

Effect of midkine on gemcitabine resistance in biliary tract cancer

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Abstract. Gemcitabine-based chemotherapy is one of the most effective and commonly used chemotherapeutic regimens for biliary tract cancer (BTC). However, development of resistance to this drug limits its efficacy. The present study aimed to explore the effects of midkine (MDK) on the resistance of BTC cells to gemcitabine. Cell viability and proliferation were measured by a Cell Counting Kit-8 assay and 5-ethynyl-2'-deoxyuridine staining, respectively. Western blot analysis was used to detect the expression of E-cadherin and vimentin. The results indicated that BTC cell lines were more resistant to gemcitabine plus MDK compared with gemcitabine alone. In terms of the underlying mechanism, MDK promoted the epithelial to mesenchymal transition (EMT) of BTC cells and the enhancing effect of MDK on gemcitabine resistance was abrogated when the EMT was blocked with small interfering (si)RNA targeting Twist. In addition, MDK promoted the expression of Notch-1, while knockdown of Notch-1 by siRNA blocked the EMT process in the BTC cell lines. Taken together, these results indicated that MDK promoted gemcitabine resistance of BTC through inducing EMT via upregulating Notch-1. It was suggested that inhibition of the EMT is a promising strategy to overcome MDK-induced drug resistance.

Introduction

Biliary tract cancer (BTC) refers to a group of cancers of the biliary tract, including gallbladder cancer, cholangiocarcinoma of intrahepatic and extrahepatic bile ducts, and cancers of

the ampulla and papilla of Vater (1,2). The incidence rate of BTC differs among geographic areas: It is high in Asia, Latin America and eastern European countries, while it is low in the US and certain western European countries (2). Complete resection has been considered the best treatment for BTC; however, most patients are ineligible for surgery due to its rapid progression and non-specific symptoms (3-5). Even patients who are treated with surgery have a poor prognosis.

Although surgery remains the only curative treatment option, chemotherapy prolongs the survival of patients with BTC (6,7). Among the chemotherapeutic drugs, gemcitabine and cisplatin have proven to be the most effective first-line drugs (8-10). However, drug resistance to gemcitabine limits its effect, and the median overall survival of patients with advanced BTC who receive chemotherapy is only ~1 year (11). Therefore, it is essential to explore the potential mechanism underlying the resistance of BTC to gemcitabine in order to enhance its effect and prolong patient survival.

Midkine (MDK), a heparin-binding growth factor, was first identified as a highly expressed factor involved in embryonic development (12). MDK has been reported to have important roles in the survival, growth and migration of cells, which may contribute to oncogenesis and tumor progression in numerous types of cancer (13-18). Several studies have demonstrated that MDK mediates drug resistance. Mirkin *et al* (19) employed a cytokine complementary DNA array to identify putative survival molecules in human neuroblastoma and osteosarcoma cells and identified MDK as a lead candidate responsible for doxorubicin resistance via regulation of the AKT pathway. Furthermore, Lorente *et al* (20) identified MDK as a pivotal factor involved in the resistance of glioma cells to the pro-autophagic and anti-tumoral action of tetrahydrocannabinol by regulation of the anaplastic lymphoma kinase receptor. Xu *et al* (21) proved that MDK, which activates AKT and extracellular signal-regulated kinase by phosphorylation, induced doxorubicin resistance in gastric cancer cells. Hu *et al* (22) indicated that MDK increased the drug-efflux ability in lymphoblastic leukemia, thereby having an important role in multidrug resistance. These results highlighted the fact that MDK may have an essential role in cancer chemotherapy resistance. However, the role of MDK in the drug resistance of BTC has remained largely elusive.

Epithelial to mesenchymal transition (EMT) is the process wherein epithelial cells lose their apical-basal polarity and

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cell-cell adhesion and transit to invasive mesenchymal cells. EMT cells exhibit decreased expression of epithelial genes (e.g., E-cadherin) and increased expression of mesenchymal genes (e.g., vimentin) (23). The link between EMT and drug resistance of cancer cells has been suggested in a previous study. Furthermore, increasing evidence has indicated that drug resistance of several cancer types, including lung (24), pancreatic (25), liver (26) and breast cancer (27), is frequently accompanied by EMT. In BTC, EMT involves the invasion and migration of BTC cells. However, evidence supporting the role of EMT in drug resistance of BTC has remained insufficient.

Therefore, the present study aimed to determine the association between MDK, EMT and gemcitabine resistance in BTC and explore the potential mechanisms underlying gemcitabine resistance.

Materials and methods

Cell culture and reagents. Two human BTC cell lines, RBE and GBC-SD, were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured according to the supplier's recommendation in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and maintained at 37°C in a humidified atmosphere of air with 5% CO₂. For hypoxia culture, cells were maintained at 37°C in a humidified atmosphere with 5% CO₂, 1% O₂ and 94% N₂. MDK was from Sigma-Aldrich (cat. no. SRP3114; Merck KGaA, Darmstadt, Germany) and was used at a concentration of 50 ng/ml. Gemcitabine (cat. no. S1714) was purchased from Selleck Chemicals (Houston, TX, USA).

Cell viability assay. RBE and GBC-SD cells were seeded into 96-well microplates at a density of 5,000 cells/well and cultured with different concentrations of gemcitabine ranging from 0.00 to 0.06 µg/ml, MDK (50 ng/ml) or a combination of the two drugs for 48 h. Cell viability was detected using the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. In brief, 100 µl medium and 10 µl CCK-8 solution were added to microplates, and the cells were incubated for 2 h. The optical density at 450 nm was determined using a MRX II microplate reader (Dynex, Chantilly, VA, USA). Cell viability in each group was determined by comparison with untreated control cells.

Cell proliferation assay. Cell proliferation was analyzed by 5-ethynyl-2'-deoxyuridine (EdU) staining using the Click-iT[®] EdU Imaging kit (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. RBE or GBC-SD cells were treated with gemcitabine alone or a combination of gemcitabine (0.03136 µg/ml for RBE; 0.1433 µg/ml for GBC-SD) and MDK (50 ng/ml) for 48 h, and then exposed to 10 µM EdU for 2 h at 37°C. The cells were then fixed with 3.7% formaldehyde for 15 min at room temperature and treated with 0.5% Triton X-100 (Sangon Biotech, Shanghai, China) for 20 min at room temperature for permeabilization. After washing twice with PBS containing 3% bovine serum albumin, the cells were treated with 0.5 ml of Click-iT[®] reaction cocktail (Invitrogen; Thermo Fisher Scientific, Inc.)

for 30 min in the dark. Subsequently, the cell DNA was stained with 1 ml 1X Hoechst 33342 (1:2,000 dilution) for 30 min. Finally, three random fields of view per slide were selected under a fluorescence microscope (Olympus, Tokyo, Japan), and the number of proliferative (EdU-positive) cells was counted.

Cell transfection for RNA interference. Human Twist small interfering RNA (siRNA) was synthesized by Shanghai GeneChem Co., Ltd. (Shanghai, China). The human Twist siRNA sequence was as follows: Twist1, 5'-GGUGUCUAA AUGCAUUCATT-3' and 5'-AUGAAUGCAUUAGACAC CTT-3'; Notch1, 5'-CCAACCCUGUCAUUGGCAATT-3' and 5'-UUGCCAUUGACAGGGUUGGTT-3'; Scrambled siRNA, 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGA CACGUUCGGAGAATT-3'. The transfection was performed by using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Western blot analysis. The interference efficiency of Twist siRNA and its effect on the expression of various proteins was determined by western blot analysis (28). The following antibodies were used: Anti-E-cadherin (cat. no. 3195), anti-vimentin (cat. no. 5741), anti-Twist (cat. no. 46702), anti-β-actin (cat. no. 8457) (all at 1:1,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-Notch-1 (cat. no. ab8925; 1:1,000 dilution; Abcam, Cambridge, UK). The corresponding secondary antibodies conjugated to horseradish peroxidase (cat. no. ab98489; 1:2,000 dilution) were obtained from Abcam. The grey value was analyzed by QuantityOne 4.62 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Three independent experiments were performed for each experiment. Experimental data were expressed as the mean ± standard deviation. Analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons among datasets were performed by using one-way analysis of variance followed by Tukey's post hoc test or a unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

MDK induces gemcitabine resistance in BTC cells. To determine whether MDK is involved in gemcitabine resistance of BTC, the effect of MDK on BTC cell viability was evaluated in the presence of different concentrations of gemcitabine. BTC cells exhibited higher cell viability after gemcitabine + MDK treatment than cells treated with gemcitabine alone (Fig. 1A and B). In addition, the EdU assay indicated that BTC cells had increased proliferation in the presence of gemcitabine and MDK compared with gemcitabine alone, which suggests that MDK induces gemcitabine resistance in BTC (Fig. 1C and D).

EMT is involved in gemcitabine resistance of BTC cells. The EMT is known to be associated with chemoresistance of cancers, and MDK was reported to induce EMT in several cancer types (29-31). Thus, the present study hypothesized that EMT may be involved in gemcitabine resistance in BTC and that MDK may promote gemcitabine resistance in BTC by regulating the EMT pathway. To prove this hypothesis,

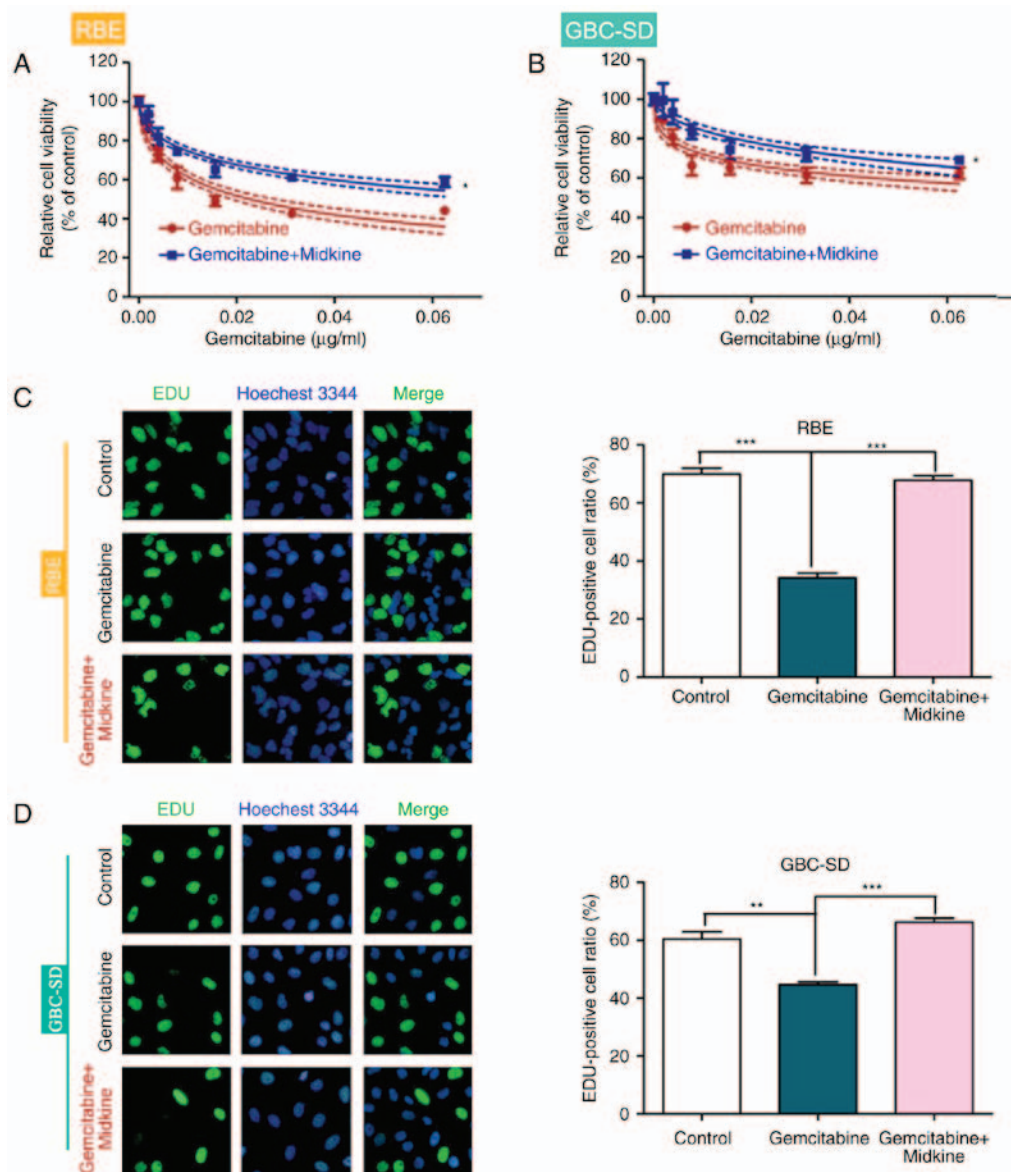


Figure 1. Comparison of BTC cell viability and proliferation with or without midkine in the presence of gemcitabine. Effect of midkine on the viability of (A) RBE and (B) GBC-SD cells cultured with different concentrations of gemcitabine (0.00, 0.02, 0.04 or 0.06 $\mu\text{g/ml}$) for 48 h. * $P < 0.05$ vs. Gemcitabine. (C) The cell proliferation rate was determined by an EdU assay. RBE cells were cultured with gemcitabine (RBE, 0.03136 $\mu\text{g/ml}$) or in combination with 50 ng/ml midkine (RBE, 0.03136 $\mu\text{g/ml}$) or vehicle. Cell nuclei were stained with Hoechst33344 (blue) (x200 magnification). The number of EdU-positive cells (green) was counted. *** $P < 0.001$ with comparisons shown by lines. (D) GBC-SD cells were cultured with gemcitabine (GBC-SD, 0.1433 $\mu\text{g/ml}$) or in combination with 50 ng/ml midkine (GBC-SD, 0.1433 $\mu\text{g/ml}$ gemcitabine) or vehicle. Cell nuclei were stained with Hoechst33344 (blue) (x200 magnification). The number of EdU-positive cells (green) was counted. ** $P < 0.01$ and *** $P < 0.001$ with comparisons shown by lines.

the efficiency of gemcitabine on the two BTC cell lines was first examined. The CCK-8 assay indicated that gemcitabine effectively inhibited the viability of BTC cells in a concentration-dependent manner. Of note, the BTC cell line RBE was more sensitive to gemcitabine than the GBC-SD cell line (Fig. 2A). Subsequently, the expression of the epithelial marker E-cadherin and the mesenchymal marker vimentin was assessed in these two cell lines, revealing that the cell line RBE had high E-cadherin levels, but low vimentin levels, whereas the opposite results were observed in the GBC-SD cell line (Fig. 2B). Accordingly, the present results indicate that EMT may be involved in gemcitabine resistance in BTC.

To confirm this result, the BTC cell lines were exposed to hypoxic conditions to induce EMT, as reported

previously (32,33). The results demonstrated that hypoxia upregulated the expression of vimentin and downregulated the expression of E-cadherin in BTC cell lines, thereby promoting EMT (Fig. 3A). As expected, BTC cell viability increased in the presence of gemcitabine under hypoxic conditions compared with that under normoxic conditions (Fig. 3B and C). In addition, gemcitabine treatment led to an upregulation of the expression of vimentin and a downregulation of the expression of E-cadherin in the BTC cell lines under normoxia conditions (Fig. 3D). To confirm the role of the EMT in gemcitabine resistance, Twist, a key regulator of EMT (34), was knocked down to block EMT in BTC cell lines. The results demonstrated that the BTC cell lines became more sensitive to gemcitabine after Twist inhibition (Fig. 3E and F). Interference efficiency of Twist1 was measured using western blot analysis (Fig. 3G).

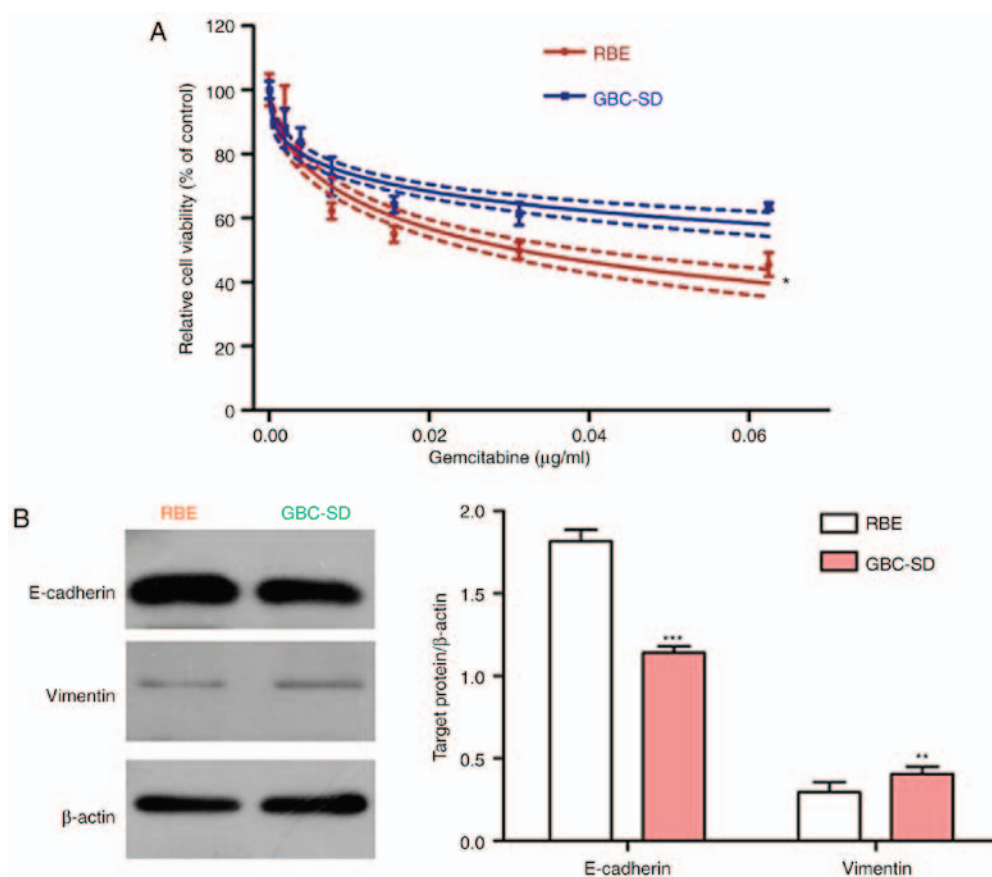


Figure 2. Effects of gemcitabine on cell viability and the expression of E-cadherin and vimentin in biliary tract cancer cell lines. (A) Comparison of the cell viability of GBC-SD and RBE cell lines cultured with different concentrations of gemcitabine was examined using the Cell Counting Kit-8 assay. * $P < 0.05$ vs. GBC-SD. (B) Expression of E-cadherin and vimentin in GBC-SD and RBE cell lines determined by western blot analysis. β -actin was used as the internal control, and the average grey values are presented in a histogram. ** $P < 0.01$ and *** $P < 0.001$ vs. RBE.

Therefore, the present results indicated that gemcitabine resistance in BTC may be mediated via the EMT.

MDK mediates gemcitabine resistance in BTC cells by regulating EMT. To further prove the abovementioned hypothesis, the association between MDK and EMT in gemcitabine resistance was examined. First, the expression of E-cadherin and vimentin was detected in the two BTC cell lines cultured in the presence of MDK. The results indicated that MDK treatment led to a significant upregulation of vimentin expression and a downregulation of E-cadherin expression in BTC cell lines (Fig. 4). Thereafter, the efficiency of MDK to induce gemcitabine resistance was examined after EMT blockage. To block EMT, Twist, the key molecule in the EMT pathway, was inhibited using siRNA. The knockdown efficiency of Twist siRNA was confirmed by western blot (Fig. 5A). As expected, cells transfected with scrambled siRNA were more sensitive to treatment with gemcitabine compared with those in the MDK+scrambled siRNA group, and after EMT inhibition with Twist siRNA, the effect of MDK to induce gemcitabine resistance in BTC cells was lost (Fig. 5B and C). Hence, the present results proved the hypothesis that MDK promotes gemcitabine resistance in BTC by regulating EMT.

Midkine induces EMT by upregulating Notch-1 expression. The Notch pathway has a significant role in EMT of cancer cells (35,36), and Notch-1 activation has been reported to

be linked to acquired chemoresistance in several cancer types (37-40). Therefore, the role of MDK in Notch-1 expression was then examined in the present study. The western blot results demonstrated that MDK significantly promoted the expression of Notch-1 in BTC cell lines (Fig. 6A). Subsequently, the expression of E-cadherin and vimentin was examined in BTC cells treated with Notch-1 siRNA or a combination of Notch-1 siRNA and MDK. The results indicated that siRNA-mediated knockdown of Notch-1 completely abolished the regulatory effect of MDK on the expression of E-cadherin and vimentin in BTC cells (Fig. 6B). The interference efficiency of Notch1 was detected by western blot analysis (Fig. 6C). These results indicate that Notch-1 is a mediator in MDK-induced EMT.

Discussion

Chemotherapy has been considered an effective adjuvant therapy for BTC; however, drug resistance limits the efficiency of chemotherapy (7,41). Therefore, further studies are required to determine the potential mechanism of drug resistance in BTC. In the present study, MDK was demonstrated to induce drug resistance in BTC via induction of the EMT through regulating the expression of the Notch-1 protein.

MDK is a growth factor that was first identified as a mediator of retinoic acid-induced differentiation (12). Further studies indicated that it was associated with drug resistance. Mirkin *et al* (19) proved that MDK was secreted

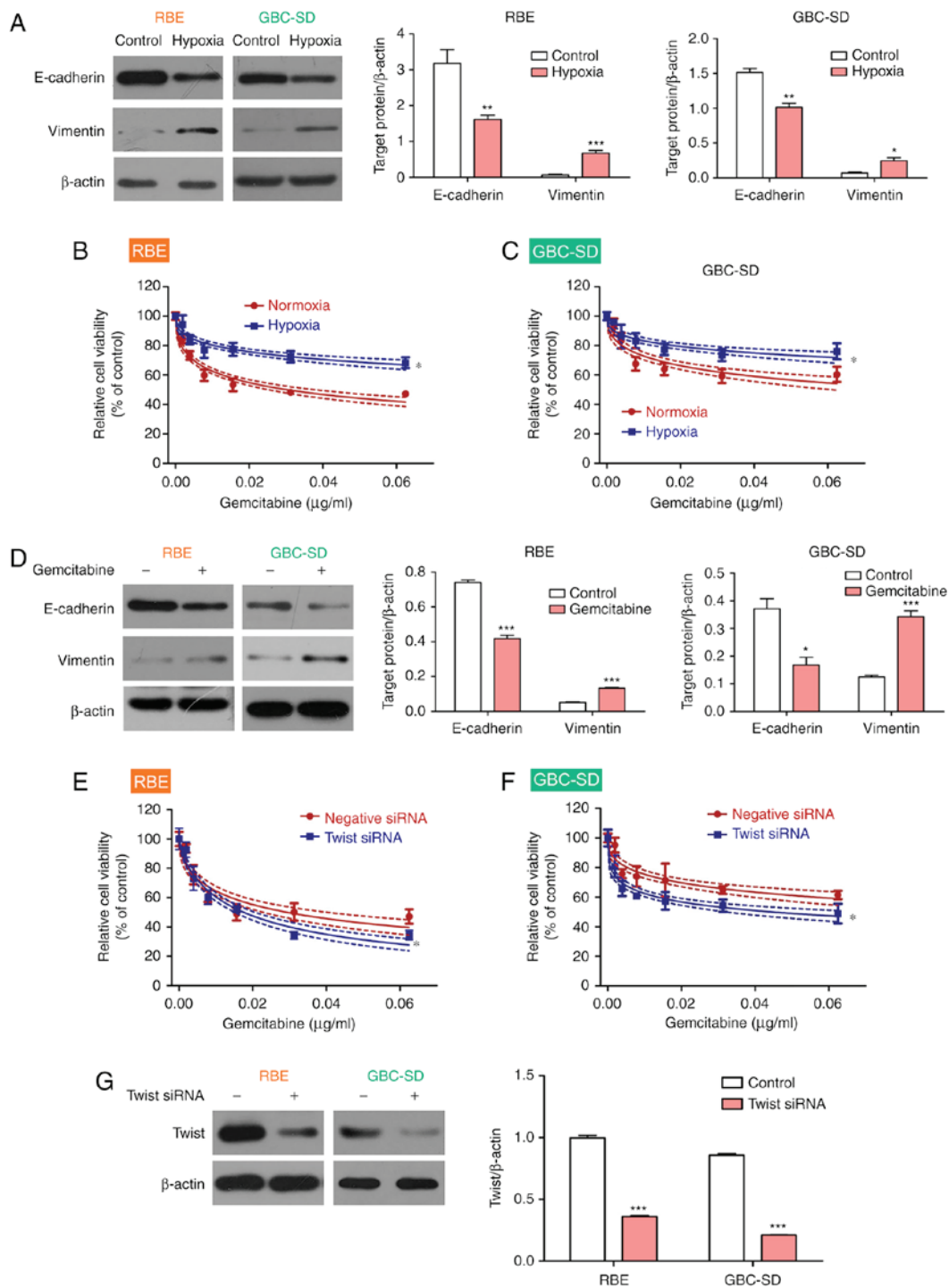


Figure 3. Effect of hypoxia and gemcitabine on the expression of E-cadherin and vimentin in biliary tract cancer cell lines. (A) Comparison of the protein expression of E-cadherin and vimentin under hypoxia and normoxia by western blot analysis. β -actin was used as the internal control, and the average grey values are presented in a histogram. * P <0.05, ** P <0.01, *** P <0.001 vs. Control. Comparison of the viability of (B) RBE and (C) GBC-SD cells cultured with different concentrations of gemcitabine under hypoxic and normoxic conditions, examined by the Cell Counting Kit-8 assay. * P <0.05 vs. Normoxia. (D) The effect of gemcitabine on the protein expression of E-cadherin and vimentin in GBC-SD and RBE cell lines under normoxia condition was determined by western blot analysis. β -actin was used as the internal control, and the average grey values are presented in a histogram. * P <0.05, *** P <0.001 vs. Control. Comparison of viability of (E) RBE and (F) GBC-SD cells treated with Twist siRNA or negative siRNA cultured with different concentrations of gemcitabine. * P <0.05 vs. negative siRNA. (G) Western blot analysis was used to detect the interference efficiency of Twist1. *** P <0.001 vs. Control.

from drug-resistant cells and protected the neighboring drug-sensitive cells from the toxicity of doxorubicin. Kang *et al* (42) identified >250 differentially expressed genes in 5-fluorouracil-, cisplatin- or doxorubicin-resistant gastric cancer cell lines by microarray analysis and determined

that MDK was overexpressed in all drug-resistant cell lines. Qi *et al* (43) reported that MDK protected murine kidney cells and cultured Wilms' tumor cells from cisplatin-induced apoptotic cell death by upregulating the expression of B-cell lymphoma 2. Regarding BTC, MDK was upregulated in

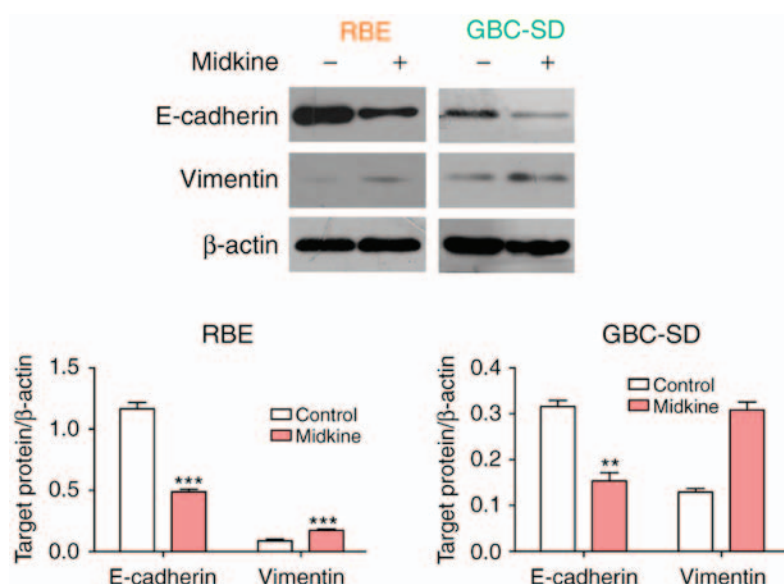


Figure 4. Effect of midkine on the expression of E-cadherin and vimentin. Protein expression of E-cadherin and vimentin in GBC-SD and RBE cell lines with or without midkine determined by western blot analysis. β -actin was used as the internal control, and the average grey values are presented in the histogram. ** $P < 0.01$ and *** $P < 0.001$ vs. Control.

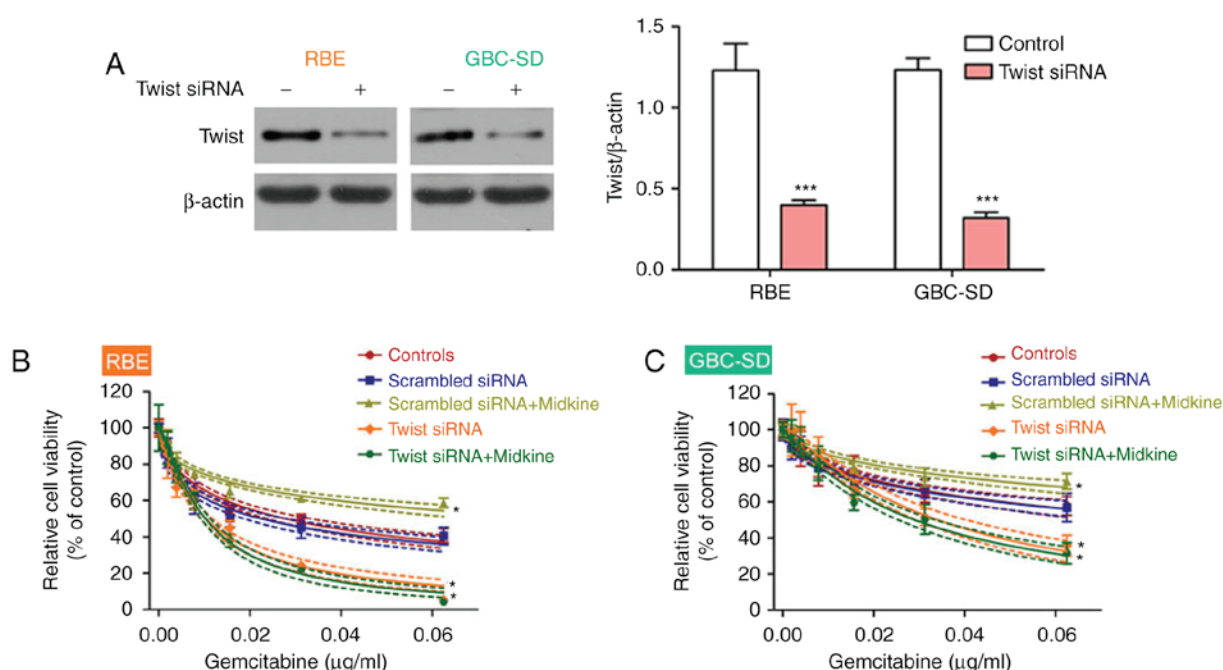


Figure 5. Comparison of the viability of the GBC-SD and RBE cell lines treated with Twist siRNA in the presence of gemcitabine with or without midkine. (A) Examination of the knockdown efficiency of Twist siRNA by western blotting. β -actin was used as the internal control, and the average grey values are presented in a histogram. *** $P < 0.001$ vs. Control. Viability assay of (B) RBE and (C) GBC-SD cells treated with Twist siRNA or scrambled siRNA, gemcitabine and optionally with midkine. * $P < 0.05$ vs. Control.

intrahepatic cholangiocarcinoma. However, little is known regarding the effect of MDK on the drug resistance of BTC. Therefore, the present study assessed this aspect and proved that MDK induced gemcitabine resistance in BTC.

The EMT is known to be involved in cancer drug resistance via various functions, including regulation of cancer cell stemness, overexpression of ATP binding cassette transporters, inhibition of epithelial growth factor receptor tyrosine kinase inhibitor-induced apoptosis and alteration of the tumor microenvironment (44). Previous studies have reported that replication

stress-induced MDK expression activates Notch-2, which drives EMT and chemoresistance in pancreatic cancer (30). The MDK-induced crosstalk of Notch2/Janus kinase 2/signal transducer and activator of transcription 3 signaling pathways regulates cell plasticity and motility, thereby contributing to EMT in human keratinocytes (31). In lung adenocarcinoma, estrogen receptor β -mediated estradiol enhanced MDK expression and increased EMT (29). Considering these previous studies, it was hypothesized that MDK may mediate gemcitabine resistance in BTC cells by regulating EMT. To

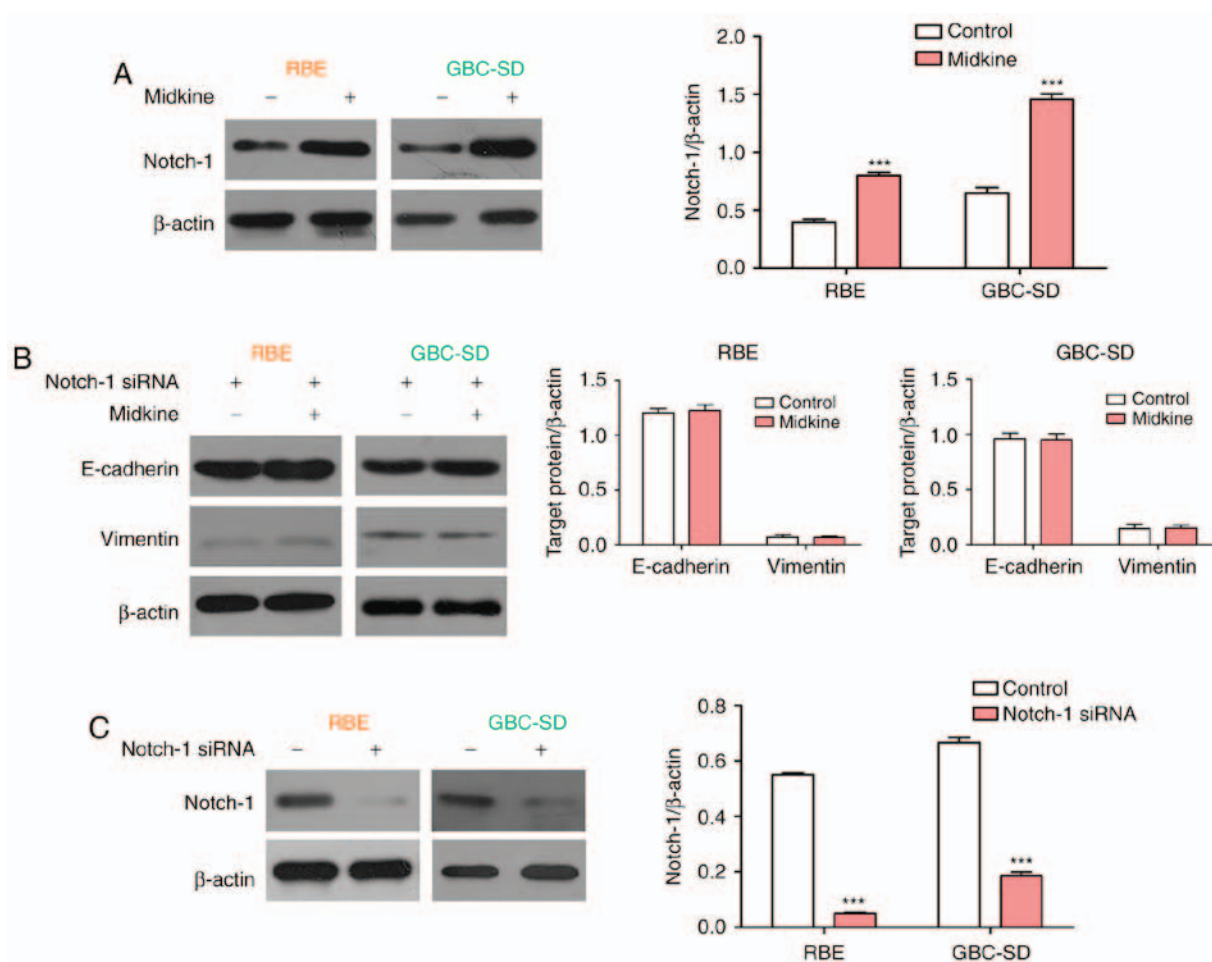


Figure 6. Effect of Notch-1 on midkine-induced epithelial to mesenchymal transition. (A) The protein expression of Notch-1 in GBC-SD and RBE cell lines treated with or without midkine was determined by western blot analysis. *** $P < 0.001$ vs. Control. (B) The protein expression of E-cadherin and vimentin in GBC-SD and RBE cell lines treated with Notch-1 siRNA in the presence of midkine was determined by western blot analysis. (C) Western blot analysis was used to detect the interference efficiency of Notch-1. β -actin was used as the internal control, and the average grey values are presented in a histogram. *** $P < 0.001$ vs. Control.

the best of our knowledge, the present study was the first to provide *in vitro* evidence to prove this hypothesis.

The Notch signaling pathway has critical roles in the development and progression of human cancers, as this pathway is critically involved in numerous cellular processes, including proliferation, survival, apoptosis, migration, invasion, angiogenesis and metastasis. Emerging evidence suggests that Notch regulates EMT, leading to tumor invasion and metastasis (35,36,45-49). Notch-1 has been reported to promote EMT in several cancer types (50-52). Although the association between MDK and Notch-2 is well known, the association between MDK and Notch-1 has remained elusive. In the present study, MDK was demonstrated to upregulate Notch-1 expression and it was revealed that MDK-induced EMT was mediated by Notch-1. These results highlight the role of Notch-1 in MDK-induced EMT.

To the best of our knowledge, the present study was the first to provide evidence that MDK enhances gemcitabine resistance in BTC cells via the Notch-1/EMT axis. Therefore, targeting MDK or blocking/reversing EMT prior to or during chemotherapy may force chemoresistant cells to revert to sensitive cells and may thus provide a tremendous benefit to patients with advanced chemoresistant cancers. Further study is required to understand the precise molecular mechanisms underlying gemcitabine resistance in BTC.

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Competing interests

The authors declare that they have no competing interests.

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