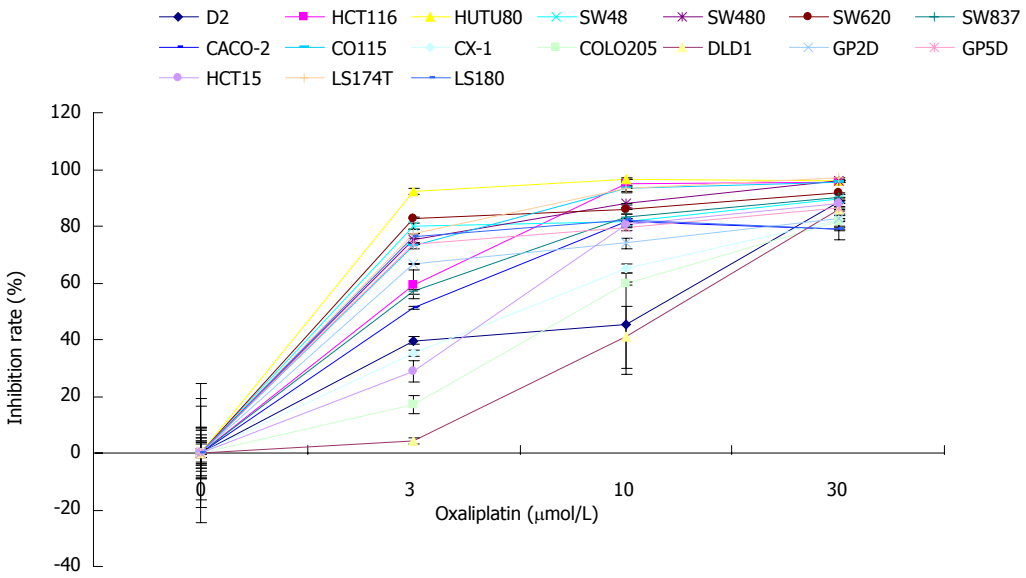

Figure 1 MTT assay. Seventeen colorectal cancer (CRC) cell lines were treated with different concentrations of oxaliplatin, and MTT assay was used to detect cell growth inhibition as described in Materials and Methods.

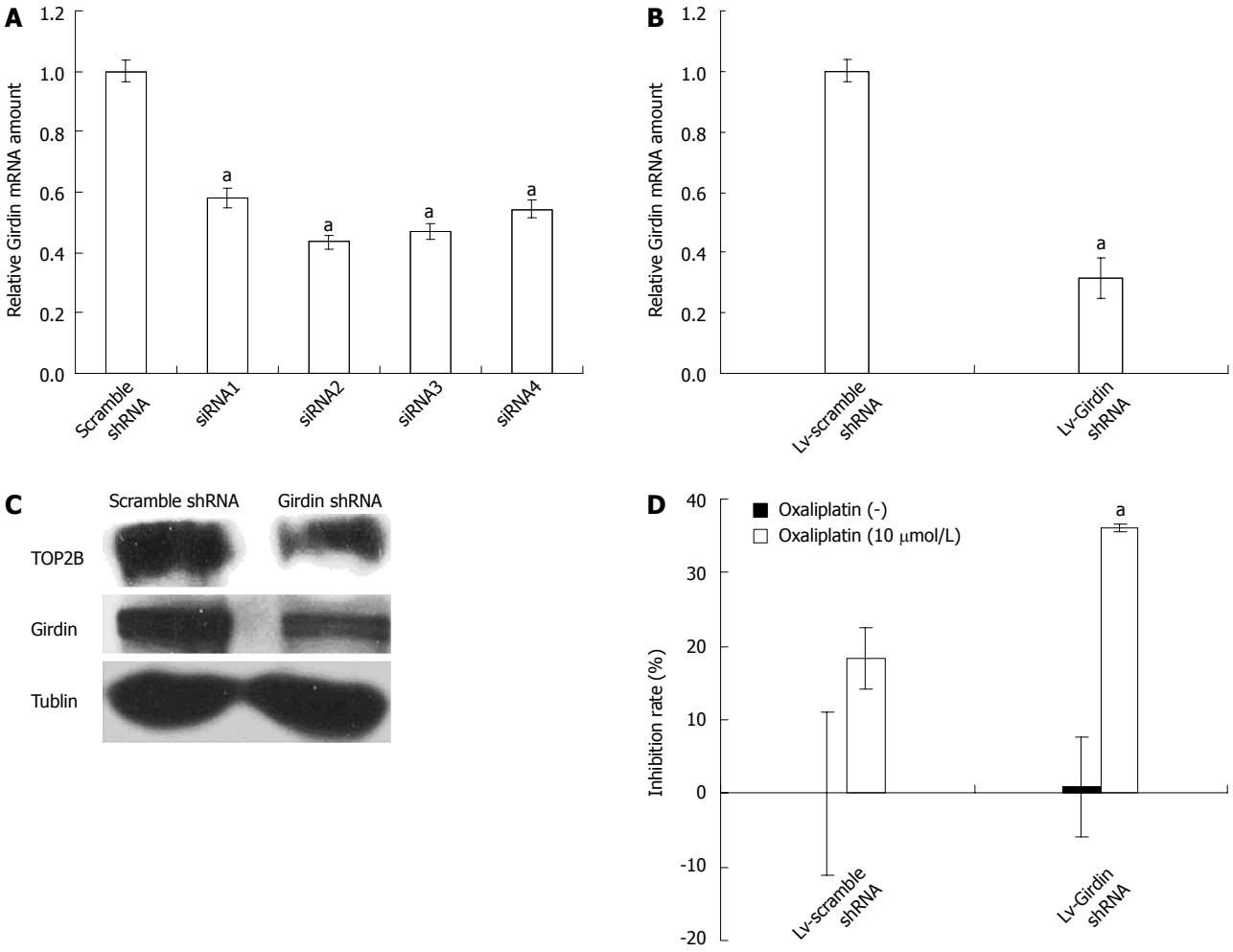

Figure 2 Effect of Girdin on DLD1 sensitivity to oxaliplatin. A: Quantification of Girdin mRNA by real-time polymerase chain reaction to validate knockdown effect of four siRNA duplexes targeting Girdin; B: Girdin mRNA level in DLD1 cells after infection with a lentivirus expressing an shRNA targeting Girdin; C: Detection of Girdin and TOP2B proteins by Western blotting; D: Growth inhibition of DLD1 cells by oxaliplatin in combination with knockdown of Girdin. $^{a}P < 0.05$ *vs* scramble shRNA.

Table 2 Top 10 downregulated and upregulated genes in Lv-Girdin shRNA infected DLD1 cell line *vs* Lv-scramble shRNA infected DLD1 cell lines

Gene name	*P* value	Ratio
Downregulated		
TOP2B	0.000148	0.780296
EIF3E	0.000154	0.794333
SCARNA9	0.000166	0.768316
SEMA3A	0.000227	0.781830
IKZF2	0.000296	0.712167
KIT	0.000311	0.792173
MIR34A	0.000358	0.783264
LRRC37A4	0.000366	0.661987
LRRC37A4	0.000391	0.66294
ARHGAP18	0.000400	0.756169
Upregulated		
PPP1R15A	0.000114	1.504745
TAP2	0.000117	1.217997
FOSL2	0.000151	1.228042
KRTAP2-1	0.000170	1.588156
TNFRSF9	0.000170	5.843542
BCL3	0.000185	1.369511
LCN2	0.000195	5.641935
CYB561D2	0.000196	1.203703
SERPINE2	0.000204	1.247110
RAB15	0.000205	1.353464

To enhance the transfection efficiency and maintain the knockdown efficacy for a relatively long time, siRNA2 was converted to shRNA sequence for the production of lentivirus mediated shRNA. Real-time PCR and Western blotting assays were performed to evaluate Girdin mRNA and protein expression after transfection. As shown in Figure 2B, Girdin relative mRNA levels in the Lv-Girdin shRNA group was 32% ± 7% compared to the scramble control ($P < 0.05$). In agreement, Western blotting data demonstrated that the Girdin protein was less expressed in the Lv-Girdin shRNA group compared with the scramble control (Figure 2C). These findings suggested that lentivirus-mediated RNAi effectively silenced Girdin expression in transfected cells.

Next, DLD1 cells were infected with the Lv-Girdin shRNA and scramble control for 72 h and exposed to 10 μmol/L oxaliplatin. We chose the concentration of 10 μM for the reason that single treatment with Girdin knockdown or with oxaliplatin less than 10 μmol/L did not show detectable inhibition effect on DLD1. As shown in Figure 2D, Lv-Girdin shRNA transfected DLD1 cells showed increased chemosensitivity to oxaliplatin ($P < 0.05$), compared with those transfected with the scramble control, indicating that Girdin silencing enhanced DLD1 cells sensitivity.

Identification of differentially expressed genes after Girdin knockdown

To identify the downstream genes of Girdin responsible for oxaliplatin resistance, we performed a global analysis of gene expression after Girdin silencing. In comparison to the scramble control, 162 and 381 genes were downregulated and upregulated in response to Girdin reduction, respectively, with ratios > 1.2 or < 0.8 ($P < 0.01$). The top 10 differentially expressed genes are listed in Table 2. Of note, TOP2B appeared in the list as downregulated with the lowest *P* value, indicating that Girdin reduction resulted in TOP2B loss and enhanced sensitivity to oxaliplatin. TOP2B downregulation after Girdin silencing was confirmed by real-time PCR and protein levels were also reduced as shown by Western blotting (Figure 3A and 2C).

TOP2B inhibitor sensitizes CRC cells to oxaliplatin

Since the action of TOP2B leads to a reversible double strand breakage (DSB) in DNA, it is used as a drug target to generate DNA damage for cancer therapy[19]. To confirm TOP2B as a sensitizing target for oxaliplatin, we examined the effect of adriamycin, a TOP2B inhibitor, on the sensitivity of DLD1 cells to oxaliplatin. As addition of adriamycin only showed an inhibitory effect on DLD1, we chose 3 μmol oxaliplatin to determine the effect of their combinational treatment. Treatment of DLD1 cells with a combination of 3 umol oxaliplatin and varying concentrations of adriamycin resulted in a shift of the dose response curve and enhanced cytotoxicity compared with single agents (Figure 3B). Similar results were also observed in other three CRC cell lines (Figure 4)

DISCUSSION

Oxaliplatin-based chemotherapy is widely used in CRC adjuvant and metastatic settings[2-4]. However, typical tumor resistance processes are the main reasons for treatment failure[20]. Thus, it is of paramount importance to search for novel strategies to enhance the chemosensitivity of CRC cells to oxaliplatin.

Induction of apoptosis is the principal mechanism by which the majority of chemotherapeutic agents exert their effects. Consequently, failure to undergo apoptosis is the likely mechanism mediating drug resistance in tumors[21]. Girdin, a nonreceptor GEF, can interact with Gαi resulting in Gβγ release and subsequently activates PI3K/AKT signaling events, which have been recognized as an important pathway for chemoresistance through its antiapoptotic effects in cancers[22]. Moreover, Girdin family proteins were shown to suppress apoptosis[15]. These findings raised the possibility that Girdin might be involved in chemoresistance of CRC cells to oxaliplatin.

In this study, lentivirus-mediated shRNA was constructed to investigate the role of Girdin in chemoresistance of CRC cells to oxaliplatin. After successful transfection with a lentivirus-mediated Girdin shRNA, Girdin mRNA and protein levels were virtually reduced and the downregulation of Girdin significantly increased chemosensitivity of CRC cells to oxaliplatin. These findings suggested that Girdin is a modulator of CRC cell chemoresistance, and therefore a potential therapeutic target. To our knowledge, this is the first study that demonstrates the ability of Girdin to enhance the chemoresistance of

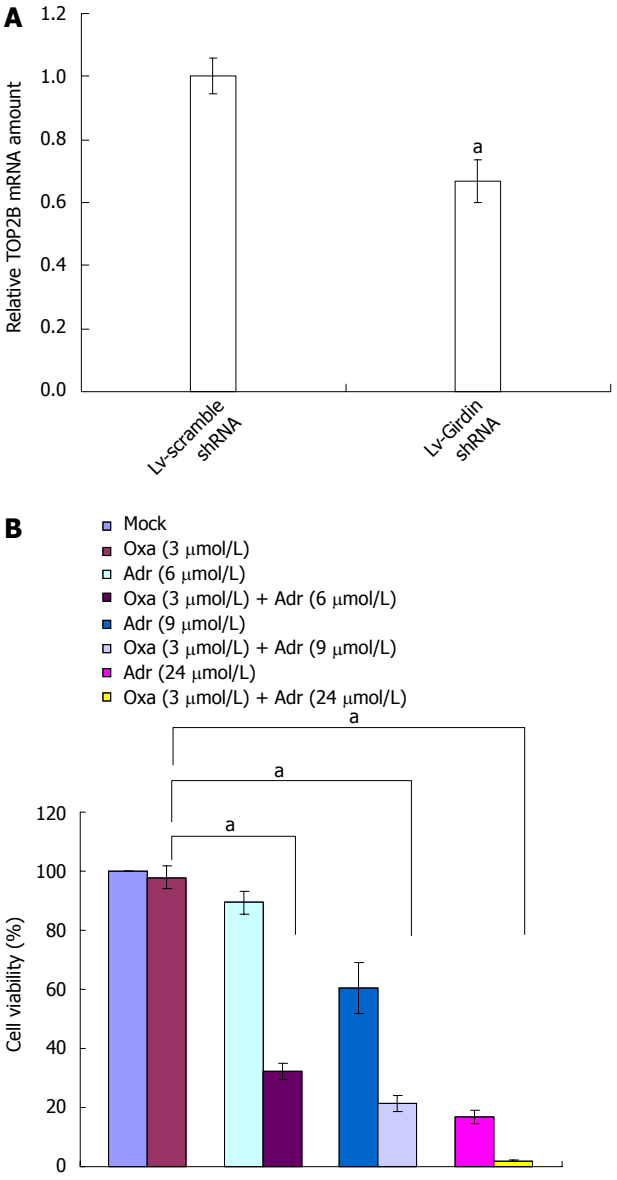

Figure 3 TOP2B as a downstream gene of Girdin is involved in the resistance of DLD1 cells to oxaliplatin. A: TOP2B mRNA level in DLD1 cells after infection with the Lv-Girdin shRNA; B: Growth inhibition of DLD1 cells by oxaliplatin in combination with adriamycin. $^{a}P < 0.05$.

CRC cells to oxaliplatin.

To identify the underlying mechanisms by which Girdin contributes to the chemoresistance of CRC cells, we performed a global analysis of gene expression after Girdin silencing by microarrays. We found that TOP2B appeared in the list of downregulated genes, with the lowest *P* value. TOP2B belongs to the TOP2 family which possesses both double strand cleavage and DNA ligation activities involved in DNA replication and DNA repair[19,23,24]. TOP2B is capable of uncoiling DNA and thus introduces a reversible DSB in DNA molecules. The DSB is stabilized by intercalative agents such as adriamycin which is a TOP2B inhibitor leading to apoptosis in a Fas-dependent or independent manner[25,26]. The microarray data described herein showed that TOP2B expression decreased upon Girdin downregulation. In addition, the

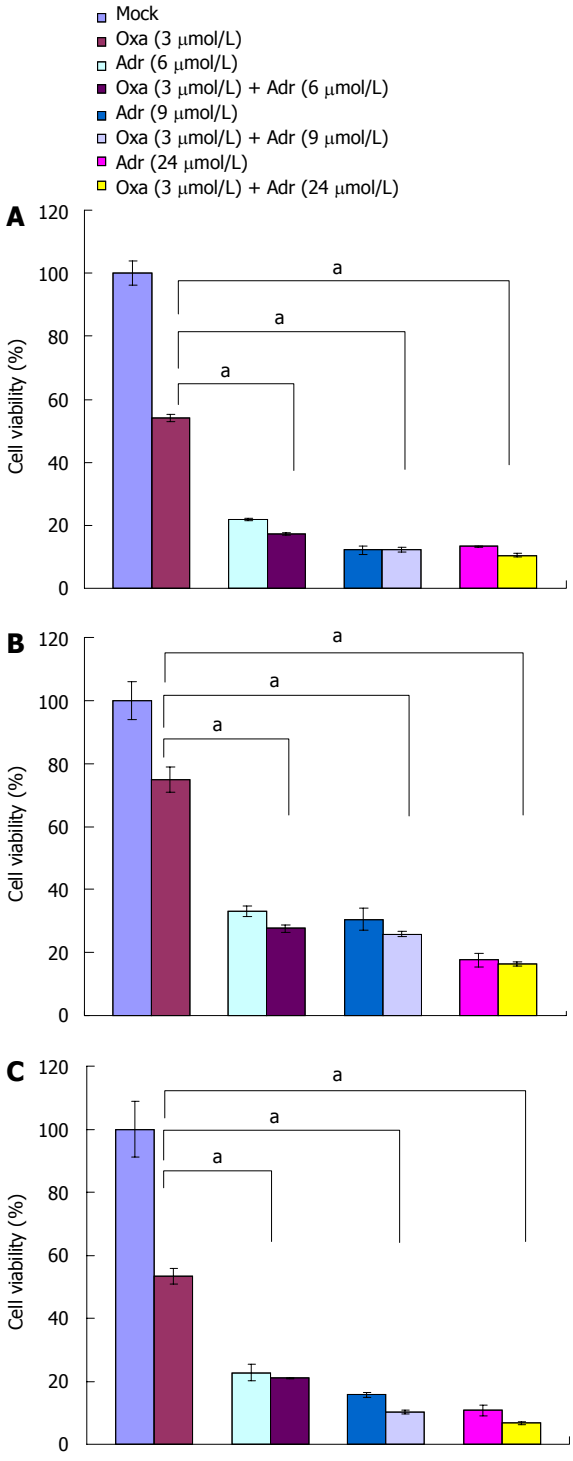

Figure 4 Growth inhibition of colorectal cancer cell lines by oxaliplatin in combination with adriamycin. A: Growth inhibition of SW620 cells; B: Growth inhibition of SW480 cells; C: Growth inhibition of HCT116 cells. $^{a}P < 0.05$.

combination of adriamycin and oxaliplatin greatly enhanced the cancer cell killing effect compared with treatment with oxaliplatin alone. These results demonstrate that TOP2B reduction is involved in the enhancement of chemosensitivity to oxaliplatin by Girdin suppression, and oxaliplatin/adriamycin combination resulted in increased cell death compared with treatments with individual agents.

In conclusion, Girdin plays a significant role in chemoresistance of CRC cells. Indeed, lentivirus-mediated shRNA targeting Girdin efficiently inhibits Girdin expression and enhances chemosensitivity to oxaliplatin in CRC. These findings provide a strong evidence for the use of Girdin knockdown in combination with oxaliplatin as a novel therapeutic strategy for CRC.

COMMENTS

Background

Oxaliplatin-based chemotherapy is widely used in the adjuvant and metastatic setting of colorectal cancer (CRC). However, treatment efficacy has reached a plateau with a response rate of 40%, which highlights the need for novel strategies to enhance the chemosensitivity of CRC cells to oxaliplatin.

Research frontiers

Girdin, a nonreceptor guanine nucleotide exchange factor (GEF), can interact with Gαi, resulting in Gβγ release and subsequent PI3K/AKT activation which has been recognized as an important pathway for chemoresistance through its antiapoptotic effects in cancers. Moreover, Girdin family proteins were found to suppress apoptosis. All of these raised the possibility that Girdin was involved in chemoresistance of CRC cells to oxaliplatin.

Innovations and breakthroughs

Previous studies have focused on the role of Girdin in the progression and metastasis of CRC cells. However, studies assessing the contribution of Girdin to chemoresistance in CRC are inexistent. Herein, the authors report for the first time that Girdin knockdown enhances chemosensitivity of CRC cells to oxaliplatin *via* reduction of TOP2B.

Applications

Lentivirus-mediated shRNA targeting Girdin efficiently inhibits Girdin expression and enhances chemosensitivity of CRC cells to oxaliplatin. These findings provide a strong evidence for the use of Girdin suppression in combination with oxaliplatin as a novel therapeutic strategy for CRC.

Terminology

Girdin, also called coiled-coil domain-containing protein 88A, is a nonreceptor guanine nucleotide exchange factor that can interact with Gαi resulting in Gβγ release and subsequently activates PI3K/AKT signaling pathway, which has been recognized as an important pathway for chemoresistance through its antiapoptotic effects in cancers. Topoisomerase 2-beta belongs to the TOP2 family which possesses both double strand cleavage and DNA ligation activities involved in DNA replication and DNA repair.

Peer review

This article is of interest as resistance to oxaliplatin is a major problem in the treatment of colorectal cancer. The study is well designed and properly developed.

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