Role of Integrin-Linked Kinase in Multi-drug Resistance of Human Gastric Carcinoma SGC7901/DDP Cells

Wei Song\textsuperscript{1,}* , Rui Jiang\textsuperscript{1,} , Chun-Ming Zhao\textsuperscript{2}

Abstract

Gastric carcinoma is a leading cause of cancer death in the world and multi-drug resistance (MDR) is an essential aspect of gastric carcinoma chemotherapy failure. Recent studies have shown that integrin-linked kinase (ILK) is involved in metastasis of human tumors, expression silencing of ILK inhibiting the metastasis of several types of cultured human cancer cells. However, the role and potential mechanism of ILK to reverse the multi-drug resistance in human gastric carcinoma is not fully clear. In this report, we focused on roles of expression silencing of ILK in multi-drug resistance reversal of human gastric carcinoma SGC7901/DDP cells, including increased drug sensitivity to cisplatin, cell apoptosis rates, and intracellular accumulation of Rhodamine-123, and decreased mRNA and protein expression of multi-drug resistance gene (MDR1), multi-drug resistance-associated protein (MRP1), excision repair cross-complementing gene 1 (ERCC1), glutathione S-transferase -π (GST-π) and RhoE, and transcriptional activation of AP-1 and NF-κB in ILK silenced SGC7901/DDP cells. We also found that there was a decreased level of p-Akt and p-ERK. The results indicated that ILK might be used as a potential therapeutic strategy to combat multi-drug resistance through blocking PI3K-Akt and MAPK-ERK pathways in human gastric carcinoma.

Keywords: Gastric carcinoma - multidrug resistance - integrin linked kinase

Introduction

Gastric carcinoma, also known as stomach carcinoma, is the fourth most common cancer in the world (Brenner et al., 2009). In China, gastric carcinoma is the second most common cancer (Zhao et al., 2010). It is reported that almost two thirds of the cases of this type of cancer occur in developing countries and 42% in China alone. Despite a major decline in incidence and mortality over the past several decades, the prognosis is generally rather poor, with 5-year relative survival below 30% in most countries. This poor prognosis makes it the second most common cause of cancer death worldwide. Surgery is the most common treatment. However, since stomach cancer is often either asymptomatic or it may cause only nonspecific symptoms in its early stages, many patients are found to have advanced disease when they get diagnosis. Chemotherapy remains a crucial treatment for these advanced patients (Shah et al., 2010). Similar to the other types of cancer, the major cause of treatment failure for gastric cancer is the development of multidrug resistance (MDR) to chemotherapy. To find out new molecular targets for improving treatment and overcoming drug resistance is of great importance. ILK (integrin-linked kinase) was discovered in 1996 by Hennigan and colleagues as a β1-integrin subunit cytoplasmic domain interactor. The following investigations confirmed it as a unique intracellular adaptor and kinase that links the cell-adhesion receptors, integrins and growth factors to the actin cytoskeleton and to a range of signaling pathways. A number of findings linked dysregulations of ILK with pathogenesis of various cancers. It has been shown that the expression of ILK is closely correlated with invasion and metastasis of gastric carcinoma and shorter survival in non-small cell lung cancer. Furthermore, the inhibition of ILK expression and activity is antitumorigenic, makes ILK an attractive target for cancer therapeutics. It has been shown that inhibition of ILK induced cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells, ILK targeting siRNA inhibited the growth and induced apoptosis in human bladder cancer cells (Gao et al., 2011), treatment with selective small molecule ILK inhibitor QLT0267 led to growth arrest and apoptosis of human anaplastic thyroid cancer cells in vitro and in vivo, and QLT0254, another ILK inhibitor, inhibited tumor growth and enhanced gemcitabine-induced apoptosis in human orthotopic primary pancreatic cancer xenografts. However, the sequences of ILK inhibition in gastric carcinoma have not yet been fully investigated. As mentioned previously, multiple drug resistance is a major obstacle in gastric carcinoma treatment and the expression of ILK was correlated with advanced gastric carcinoma, it is worth to exploring the potential therapeutic value of targeting ILK in this disease. In the present study, we...
showed that downregulation of ILK by siRNA arrested the growth and reversed the multiple drug resistance of human gastric cell line SGC7901/DDP.

Materials and Methods

Cell culture and establishment of cisplatin-resistant cell line

The human gastric carcinoma cell line SGC7901 was purchased from Cell Bank of Chinese Academy of Science. The multiple drug resistance cell line SGC7901/DDP was established by continuing exposure of the parental cells to gradient increasing level of cisplatin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. The culture medium was RPMI-1640 supplemented with 10% fetal bovine serum (FBS).

Construction of ILK siRNA expression plasmids

The ILK-siRNA (short hairpin RNA) expression plasmids were constructed as described by Wong et al. (Wong et al., 2007). Briefly, the siRNA sequences of ILK corresponded to positions 472 to 492 of the human ILK gene. The sense oligonucleotide containing the ILK targeting sequence was 5'-GATCCCCCTCTCAACCGTA TTTCACTCTT-3', and the antisense oligonucleotide was: 5'-AGCTTAAAAATCTCAACCGTATTCT ACCTCT TTTG-AAGATGGAATACGGTTG. The vector used here was pSUPERIOR-neo plasmid (OligoEngine).

Transfecting ILK siRNA into the cells

Confluent SGC7901/DDP cells were washed with PBS and harvested by trypsin/EDTA. Cell viability was determined by Trypan blue dye exclusion test and should be greater than 90%. For plasmids transfection, 105 cells were seeded in 24-well plates for 24hrs in culture medium. 5 μg of purified recombined pSUPERIOR-ILK-siRNA-neo plasmids were transfected into 70-80% confluency SGC7901/DDP cells. Six hours after transfection, the medium was replaced by fresh culture medium. After another 24 hrs period of culture, the cells were plated into 6-well plates with selection medium, containing 600 μg/ml G418. This concentration of G418 was determined pre-study and was the lowest concentration that could maintain at 37°C in a humidified atmosphere of 5% CO₂. The culture medium was RPMI-1640 supplemented with 10% fetal bovine serum (FBS).

MTS assay to determine the drug sensitivity of the cells

0.1 ml of 5×10⁶/ml cells were seeded into the 96-well plate, and incubated overnight to allow the cells to adherence. Then varied concentrations of cisplatin were added to each well and the cells were incubated for another 72hrs. At the end of the incubation, CellTiter 96 Aqueous One Solution Reagent (Promega) was added according to the manufacturer’s instructions. 4hrs later the cell viability was determined by measuring the absorbance at 490nm using a BioRad microplate-reader.

Real-time PCR analysis

More than 10⁶ cells were harvested for RT-PCR analysis. The total mRNA was extracted from the cells by the Dynabeads mRNA Direct Kit (Invitrogen) according to the manufacturer’s instruction manual. Total mRNA was then reverse transcribed for 1hr at 42°C in incubation buffer containing 250 μM of each deoxynucleotide triphosphate, 5 μM oligo (dT)20, 25 units of RNase inhibitor, and 20 units of avian myeloblastosis virus reverse transcriptase (Roche Diagnostics). The transcription level of MDR1, MRP1, ERCC1, GST-π, and RhoE were detected by semiquantitative real-time PCR using the iCYCLER iQ detection system (Bio-Rad). The PCR condition was as following: decontamination at 95°C for 10 min, followed by cycles at 95°C for 20 sec. and at hybridization T℃ for 40 sec. GAPDH was used as an internal control for loading. The full details were shown in Table 1.

Western blotting

Transfected clones and parental SGC7901/DDP cells were cultured in culture medium to reach 70%-80% confluency in 6-well plates. Cells were eliminated by trypsinization and the whole proteins were obtained by RAPI lysis buffer (Millipore) extraction and then centrifugation at 12,000 g for 10 min. Total protein concentrations of the supernatants were measured by the BCA method (Sigma Aldrich). Proteins (15 μg) were separated on 12% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked for 2 hrs at room temperature in TNT buffer (10 mM Tris-HCl and 150 mM NaClpH 7.4, 0.1% Tween-20) with 5% non-fat dried milk, followed by incubation overnight at 4°C with rabbit anti-MDR1, -MRP1, -ERCC1, -GST-π, -RhoE, -ERK, -p-ERK, -Akt, -p-Akt, -β-actin antibody (Santa Cruz). All primary antibodies were diluted according to the instruction manual. Membranes were washed and incubated for 1 hr with peroxidase-labelled anti-rabbit IgG (Santa Cruz, diluted at 1:2000). Finally, membranes were washed three times in TNT and exposed to the ImmobilonTM Western chemiluminescent HRP substrate (Millipore) for 1 min, and then exposed autoradiography film for 1–5 min in the dark.

Flow cytometry to analyze intracellular Rhodamine-123 accumulation, cellular p-glycoprotein expression and cell apoptosis

Table 1. Oligonucleotide Sequences Used for Real-time PCR

<table>
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<tr>
<th>Gene</th>
<th>Sequences (5’-3’)</th>
<th>Hybridization T℃</th>
<th>Cycles</th>
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<td>ILK</td>
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<td></td>
<td>Reverse: CAATCCGGTTTGTTC</td>
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<tr>
<td>MDR1</td>
<td>Forward: AAAAAGTCAACTGTAACCTCTTAC</td>
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<tr>
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<td>65</td>
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<tr>
<td></td>
<td>Reverse: GTGGAGGTGAGGAAACAG</td>
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<tr>
<td>GST-π</td>
<td>Forward: TGGGCATCTGAAGGCTTTG</td>
<td>60</td>
<td>35</td>
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<tr>
<td></td>
<td>Reverse: GATCTGGTATCCACAGATGAA</td>
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<tr>
<td>RhoE</td>
<td>Forward: GTCCTCAACGTGTTGATCGGTTT</td>
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<tr>
<td></td>
<td>Reverse: GTGAACTGCG-TACGGTTGCTT</td>
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<tr>
<td>GAPDH</td>
<td>Forward: GAAGGTTAGGTTGAGATGTC</td>
<td>60</td>
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<td>Reverse: GAAGATGTTGATGGGATTC</td>
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The activities of AP-1 or NF-κB were determined by reporter gene system according to the instruction manual with moderate modification. Briefly, the assays were performed in a 96-well plate. Nearly 8×10^4 viable cells were seeded into each well in triplicate and were incubated overnight to allow the adherence of cells. Then transfected each well of the cells with 0.012 pGL 4.32[luc2P/AP1 RE/Hygro] vectors (Promega) or 0.1μg pGL 4.32[luc2P/NF-kB-RE/Hygro] plasmids (Promega) respectively. The former vectors contain six copies of an AP-1 response element (AP1 RE) and the latter plasmids four copies of an NF-κB response element (NF-κB-RE) that drive transcription of the luciferase reporter gene luc2P (Photinus pyralis). After 24 hrs of transfection, the luciferase activity was analyzed by Bright-GloTM Luciferase Assay System (Promega).

**Results**

**ILK-siRNA expression plasmids transfection resulted in downregulation of ILK mRNA and protein expression**

Human gastric carcinoma SGC7901/DDP cells were transfected with recombinant plasmids encoding ILK-siRNA, or with negative control plasmids. Two ILK-siRNA transfected and one negative transfected cell clones with the strongest growth rate in G418 selectivity medium were obtained. To ensure the siRNA transfection was successful, the total mRNA and proteins were extracted from the transfected and parental cells. RT-PCR and western blotting were performed to determine the level of ILK mRNA and protein. As shown in Figure 1A, when compared to the parental cells, while the negative plasmid exhibited no interference effect, the ILK scripts were down-regulated in the two ILK-siRNA transfected cloned by 28.6% and 11.4%, respectively. Furthermore, the ILK protein level was also strongly decreased in the positive transfected but not in the negative transfected cells, as shown in Figure 1B. Therefore, these three clones of cells were successfully transfected as expected and were used in the following investigations.

**ILK silencing increased the cisplatin sensitivity and apoptosis rate of the cells**

SGC7901/DDP was obtained after 8 months of continuing exposure of the parental cells to the cisplatin. Generally, the growth rate of SGC7901/DDP was similar to the parental cells (data not shown). However, the drug sensitivity of SGC7901/DDP cells were determined by MTS assay in SGC7901/DDP cells. Values are presented as the means ± SD from at least three independent experiments, bars indicate SD, n= 5

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<th>0.4</th>
<th>0.8</th>
<th>1.6</th>
<th>3.2</th>
<th>6.4</th>
<th>12.8</th>
<th>25.0</th>
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Data were expressed as means±SD, statistical analysis was carried out using Student’s t-test (two tailed) and p<0.05 indicates statistical significance.
resistance to cisplatin of this cell line was significantly increased. We concerned was whether the ILK silencing could reverse the drug resistance property of SGC7901/DDP. We used MTS assay to determine the cells sensitivity to cisplatin, which was highly resisted by SCG7901/DDP after transfected (Figure 2). The drug sensitivity was expressed by IC$_{50}$. As shown in Table 2, we could see the IC$_{50}$ values of the positive transfected cells were lower than that of the parental cells, while that of the negative transfected cells was nearly unchanged. RI (resistance index) = IC$_{50}$(drug resistance cell)/IC$_{50}$(parent cell). Furthermore, Annexin V/PI assay showed that cisplatin treatment resulted in a significantly increase of apoptosis rate in the ILK silencing cells (Figure 3). Took together, these results indicated that ILK silencing reversed the cisplatin resistance of the cells.

ILK silencing increased the intracellular accumulation of Rhodamine-123 and decreased cellular p-glycoprotein expression

We supposed that the increase sensitivity toward cisplatin by ILK silencing was associated with increased accumulation of intracellular drug and decreased cellular p-glycoprotein expression, since drug efflux is a major drug resistance mechanism of SGC7901/DDP. Rhodamine-123 is a fluorescent pigment that shares the membrane transporter protein (for example p-glycoprotein) with many drugs, and is frequently used to reflect the intracellular drug accumulation potency.

The flow cytometry analysis results showed that the ILK silencing cells did contain a greater contain of fluorescent activity, which mean more Rhodamine-123 retained in the cell. Meanwhile, there was less p-glycoprotein expression in ILK silencing cells compare with parent and negative control cells (Figure 4).

ILK silencing downregulated the expression of MDR1 and MRP1

MDR1 and MRP1 are two major membrane transporter proteins that involved in efflux activities and lead to multiple drug resistance. It has been shown that SGC7901/DDP cells express a high level of MDR1 and MRP1; we would like to investigate whether ILK silencing could downregulate the expression of these proteins to restore the intercellular accumulation of Rhodamine-123. All of the flow cytometry analysis, RT-PCR and western blotting results showed that, oppose to the high expression level observed in the parental cells, the MDR1 and MRP1 were decreased in the positive transfected cells (Figure 5A).

ILK silencing downregulated the expression of ERCC1, GST-π and RhoE

Apart from MDR1 and MRPl, other molecules are also involved in the tumor resistance to cisplatin. ERCC1 is a protein that functions in nucleotide excision repair of damaged DNA. It has been reported that overexpression of ERCC1 are associated with tumor resistance to chemotherapies or radiotherapies (Kim et

| Table 2. The Reversal of ILK Silencing on Cisplatin Resistance for SGC7901/DDP Cell Lines |
|-----------------------------------------------|--------------------------------------------------|
| IC$_{50}$(g/L) | RI |
| SGC7901       | 0.245                           |       |
| SGC7901/DDP parent | 2.862                  | 11.7 |
| SGC7901/DDP negative control          | 2.773                  | 11.3 |
| SGC7901/DDP ILK-siRNA-1                             | 0.523                  | 2.1   |
| SGC7901/DDP ILK-siRNA-2                             | 0.476                  | 1.9   |
The ILK silencing downregulated the phosphorylation of AKT and ERK

It has been reported that expression of ILK, but not of a kinase-deficient ILK mutant, increased activating Ser473 phosphorylation of AKT. Additionally, as mentioned previously, AKT was a linker between ILK and NF-κB. Early reports showed that ILK could regulate the ERK pathway as well, but until the recent study, the interaction pathway as well, but until the recent study, the interaction

Discussion

A large number of evidences have identified ILK as a potential oncogene. However, the role of ILK in gastric carcinoma with multiple drug resistance is largely unknown. The study reported here showed that ILK silencing could reverse the drug resistance of SGC7901/DDP, a human gastric carcinoma cell lines with multi-drug resistance. A panel of molecules associated with multiple drug resistance and tumorigenicity were solidly regulated

Figure 5. The Effect of ILK Silencing on Expression of Multi-drug Resistance Relative Gene in SGC7901/DDP Cells.

A. The total cell lysate was separated by SDS-PAGE gel followed by Western blot with anti-MDR1 antibody, anti-MRP1 antibody or anti-β-actin antibody. Parent SGC7901/DDP cells, negative control cells, and ILK silencing cells (ILK-siRNA-1 and ILK-siRNA-2, respectively). β-actin was used as an internal control for loading. The experiment shown is representative of three independent experiments with similar results. The graph representing the analysis of MDR1 and MRP1 mRNA expression in SGC7901/DDP cells. Data presented are means ± SD values. # compared to control group, p > 0.05; * compared to control group, p < 0.05. GAPDH was used as an internal control for loading, bars indicate SD, n= 5. B. The total cell lysate was separated by SDS-PAGE gel followed by Western blot with anti-ERCC1 antibody, anti-GST-π antibody, anti-RhoE antibody or anti-β-actin antibody. Parent SGC7901/DDP cells, negative control cells, and ILK silencing cells (ILK-siRNA-1 and ILK-siRNA-2, respectively). β-actin was used as an internal control for loading. The experiment shown is representative of three independent experiments with similar results. The graph representing the analysis of ERCC1, GST-π, and RhoE mRNA expression in SGC7901/DDP cells. Data presented are means ± SD values. # compared to control group, p > 0.05; * compared to control group, p < 0.05. GAPDH was used as an internal control for loading, bars indicate SD, n= 5.

Figure 6. The Effect of ILK Silencing on Multi-drug Resistance Relative Signal Pathway Molecules in SGC7901/DDP Cells.

A. The total cell lysate was separated by SDS-PAGE gel followed by Western blot with anti-p-AKT antibody, anti-p-ERK antibody, anti-total-AKT antibody or anti-total-ERK antibody. Parent SGC7901/DDP cells, negative control cells, and ILK silencing cells (ILK-siRNA-1 and ILK-siRNA-2, respectively). Total-AKT or total-ERK were used as an internal control for loading. The experiment shown is representative of three independent experiments with similar results. B. Cells were transfected with pAP-1-luc or pNF-xB-luc and the transcriptional activation was detected by luciferase reporter assay. Data presented are means ± SD values. # compared to control group, p > 0.05; * compared to control group, p < 0.05. pRL-SV40 as an internal control, bars indicate SD, n= 5.

The transcriptional activities of AP-1 and NF-xB were decreased after ILK silencing

It has been shown that ILK could indirectly modulate the activity of AP-1 and NF-xB through regulation of GSK3 activity and AKT respectively. The transcription factors of AP-1 and NF-xB are involved in the expression and regulation of a number of important cellular processes that could lead to angiogenesis, tumor invasion and proliferation. As the ILK silencing showed anti-tumor activities, we would like to know if AP-1 and NF-xB were also involved. The reporter gene system assays showed that the activities of these two transcription factors were significantly decreased after ILK silencing (Figure 6B).
by ILK silencing, which supported the central position of ILK and further demonstrated targeting ILK as a potential strategy in gastric carcinoma treatment.

Cisplatin is a major chemotherapy drug in gastric carcinoma treatment (Piacentini et al., 2012). Drug resistance is a common problem faced in gastric carcinoma care and therefore new treatment strategies are in urgent. The SGC7901/DDP cells were established after continuing exposure of SGC7901 to cisplatin and had a stable resistance to cisplatin compared to the parental cells. Our previously study (no shown here) demonstrated that ILK was solidly expressed in this cisplatin-resistant cell line, which made the present study possible. We reconstructed SGC7901/DDP cells by trasfection with ILK-siRNA expression plasmids and obtained two clones of cells with high growth rate in the G418 selective medium. The success of ILK-siRNA trasfection in the clones was confirmed by western blotting and RT-PCR which showed the ILK expression was significantly downregulated. We first investigated the influence of ILK silencing on the drug sensitivity of the cells. The MTS assay strongly suggested that ILK silencing restored the sensitivity of the cells to cisplatin. Accordingly, cell apoptosis, a typical consequence of anti-tumor effect by cisplatin, was notably increased after cisplatin treatment to the ILK silencing cells.

Drug efflux from the tumor cells is a major mechanism of tumor resistance to chemotherapy. Membrane transporters, i.e. MDR1 and MRP1, which transport drugs from intracellular compartment to the extracellular one, are frequently noticed to overexpression in this drug resistance environment (Keppler et al., 2011). The increase of intercellular accumulation of Rhodamin-123 determined by flow cytometry provided indirect evidence that the restore of drug sensitivity was at least partly attributed to the reduction of drug efflux effect of the resistant cells. The substantial expression of MDR1 and MRP1 mRNAs and proteins in the SGC7901/DDP, and in contrast, significant downregulation in ILK silencing clones, provided further proof to our hypothesis.

Apart from the MDR1 and MRP1, other molecules are also involved into the tumor resistance to cisplatin (Lainie et al., 2008). ERCC1 could repair the DNA damage caused by cisplatin, and its overexpression reasonably resulted in cisplatin resistance. GST-π could catalyze the conjugation of cisplatin to the GSH, which attenuated the potency of cisplatin to form adduct with DNA. It has recently been shown that RhoE enhanced multidrug resistance of gastric cancer cells by suppressing Bax. When compared to the SGC7901 cells, the expression of ERCC1, GST-π and RhoE were upregulated in SGC7901/DDP cells, indicating that they were dedicated to the drug resistance as well. Further investigations showed that after ILK silencing, the expression of these panel of molecules were downregulated simultaneously. Of great interest was how ILK modulated these molecules. It has been shown that the expression of ERCC1 (Li et al., 2012) and GST-π (Zhang et al., 2009) is regulated by MAPK/ERK pathway, and the RhoE has a direct interaction with ILK (Pereira et al., 2009; Berti et al., 2011). The western blotting results confirmed that the phosphorylation of ERK was attenuated by ILK silencing, which might result in the negative regulation of ERCC1 and GST-π.

Other important molecules that are involved in tumorigenicty include AKT (Engelman et al., 2009), NF-κB, and AP-1 (Wagner et al., 2009), which could promote one or several of functions including cell survival, proliferation, tumor invasion and angiogenesis. As mentioned previously, it has been shown that ILK could positively regulate AKT and AP-1 directly, and NF-κB indirectly through AKT. Identical to these previous reports, we found that the phosphorylation of AKT, and transcriptional activity of AP-1 and NF-κB were downregulated by ILK silencing. These modulations should also contribute to the increase of apoptosis and sensitivity to cisplatin treatment of transfected cells. It is rather difficult to figure out the proportional contribution of each aspect discussed above to the reverse of drug resistance. However, we supposed that the downregulation of MDR1 and MRP1 which led to intracellular drug accumulation, the downregulation of ERCC1 and GST-π, which led to reduction of cell detoxification ability, and finally the downregulation of RhoE, played a more straightforward role. In contrast, the AP-1, NF-κB, ERK and AKT were more upstream and had a more comprehensive role.

In conclusion, our study showed that ILK played an important role in the drug resistance of SGC7901/DDP. Targeting ILK could reverse the drug resistance and promote tumor cells to apoptosis and growth arrest, which confirmed it as an optimal target for gastric carcinoma treatment, especially for those with drug resistance.

References
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