

Original Article

MiR-125b-mediated BAK1 downregulation contributes to drug-resistance in gastric cancer cells

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Abstract: Objective: To investigate the roles of microRNAs-125b (miR-125b) in drug resistance of gastric cancer cells, and assess the mechanisms involved. Methods: The resistance of SGC-7901/DDP cells to chemotherapy was studied. Apoptosis of the cancer cells was induced by fluorouracil (5-FU). MiR-125b inhibitor and BAK1 siRNA (20 nM) were applied to decrease miR-125b and BAK1 expression. 48 h after miR-125b and/or BAK1 silencing, the cells were treated by 5-FU for 24 h, and cell viability was then assayed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT). Apoptosis and cell cycle distribution were detected by flow cytometry with annexin V and/or propidium iodide staining. Expression of miR-125b and BAK1 was assessed by real-time PCR. Expression of p53 and Bcl-2 was detected by Western blotting. Results: Our data showed that 5-FU inhibited the proliferation of SGC-7901/DDP cells in a dose-dependent manner. BAK1 expression was promoted after miR-125b inhibition in 5-FU-treated and -untreated cells. The expression of p53 and Bcl-2 was not altered after miR-125b inhibition. Interestingly, miR-125b inhibition promoted 5-FU-induced apoptosis, proliferation-inhibition and cell cycle arrest. By contrast, co-application of BAK1 siRNA with miR-125b inhibitor reversed the effects of miR-125b inhibition on sensitivity to chemotherapy. Conclusion: miR-125b played a critical role in alleviating drug resistance in gastric cancer cells through decreasing BAK1 expression. MiR-125b and BAK1 could be potential targets to chemosensitize gastric cancer cells.

Keywords: Drug-resistance, miR-125b, proliferation, apoptosis, BAK1

Introduction

Gastric cancer is one of the heterogeneous malignancies, causing tremendous therapeutic burden in China. Age, diet, and stomach disease are the potential factors determining the development and progression of gastric cancer [1]. Although a series of biomarkers are available for the diagnosis of gastric cancer [2-4], malignant cancer cells are difficult to be eliminated once the cancer is confirmed. Multiple signaling pathways are involved in the development of gastric cancer, for example, the mitogen-activated protein kinase (MAPK), Wnt and p53 signaling pathways [5-7]. Therefore, specific compounds for antagonizing those signaling pathways have been designed to kill the cancer cells [8, 9]. However, a question regarding drug resistance is raised, which disturbs the application of chemotherapeutic drugs.

MiRNAs were found to regulate the development of many types of cancers and have become potential therapeutic candidates for cancers. MiR-125b was proposed as the oncogene or anti-oncogene in several types of cancers [10]. Moreover, abnormal expression of miR-125b was found in gastric cancer [11-13]. Experimental evidence showed that miR-125b promoted cellular proliferation, and migration and invasion of gastric cancer cells [12, 14], though the specific target molecule of miR-125b has not been confirmed. Bcl-2 homologous antagonist/killer 1 (BAK1) belongs to the Bcl-2 protein family. In mammalian cells, Bcl-2 family members form oligomers or heterodimers and act as anti- or pro-apoptotic regulators that are involved in a wide variety of cellular activities [15]. BAK1 is known to localize to mitochondria, and functions to induce apoptosis through accelerating the opening of the

MiR-125b contributes to chemoresistance of gastric cancer cells

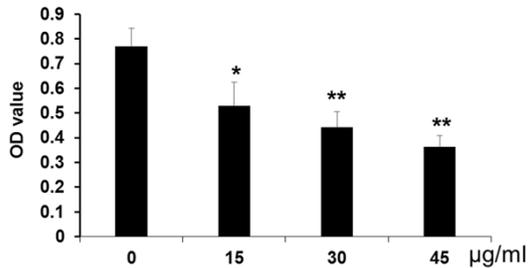


Figure 1. 5-FU inhibits cell growth in a concentration-dependent manner. The data are expressed as mean \pm SD. * $P < 0.05$ compared with control (0 $\mu\text{g/ml}$). ** $P < 0.01$ compared with control. Student's *t*-test.

mitochondrial voltage-dependent anion channel, which leads to a loss in membrane potential and the release of cytochrome *c* [16]. In addition, BAK1 also interacts with the tumor suppressor p53 after exposure to cellular stress [17]. Moreover, BAK1 was regulated by miR-125b in the development of chronic myeloid leukemia [18]. In addition, the regulation of BAK1 by miR125b was also reported to mediate apoptosis in neuronal cells [19, 20] and cardiac cells [21].

Considering the critical role of miR-125b in cancer development, we were interested in investigating whether miR-125b influences BAK1 to regulate the efficacy of chemotherapy in gastric cancer. SGC-7901/cisplatin (DDP) is a type of gastric cancer cell line widely applied to study chemoresistance. In this study, we selected this cell line and employed genetic method to inhibit miR-125b or silence BAK1 expression to investigate the drug resistance of SGC-7901/DDP to 5-FU.

Materials and methods

Cell culture

SGC-7901/DDP was produced from parental SGC-7901 by persistence gradient exposure to cisplatin for about 12 months (Keygen Biotech Co., Ltd., Nanjing, China) and cultured in Dulbecco's minimum essential medium (DMEM) (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin (Sigma, St. Louis, MO, USA) in 5% CO_2 at 37°C. Cells that were 60% confluent were applied in the following experiments. 5-FU (Sigma, USA) at the concentrations of 0-45 $\mu\text{g/ml}$ was applied to treat cancer cells for 24 h. In subsequent experiments,

15 $\mu\text{g/ml}$ 5-FU was applied to study the function of miR-125b-mediated BAK1 in chemotherapy of SGC-7901/DDP to 5-FU. A miR-125b inhibitor (0.1 nM, Biomics Biotech, Nantong, China) was designed to decrease miR-125b expression. After treatment for 48 h, the cells were treated by 5-FU for another 24 h. In BAK1 silencing experiment, BAK1 siRNA was designed to knock down BAK1 expression 48 h prior to 5-FU treatment. The sequence of BAK1 siRNA was designed by GenePharm (Shanghai, China).

MTT assays

Cancer cells in the logarithmic growth phase were seeded in a 96-well culture plate. MiR-125b inhibitor or BAK1 siRNA was transfected. After treatment with 5-FU, 20 μL MTT (5 mg/mL, Gibco, USA) was added into the 200 μL culture medium of each well. Four hours later, the medium was removed, and 150 μL DMSO (Sigma, USA) was added into each well in order to dissolve the precipitation. Absorbance (A) was measured at 570 nm using an automated microplate reader (iMark, USA).

Real time-PCR (RT-PCR)

Total RNA was extracted using the Trizol kit according to the instruction of the manufacturer (Dalian Baosheng, Dalian, China) and the purity of RNA was confirmed by optical density (OD) 280/OD260. Thereafter, RNA was amplified using an one-step RT-PCR kit (Dalian Baosheng, Dalian, China), and the PCR products were detected by 2% agarose gel electrophoresis. The primers were added into a 25- μL PCR reaction system which was run at 94°C for 60 s, 98°C for 10 s, and 72°C for 60 s for a total of 30 cycles. The primers were synthesized by Shanghai Biotech (Shanghai, China) and are listed as follows: miR-125b upstream primer: 5'-CCACCCAACCTCCTTC-3'; downstream primer: 5'-CACGCTGCTTCTCCTG-3'; BAK1 upstream primer: 5'-TCAGGCTTTGCGATTT-3'; downstream primer: 5'-CTCGGGCACTTATTGG-3'; GAPDH upstream primer: 5'-AGCCACATCGCTCAGACA-3' and downstream primer: 5'-TGGACTCCACGACGTACT-3'.

Cell transfection

When the cell confluence reached 50-70%, Lipofectamine™ 2000 (Invitrogen, USA) was applied to transfect scramble control (SC) and

MiR-125b contributes to chemoresistance of gastric cancer cells

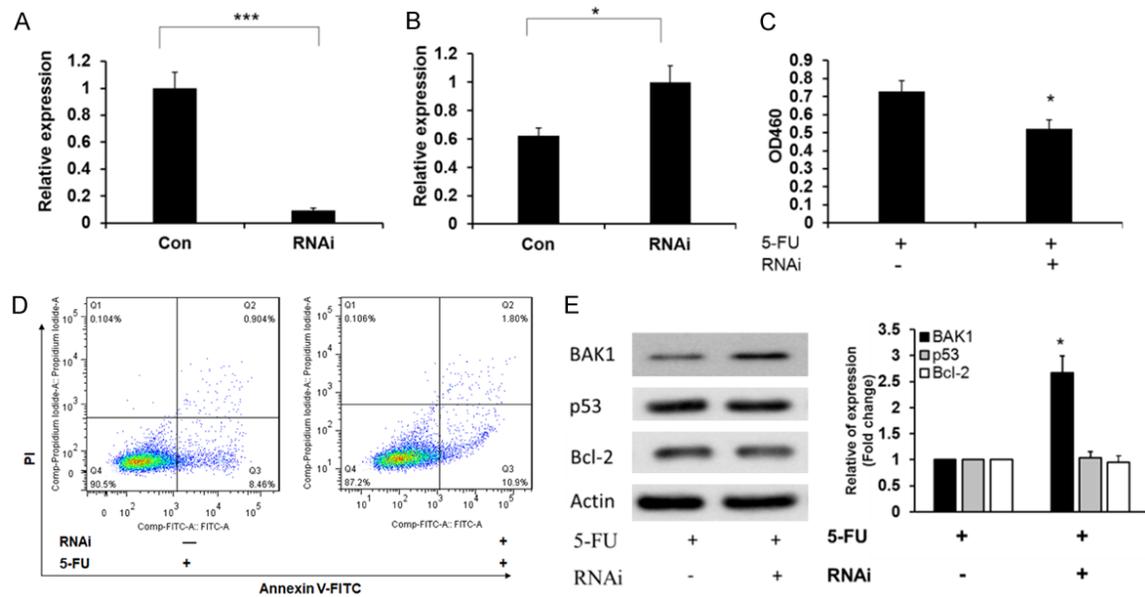


Figure 2. MiR-125b inhibitor promotes BAK1 expression and chemotherapy of 5-FU in gastric cancer cells. A. miR-125b inhibitor significantly decreases miR-125b expression. B. miR-125b inhibitor promotes BAK1 expression. C. miR-125b inhibitor promotes 5-FU-induced proliferation-inhibition. D. miR-125b inhibitor promotes 5-FU-induced apoptosis. E. 5-FU/miR-125b inhibitor does not affect p53 and Bcl-2 expression, but promoted BAK1 expression in 5-FU treated cells. Left panel showed the representative blots; Right panel were the quantification data. * $P < 0.05$ compared with control. *** $P < 0.001$ compared with control. Student's *t*-test.

miR-125b inhibitor. Six h later, the medium was changed into DMEM. MiR-125b inhibitor and SC were designed by Shanghai GenePharma. The cells were then cultured in DMEM containing 10% FBS for another 48 h. RT-PCR was applied to detect miR-96 expression.

Flow cytometry

After treatment for 24 h, the cells were collected for Annexin V/propidium iodide (PI) staining (Beyotime, Jiangsu, China) and detected within 1 h by FACSCalibur (BD Co., New York, USA).

After treatment for 24 h, the cells were collected for PI staining and detected within 1 h by FACSCalibur (BD Co., New York, USA). The cell cycle was analyzed.

Western blotting assays

After treatment for 24 h, the cells were collected for biochemical experiments. Protein concentration was quantified by BCA method (Beyotime, Jiangsu, China), and an equal amount of protein (20 μ g) was loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (SDS-PAGE). After electropho-

resis, proteins were transferred to the nitrocellulose membrane. Non-specific protein binding was blocked by 4% defat milk. The membranes were incubated with antibodies against the following proteins: P53 (1:2000, mouse monoclonal antibody) (Abcam, USA), Bak1 (1:2000, monoclonal antibody) (Abcam, USA), Bcl-2 (1:2000, monoclonal antibody) (Abcam, USA) and GAPDH (1:2000, monoclonal antibody) (Beyotime, China). After washing, the membranes were incubated with peroxidase-labeled secondary antibody (1:4000, goat anti mouse, Abcam, USA). The signal was detected using an enhanced chemiluminescence detection kit (Amersham ECL RPN 2106 Kit, Amersham Pharmacia Biotech, QC, Canada) and scanned by ChemiDoc™ XRS (Bio-Rad, Hercules, CA, USA). The densities of the blots were analyzed by Quantity One Software.

Statistical analysis

The data were presented as mean \pm standard deviation (S.D.). Statistical analyses of the data were performed using Student's *t*-test. $P < 0.05$ was considered as significant difference.

MiR-125b contributes to chemoresistance of gastric cancer cells

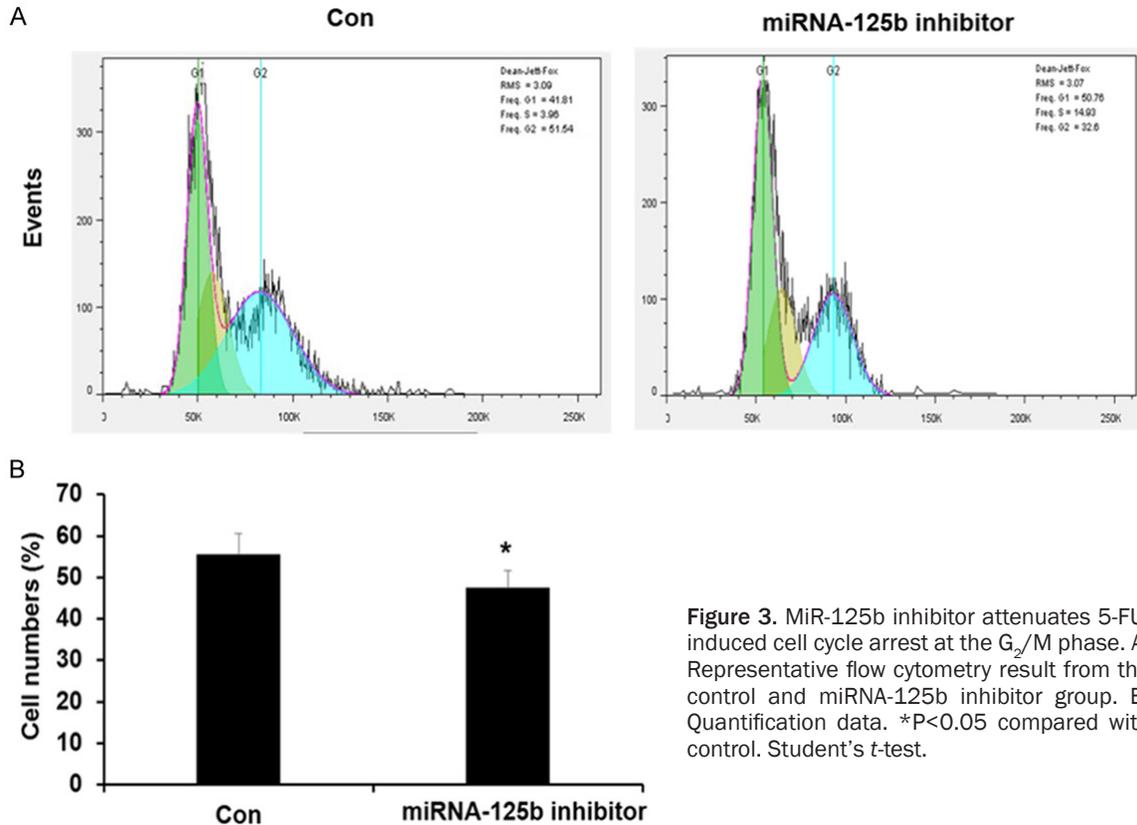


Figure 3. MiR-125b inhibitor attenuates 5-FU-induced cell cycle arrest at the G₂/M phase. A. Representative flow cytometry result from the control and miRNA-125b inhibitor group. B. Quantification data. *P<0.05 compared with control. Student's *t*-test.

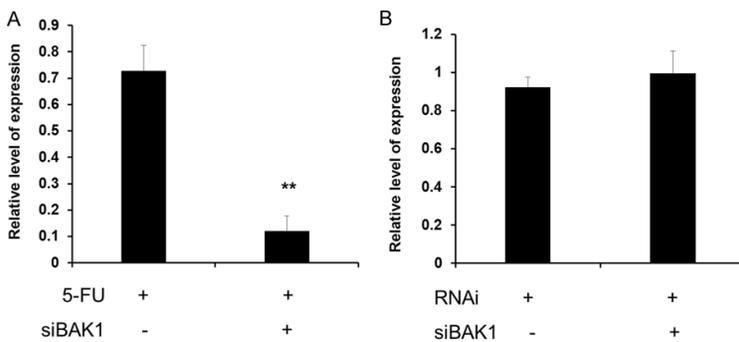


Figure 4. siBAK1 decreases BAK1 expression, but does not affect miR-125b expression. A. BAK1 expression; B. miR-125b expression. **P<0.01 compared with siBAK1(-). Student's *t*-test.

Results

MiR-125b inhibitor promoted BAK1 expression and chemotherapy of 5-FU in gastric cancer cells

SGC-7901/DDP cell line was treated by different concentrations of 5-FU (0-45 μ g/mL) for 24 h. As shown in **Figure 1**, 5-FU inhibited SGC-7901/DDP proliferation in a concentration-dependent manner. Fifteen μ g/mL 5-FU

caused a 25% inhibition. In the subsequent experiments, 15 μ g/mL 5-FU was used.

To investigate the effect of miR-125b on chemoresistance, we designed a miR-125b inhibitor. As shown in **Figure 2A**, the miR-125b inhibitor significantly decreased miR-125b expression. Moreover, the miR-125b inhibitor promoted BAK1 expression (**Figure 2B**). We also detected the effects of miR-125b inhibitor on 5-FU-mediated proliferation-inhibition,

apoptosis and cell cycle distribution. As shown in **Figure 2C**, 5-FU at 15 μ g/mL caused a 25% inhibition of cell proliferation. However, the miR-125b inhibitor enhanced the proliferation-inhibition to 42%. As shown in **Figure 2D**, the miR-125b inhibitor promoted 5-FU-induced apoptosis. The apoptotic rate in the 5-FU-treated group was 9%, while with the addition of the miR-125b inhibitor, 5-FU-induced apoptotic rate increased to 12%. 5-FU The miR-125b inhibitor did not affect p53 and Bcl-2 expres-

MiR-125b contributes to chemoresistance of gastric cancer cells

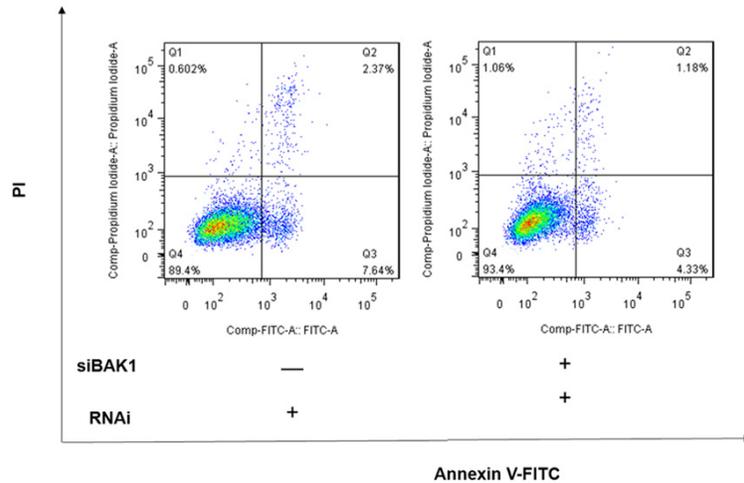


Figure 5. BAK1 siRNA reverses the effect of miR-125b inhibitor on 5-FU-induced apoptosis.

Importantly, the effect of the miR-125b inhibitor on 5-FU-induced apoptosis was reversed by BAK1 silencing compared with the miR-125b inhibitor (10% vs. 5.5%) (**Figure 5**).

BAK1 siRNA reversed the effect of miR-125b inhibitor on 5-FU-induced proliferation-inhibition

We also examined cellular proliferation. As shown in **Figure 6**, BAK1 siRNA also attenuated the effects of miR-125b inhibitor on 5-FU-induced cell proliferation inhibition (0.56 vs. 0.76).

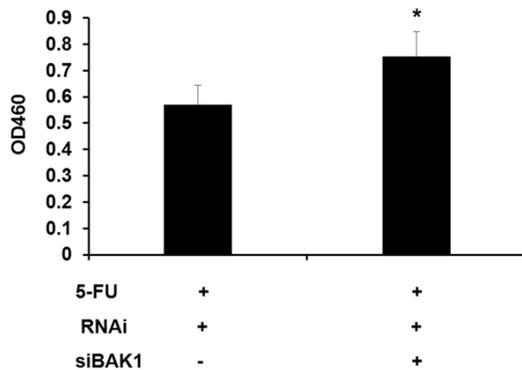


Figure 6. BAK1 siRNA reverses the effect of miR-125b inhibitor on 5-FU-induced proliferation-inhibition. * $P < 0.01$ compared with siBAK1(-). Student's *t*-test.

BAK1 siRNA reversed the effect of miR-125b inhibitor on cell cycle distribution

As shown in **Figure 7**, BAK1 siRNA reversed the effect of miR-125b inhibitor on cell cycle distribution. By contrast, BAK1 siRNA did not affect p53 and Bcl-2 expression (**Figure 8**).

Discussion

Chemoresistance is a daunting challenge for chemotherapy of various cancers. Much attention has been paid on this study area. However, the mechanisms were still unconfirmed. In this study, a chemoresistant gastric cancer cell line was used to study the mechanisms of chemoresistance in gastric cancer cells. We found that miR-125b inhibitor effectively promoted the chemotherapeutic effects of 5-FU. Importantly, we disclosed that chemoresistance was through miR-125b-mediated BAK1 expression.

MiRNAs have been proposed as oncogenes or anti-oncogenes. Therefore, miRNA inhibitors or mimics are widely designed to treat cancers. For example, miR-194 was reported to inhibit gastric cancer cell proliferation [22]. MiR-502 promoted gastric cancer development [23]. MiR-187 regulated gastric cancer progression by targeting tumor suppressor CRMP1 [24]. MiRNAs were also proposed to regulate chemoresistance. MiR-30a decreases multidrug resistance (MDR) of gastric cancer cells [25]. In our study, we disclosed that miR-125b also functioned as an important factor in mediating

sion, while promoted BAK1 expression—in 5-FU-induced apoptosis (**Figure 2E**). Cell cycle distribution analysis showed that the miR-125b inhibitor arrested the cell cycle at G₂/M phase (**Figure 3**).

BAK1 siRNA reversed the effect of miR-125b inhibitor on 5-FU-induced apoptosis

As miR-125b inhibitor promoted BAK1 expression, we therefore aimed to determine the roles of BAK1 in the process. A BAK1 siRNA was designed to decrease BAK1 expression. As shown in **Figure 4A**, BAK1 was remarkably down-regulated after application of BAK1 siRNA. By contrast, silencing of BAK1 did not affect miR-125b expression (**Figure 4B**). Im-

MiR-125b contributes to chemoresistance of gastric cancer cells

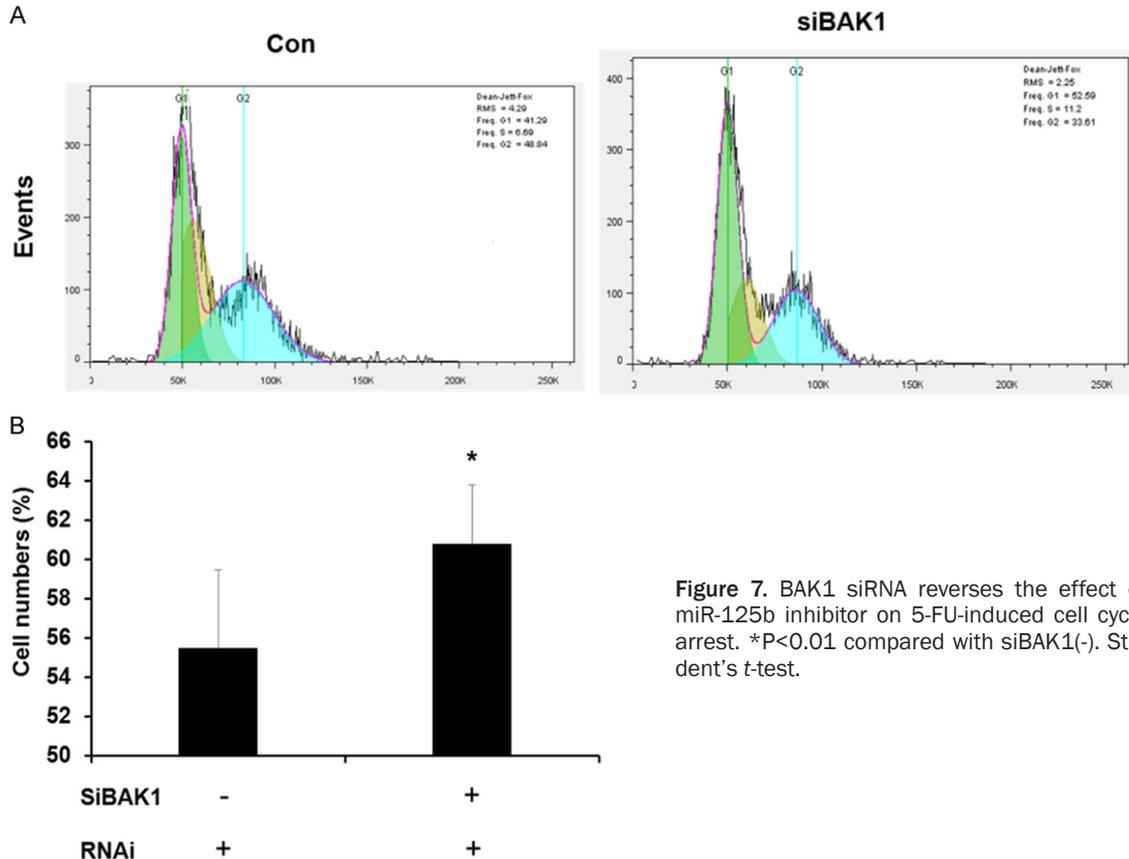


Figure 7. BAK1 siRNA reverses the effect of miR-125b inhibitor on 5-FU-induced cell cycle arrest. * $P < 0.01$ compared with siBAK1(-). Student's *t*-test.

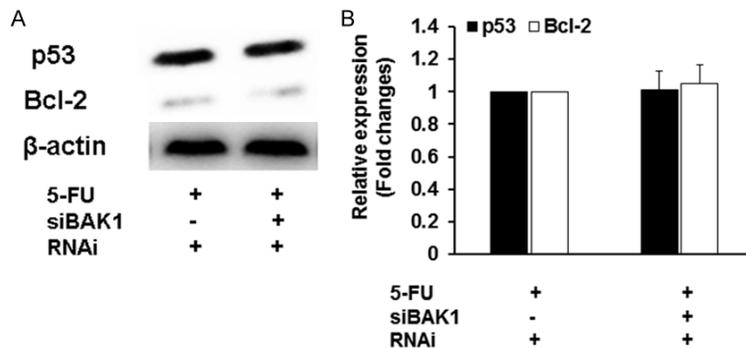


Figure 8. siBAK1 does not affect p53 and Bcl-2 expression in 5-FU treated cells. A. Representative blots of p53 and Bcl-2. B. Quantification data of p53 and Bcl-2 expression.

chemotherapeutic response. We showed that 5-FU inhibited cell proliferation of gastric cell line in a concentration-dependent manner. Using a miR-125b inhibitor, 5-FU-induced proliferation-inhibition and apoptosis were facilitated in SGC-7901/DDP cell line. Moreover, cell cycle was prominently arrested at the G_2/M phase in miR-125b inhibitor treated cells compared with control. Those data implicated that miR-125b was an important factor contributing

to chemoresistance in gastric cancer cells. MiR-125b inhibitor promoted the chemotherapeutic effects of 5-FU on the proliferation and apoptosis of SGC-7901/DDP cell line.

MicroRNA-125b was reported to regulate p53 mRNA translation in response to genotoxic stress [26]. However, in our study, p53 protein expression was not affected in gastric cancer cells after miR-125b level was reduced. These data implicated that p53 was not

the target of miR-125b in regulating chemoresistance, at least in gastric cancer cells. It also suggested that microRNA-125b induced cancer cell apoptosis through suppression of Bcl-2 expression [27]. In our condition, Bcl-2 was also excluded as the downstream target of miR-125b.

BAK1 is a direct target of miR-125b [28]. As reported previously, BAK1 was an important

cancer related gene, performing functions in regulating cell proliferation, apoptosis and migration [29, 30]. Moreover, miR-125b confers chemoresistance in several types of cancer cells, including breast cancer [28], acute promyelocytic leukemia [31], and glioblastoma [32], etc. In those studies, BAK1 has been reported to be suppressed after chemotherapy. Hence, downregulation of BAK1 was likely implicated in chemoresistance. In our study, we also proposed that miR-125b-mediated downregulation of BAK1 contributed to chemoresistance in gastric cancer cells. BAK1 siRNA reversed miR-125b inhibitor-sensitized gastric cancer cells to 5-FU. In combination with other publications [31, 32], miR-125b-regulated BAK1 expression confers a general function in chemoresistance in cancer treatments.

Conclusion

In our study, we reported that miR-125b inhibitor chemosensitized gastric cancer cells. The potential mechanisms were related to upregulation of BAK1 expression, but not Bcl-2 or p53. These data might be of implications in the chemotherapy of gastric cancer.

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Disclosure of conflict of interest

None.

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