LncRNA HOXB-AS3 Promotes Proliferation, Migration, Invasion, and Epithelial-Mesenchymal Transition of Gallbladder Cancer Cells by Activating the MEK/ERK Pathway

Jiayan Wu
Sun Yat-sen University Sixth Affiliated Hospital
Hongquan Zhu
Sun Yat-sen University Sixth Affiliated Hospital
Jiandong Yu
Sun Yat-sen University Sixth Affiliated Hospital
Zhiping Chen
Sun Yat-sen University Sixth Affiliated Hospital
Zeyu Lin
Sun Yat-sen University Sixth Affiliated Hospital
Yongling Liang
Sun Yat-sen University Sixth Affiliated Hospital
Qin Chen
Wuxi No 2 People's Hospital
Guolin Li
Sun Yat-sen University Sixth Affiliated Hospital
Yun-Le Wan (wanyl036@sina.com)
Sun Yat-sen University Sixth Affiliated Hospital https://orcid.org/0000-0002-4354-3430

Research Article

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Abstract

OBJECTIVE: Long non-coding RNA HOXB-AS3 has been implicated in tumor progression in a variety of carcinomas. However, its biological role in gallbladder cancer (GBC) is unknown. The biological function and underlying mechanism of the lncRNA HOXB-AS3 for GBC were investigated in this study.

MATERIALS AND METHODS: To investigate the function of lncRNA HOXB-AS3 in GBC, the level of lncRNA HOXB-AS3 in GBC cells was detected by quantitative reverse-transcription polymerase chain reaction. The cell viability was tested by cell counting kit-8 assay and colony formation assay. Flow cytometry was performed to investigate cell apoptosis and cell cycle. In addition, cell migration ability was assessed by wound healing assay and cell invasion ability by transwell invasion assay.

RESULTS: It was found that HOXB-AS3 was obviously elevated in GBC tissues and cells. However, inhibition of HOXB-AS3 could depress NOZ and GBC-SD cell viability as well as induce cell apoptosis. Also, the gallbladder cancer cell cycle was blocked in the G1 phase. Meanwhile, NOZ and GBC-SD cell migration, invasion, and epithelial-mesenchymal transition were obviously suppressed by knockdown of HOXB-AS3. What is more, we found that HOXB-AS3 might promote gallbladder progress by activating the MEK/ERK pathway.

CONCLUSION: The results show that lncRNA HOXB-AS3 serves as a key regulator in GBC progression, which provides a new treatment strategy for GBC.

Introduction

- Gallbladder cancer (GBC), the sixth most common gastrointestinal malignancy worldwide, is the most common biliary tract neoplasm, with a worldwide incidence of approximately 2 per 100,000 [1–5]. However, GBC shows a unique geographical distribution pattern, with Chile, India, Pakistan, Japan, and Korea reporting more cases than the other countries in the world [6–8]. Meanwhile, due to a lack of specific symptoms, signs, or biomarkers in early stages, the majority of patients with gallbladder cancer are often diagnosed at advanced stages [9]. Currently, complete surgical resection is the only potentially curative strategy for GBC patients [10], with radiotherapy, chemotherapy, and molecular targeted therapy as palliative treatments. What a pity that the overall survival rate (OS) of GBC patients has not improved significantly in the past few decades [11–13]. Hence, it is urgent to investigate the underlying pathogenesis of GBC and improve the understanding of mechanism-based treatment for it.
- Long non-coding RNAs (lncRNAs) are defined as a class of RNAs with no or weak protein-coding abilities, composed of more than 200 nucleotides (nts) in length [14–16]. LncRNAs have been reported to play important roles in many biological processes: cell proliferation, apoptosis, invasion and metastasis [17–19], regulating gene expression at the transcriptional, post-transcriptional and translational levels [20–23]. HOXB cluster antisense RNA 3 (HOXB-AS3) was annotated as a lncRNA in

the NONCODE databases. It encodes a 53-amino acid peptide, first reported by J. Huang, and can suppress colon carcinoma tumorigenesis by metabolic reprogramming [24]. Subsequently, X Zhuang and colleagues proved that HOXB-AS3 promotes tumorigenesis in ovarian cancer by activating the Wnt/β-catenin pathway [25]. In hepatocellular carcinoma, XM Zhang et al. demonstrated that HOXB-AS3 could promote cancer proliferation [26]. However, the potentially molecular mechanism in the pathogenesis of lncRNA HOXB-AS3 in gallbladder cancer remained unknown.

- Genes in the MEK/ERK pathway play a vital role in a variety of human cancers. For instance, mitogen-activated protein kinase (MAPK) superfamily members are related to cell proliferation, survival, and apoptosis [27]. However, whether HOXB-AS3 is able to regulate the MEK/ERK signaling pathway is still unknown.
- In our study, we hypothesized that lncRNA HOXB-AS3 promotes gallbladder carcinogenesis by activating the MEK/ERK pathway.

Materials And Methods

Expression profile information

- The datasets GSE74048 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE74048) were downloaded from the GEO database. This datasets GSE74048 included the lncRNA expression profile of human gallbladder carcinoma identified by microarray in 3 pairs of human GBC tissues and the matched peri-carcinomatous tissues. Furthermore, quantitative real-time polymerase chain reaction was used for the validation of the microarray data. Then, using the Perl programming language software (version 5.34.0) and the R programming language software (version 4.0.5), the expression data of lncRNA HOXB-AS3 were collected.

Cell culture

- Human gallbladder cancer cell lines including GBC-SD and NOZ and human intrahepatic biliary epithelial cell (HIBEpic) were purchased from JennioBio (China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, USA). All cell lines were cultured with DMED supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, USA) at 37˚C with 5% CO2.

Cell infection

- The GBC-SD and NOZ in the logarithmic growth phase were seeded into 6-well plates at a density of 1 x 10^6 cells/well the day before transfection. HOXB-AS3 small interfering RNA (siRNA) was constructed by HanyiBio (China) to knockdown HOXB-AS3. All the siRNAs were transfected using lipofectamine 3000 (Invitrogen, ThermoFisher Scientific, USA) with DMEM medium without FBS

according to the manufacturer's instructions. The sequences of siHOXB-AS3 and siNC were as follows: siHOXB-AS3-1: 5'-UGCUUGUCUGGAGAUGGAGC-CA-3'; siHOXB-AS3-2: 5'-CAAAGGGAGA-

- CAAAGUCAATT-3'; siHOXB-AS3-3: 5'-GGGUAGACUUCGCUUGAAATT-3'; siHOXB-AS3-4: 5'-CGACAAAGGGAGACAAAGUTT-3'; siNC: 5'-UUCUCCGAACGUGUCACGU-3'. After 48-72h later, the examination of HOXB-AS3 expression level and subsequent experiments were performed.

Cell counting kit-8 (CCK-8) assay

- NOZ and GBC-SD cells were seeded in 96-well plates (5 x 10^3 per well) overnight. Then, cells were transfected with lncRNA HOXB-AS3 siRNA1, lncRNA HOXB-AS3 siRNA2, or negative control (siNC) for 0, 24, 48, or 72hrs. 10μL CCK-8 reagent (LiankeBio, China) was added to each well and further incubated for 3 hours at 37°C. Finally, the optical density (OD) of each well was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, USA).

Colony formation assay

GBC-SD and NOZ cells were seeded in 6-well plates at 1000 cells/well and cultured in complete DMEM at 37˚C overnight. Then, cells were treated with lncRNA HOXB-AS3 siRNA1, lncRNA HOXB-AS3 siRNA2 or negative control (siNC). The culture medium was changed regularly for 14 days. The colonies were fixed in 20% methanol at room temperature for 20 min and stained with 1% crystal violet (Beyotime, China) dissolved in methanol for 20 min at room temperature and counted under an inverted light microscope (Olympus, Japan).

Flow cytometric analysis of cell apoptosis

- Cell apoptosis was determined using flow cytometric analysis. NOZ and GBC-SD cells transfected with si-HOXB-AS3 or si-NC were cultured in 6-well plates for 72 h.The cells were digested by pancreatin that not contain ethylenediamine tetraacetic acid. A total of 1 x 10^5 cells/ml of suspended cells were collected. Hundred microliters of 1× Annexin buffer were used to suspend the cells. Then, cells were double-stained with annexin V-fluorescein iso-thiocyanate (FITC) and PI using an FITC annexin V Apoptosis Detection kit (LiankeBio, China). The cells were immediately examined by flow cytometry (Beckman, USA) according to the manufacturer's protocol. The ratio of cell apoptosis was calculated with Flowjo software (version 10.6.2).

Flow cytometric analysis of the cell cycle

- NOZ and GBC-SD cells transfected with si-HOXB-AS3 or si-NC were cultured in 12-well plates for 48 h. The cells were digested by pancreatin that not contain ethylenediamine tetraacetic acid. A total of 2 x 104 cells/ml of suspended cells were harvested. The harvested cells were stained with propidium iodide (LiankeBio, China). Cell cycles were divided into G1/G0, S, and G2/M stages, and the ratio of all the stages was calculated with Modfit software (version 3.1).

Wound healing assay

- Cells were seeded into a 6-well plate and cultured in DMEM supplemented with 10% FBS until the cells were grown to 100% confluence. A 200 μl pipette tip was used to create a scratch wound in the cell monolayer. DMEM without FBS was added to the 6-well plate. At 0 and 72 h, cell migration was observed and photographed. Image J was used to quantify the wound distance at 0 and 72 h. The percentage of gap closure was used as an indicator of cell migration. The gap closure percentage is calculated as (average distance at 0 h - average distance at 72 h)/(average distance at 0 h) x100%.

Transwell invasion assay

- At 24 h post-transfection, NOZ and GBC-SD cells were trypsinized and resuspended in DMEM without FBS. Matrigel (Solarbio, China) was used to precoat the membrane of the upper chambers at 37˚C for 1 h. Cells (1 x 105) in serum-free medium were plated into the upper chamber, and medium containing 10% FBS was plated into the lower chamber. Following incubation at 37˚C for 24 h, cells were rinsed with PBS, fixed with 4% paraformaldehyde at room temperature for 25 min, and stained with 0.1% crystal violet at 37˚C for 25 min. Invading cells were observed using a light microscope (Olympus, Japan) in five randomly selected fields of view.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

- Total RNA of HIBEpic, NOZ, or GBC-SD cells was extracted with Trizol (invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's protocol, and a biological spectrometer (Thermo Fisher Scientific, USA) was used to detect the concentration of RNA. Afterward, the RNA was reverse-transcribed to cDNA for 20 min at 42˚C with a hifiscript cDNA synthesis kit (CWBio, China) according to the manufacturer's instructions. The qRT-PCR was performed using a Roche Real-Time PCR System with a SYBR Premix Ex Taq II (CWBio, China). The primers used were as follows: HOXB-AS3 forward, 5’-CCCTCCCTCCAAGTCCAGTAAGAAG

-3’ and reverse, 5’-TGTAGTGGCTCCATCTCCAGACAAG-3’; and GAPDH 5’-CACGAGGCA-

- TTGCTGCTGATGAT-3’ and reverse, 5’-GAAGGCTGGGGCTCATTT-3’.The expression levels of HOXB-AS3 were calculated and normalized to GAPDH using the 2-ΔΔct method.

Western blot analysis

- At 72 h post-transfection, total protein was extracted from cells from each group using a total protein extraction kit (CWBio, China). Protein concentration was determined using an enhanced BCA protein assay kit (Beytime, China). Proteins (10 μg per lane) were separated by SDS-PAGE on 10% gels and

transferred to PVDF membranes. After blocking with rapid blocking buffer (Epizyme, China) for 20 min at room temperature, the membranes were incubated with the primary antibodies overnight at 4˚C. The following primary antibodies were used: Anti-ERK (1:1,000, CST), p-ERK (1:1,000; CST), MEK (1:1,000; CST), p-MEK (1:1,000; CST), GAPDH (1:1,000; CST) E-cadherin (1:1,000; CST), vimentin (1:1,000; CST), and N-cadherin (1:1,000; CST) from cell Signaling Technologies. Primary antibody incubation was followed by incubation with a horseradish-peroxidase-conjugated secondary antibody (dilution 1:5,000; CWBio, China) at room temperature for 1 h, and the protein blots on the membrane were imaged by a chemiluminescence kit (Biosharp, China). Image J software (version 1.8.1) was used to semi-quantify the data.

Statistical analysis

- All the experiments were performed at least three times. Data were presented as the mean ± standard deviation of at least three independent experiments. GraphPad Prism software (version 8.0) was used for the analysis. Statistical analyses were performed using one-way ANOVA with Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of HOXB-AS3 was increased in gallbladder carcinoma

- HOXB-AS3 was greatly up-regulated in 3 gallbladder cancer tissues than that matched peri-carcinomatous tissues by microarray based on the GEO databases (Fig. 1a). Furthermore, HOXB-AS3 expression in human intrahepatic biliary epithelial cell (HIBEpic) and gallbladder cancer cell lines including GBC-SD and NOZ were determined. In Fig. 1b, we found that HOXB-AS3 was significantly up-regulated in gallbladder cancer cell lines compared with HIBEpic cell. These results demonstrated that HOXB-AS3 was highly expressed in GBC, supporting a vital role in gallbladder cancer progression.

HOXB-AS3 down-regulation inhibited gallbladder cancer cell proliferation

- To explore the role of HOXB-AS3 in GBC progression, four small interfering RNA (siRNA) targeted at HOXB-AS3 and one non-specific siRNA (si-NC) were used. As shown in figure 2, the expression of HOXB-AS3 was significantly down-regulated following transfection with siRNA in NOZ and GBC-SD cells. What is more, we observed that the silencing of HOXB-AS3 significantly depressed cell proliferation both in NOZ and GBC-SD cell lines, as proved using CCK-8 assay (Fig. 2c,d). Then, as shown in figure 2e,f, NOZ and GBC-SD cell colony formation was greatly repressed by the down-

regulation of HOXB-AS3. These suggests that HOXB-AS3 promotes gallbladder cancer cell proliferation.

Decrease of HOXB-AS3 blocked cell cycle progression and triggered cell apoptosis

- Then, as exhibited in figure 3a,b, NOZ and GBC-SD cell cycle were blocked in the G1 phase because of inhibition of HOXB-AS3 in vitro. Also, NOZ and GBC-SD cell apoptosis was induced by HOXB-AS3 siRNA in figure3c,d. In total, these results suggest that HOXB-AS3 might play an important role in gallbladder cancer cell cycle progression and cell apoptosis.
- **Knockdown of HOXB-AS3 inhibited GBC cell migration, invasion, and EMT.**
- Following that, the wound healing and transwell assays were used to test the effects of HOXB-AS3 on metastasis-associated cell behaviors such as cell migration and invasion. As displayed in figure4a,b, HOXB-AS3 siRNA greatly depressed the wound closure in NOZ and GBC-SD cells. And, in figure4c,d, transwell assays demonstrated that loss of HOXB-AS3 dramatically depressed NOZ and GBC-SD cell invasion. Additionally, in the present study, HOXB-AS3 knockdown notably decreased N-cadherin and vimentin but increased E-cadherin expression in NOZ and GBC-SD cells (Fig. 4e and 4f). These indicated that NOZ and GBC-SD cells may promote the migration and invasion of GBC cells via EMT.
- **MEK/ERK pathway was involved in the HOXB-AS-induced proliferation of GBC cells.**

At last, in figure5a,b, inhibition of the expression of HOXB-AS reduced the levels of MEK

- and ERK phosphorylation, but had no effect on the expression of total MEK and ERK in NOZ and GBC-SD cells. These data suggest that lncRNA HOXB-AS3 might promotes proliferation, migration, invasion, and epithelial-mesenchymal transition of gallbladder cancer cells by activating the MEK/ERK pathway.

Discussion

- GBC is still a highly lethal, malignant threat to our health [28]. Increasing studies have revealed that a number of lncRNAs are involved in the development of GBC. For instance, lncRNA OIP5-AS1 contributes to gallbladder cancer cell invasion and migration [29]. LncRNA FOXD2-AS1 inhibits the progression of gallbladder cancer by mediating methylation of MLH1 [30]. LncRNA EPIC1 promotes proliferation and inhibits apoptosis of gallbladder cancer cells by interacting with LET [31]. LncRNA LINC01410 promoted gallbladder tumor progression via the ErbB signaling pathway by targeting STAT5 [32]. But whether lncRNA HOXB-AS3 played a role in gallbladder cancer progression has not been reported before.

- In our study, lncRNA HOXB-AS3 expression was significantly up-regulated in NOZ and GBC-SD cells,compare with human intrahepatic biliary epithelial cell (HIBEpic). Additionally, HOXB-AS3 knockdown greatly inhibited GBC-SD and NOZ cell proliferation, invasion, migration, and epithelial-mesenchymal transition (EMT), and promoted apoptosis compared with the control group. Furthermore, our results implied that lncRNA HOXB-AS3 might affect GBC progression via the MEK/ERK signaling pathway.
- The present study demonstrated that HOXB-AS3 knockdown inhibited MEK and ERK phosphorylation in NOZ and GBC-SD cells compared with that in the control group. Previous studies have reported that MEK/ERK signaling is related to the oncogenesis of many human tumors, including gastric cancer, retinoblastoma, and pancreatic cancer[33–35]. And MEK/ERK pathway play an important role in cellular functions such as proliferation, differentiation, survival and migration [36]. Although a number of studies have demonstrated the vital role of lncRNAs in oncogenesis processes, the underlying mechanisms remain unclear. In our study, we found that lncRNA HOXB-AS3 promoted the proliferation and invasion of GBC might by activating the MEK/ERK signaling pathway. However, no evidence was observed to indicate whether HOXB-AS3 directly phosphorylated MEK or ERK. The specific regulatory mechanism of lncRNA HOXB-AS3 on MEK/ERK signaling in GBC needs to be further investigated.
- Furthermore, tumor cells might lead to its invasion and distant metastasis through epithelial-mesenchymal transition (EMT), with a poor prognosis. X.H.et al. reported that HOXB-AS3 could enhance EMT and activate the Wnt pathway in ovarian cancer [25]. The characteristics of EMT are down-regulation of epithelial proteins such as E-cadherin and up-regulation of mesenchymal protein, including E-cadherin and vimentin. In the present study, we found that knockdown of HOXB-AS3 up-regulated the levels of the epithelial protein E-cadherin and down-regulated the mesenchymal protein markers E-cadherin and vimentin. The effects of HOXB-AS3 on metastasis-associated cell behaviors, including cell migration and invasion, were tested by the wound healing and transwell assays. As displayed in Figure 4a, 4b, HOXB-AS3 siRNA greatly depressed the wound closure in NOZ and GBC-SD cells. And, in Figure 4c, 4d, transwell invasion assays demonstrated that loss of HOXB-AS3 dramatically depressed NOZ and GBC-SD cell invasion. Consequently, in the present study, HOXB-AS3 knockdown notably decreased N-cadherin and vimentin but increased E-cadherin expression in NOZ and GBC-SD cells (Figure 4e, 4f). This indicated that NOZ and GBC-SD cells might promote the migration and invasion of GBC cells via EMT.

Conclusion

- In conclusion, we revealed that HOXB-AS3 promoted the cell viability, migration, invasion, and EMT of GBC cells might by activating the MEK/ERK pathway. These results indicate that HOXB-AS3 might contribute to GBC tumorigenesis and metastasis and thus be a potential therapeutic target for GBC intervention.
- **Figure legends**

- **Fig. 1** LncRNA HOXB-AS3 was up-regulated in human gallbladder cancer tissues and cell lines (a) Compared with para-carcinoma tissues, HOXB-AS3 was significantly up-regulated in gallbladder cancer tissues. (b) The relative expression levels of HOXB-AS3 in human gallbladder cancer cells (NOZ and GBC-SD) and human intrahepatic biliary epithelial cell (HIBEpic). HOXB-AS3 expression was normalized against GAPDH expression. Compared with HIBEpic cell, HOXB-AS3 was significantly up-regulated in gallbladder cancer cell lines.Three independent experiments were performed, and the data are presented as the mean ± SD. *p < 0.05, ***p < 0.001.
- **Fig. 2** HOXB-AS3 promotes GBC cell proliferation (a,b) The HOXB-AS3 expression levels were reduced by small interfering RNA (siRNA) targeting HOXB-AS3 with qRT-PCR analysis. HOXB-AS3 expression levels were normalized to GAPDH. Compared with the negative control group, the expression levels of HOXB-AS3 were significantly reduced by siRNA targeting HOXB-AS3 in NOZ and GBC-SD. However, there was no significant difference between siNC group and siHOXB-AS3#3 group or siHOXB-AS3#4 group in GBC-SD cell. (c,d) Cell growth rates were evaluated with CCK-8 assay in NOZ and GBC-SD cells following HOXB-AS3 interference, and the results showed that knockdown of HOXB-AS3 significantly inhibited the growth of GBC-SD cells. (e,f) The colony formation assay was used to analyze the colony formation capacity of GBC cells, and the results showed that HOXB-AS3 knockdown significantly inhibited the colony formation capacity of NOZ and GBC-SD cells. Three independent experiments were performed, and the data are presented as the mean ± SD. *p < 0.05, **p < 0.01, ns: not significant.
- **Fig. 3** Flow cytometry analysis was performed to assess the effect of HOXB-AS3 on the cell cycle and cell apoptosis of GBC cells (a,b) Inhibition of HOXB-AS3 significantly increases G1 phase and decreases S phase in NOZ and GBC-SD cells compared with the negative control group. (c,d) Compared with the negative control group, inhibition of HOXB-AS3 significantly induces cell apoptosis in NOZ and GBC-SD cells. Three independent experiments were performed, and the data are presented as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.
- **Fig. 4** Down-regulation of HOXB-AS3 suppresses the migration, invasion, and EMT of human gallbladder cancer cells (a, b) Wound healing assay was performed to determine the cell migration in NOZ and GBC-SD cells. Compared with the negative control group, HOXB-AS3 knockdown significantly inhibited the migration ability of NOZ and GBC-SD cells. (c, d) Transwell invasion assay was performed to detect the cell invasive ability of GBC cells. The results showed that down-regulation of HOXB-AS3 significantly inhibited the invasive ability of NOZ and GBC-SD cells. (e, f) Differences among epithelial-mesenchymal transition marker expression in NOZ and GBC-SD cells following transfection with siRNA were detected by western blotting. Compared with the negative control group, down-regulation of HOXB-AS3 significantly increased the epithelial protein E-cadherin and decreased the mesenchymal protein N-cadherin and vimentin in NOZ and GBC-SD cells. Three independent experiments were performed, and the data are presented as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 5 Effects of HOXB-AS3 on MEK/ERK pathway (a) p-ERK and p-MEK protein expression in NOZ cells.Inhibition of the expression of HOXB-AS reduced the levels of MEK and ERK phosphorylation, but

had no effect on the expression of total MEK and ERK in NOZ cell. (b) Protein expression of p-ERK and p-MEK in GBC-SD cell. Inhibition of the expression of HOXB-AS reduced the levels of MEK and ERK phosphorylation, but had no effect on the expression of total MEK and ERK in GBC-SD cell. Three independent experiments were performed, and the data are presented as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

*All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [*Jiayan Wu*], [*Hongquan Zhu*] and [*Yongling Liang*]. The first draft of the manuscript was written by [*Jiayan Wu*] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript*

Data Availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University.

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Figures

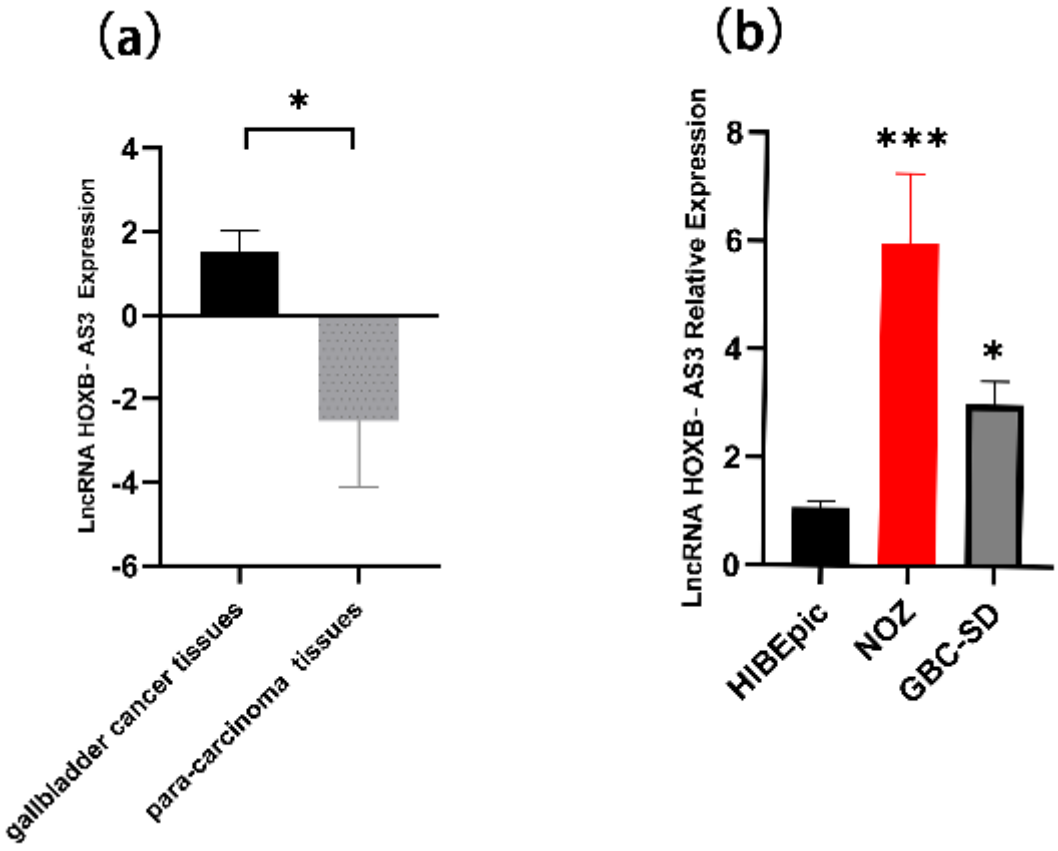

Figure 1

LncRNA HOXB-AS3 was up-regulated in human gallbladder cancer tissues and cell lines (a) Compared with para-carcinoma tissues, HOXB-AS3 was significantly up-regulated in gallbladder cancer tissues. (b) The relative expression levels of HOXB-AS3 in human gallbladder cancer cells (NOZ and GBC-SD) and human intrahepatic biliary epithelial cell (HIBEpic). HOXB-AS3 expression was normalized against GAPDH expression. Compared with HIBEpic cell, HOXB-AS3 was significantly up-regulated in gallbladder

cancer cell lines.Three independent experiments were performed, and the data are presented as the mean ± SD. *p < 0.05, ***p < 0.001.

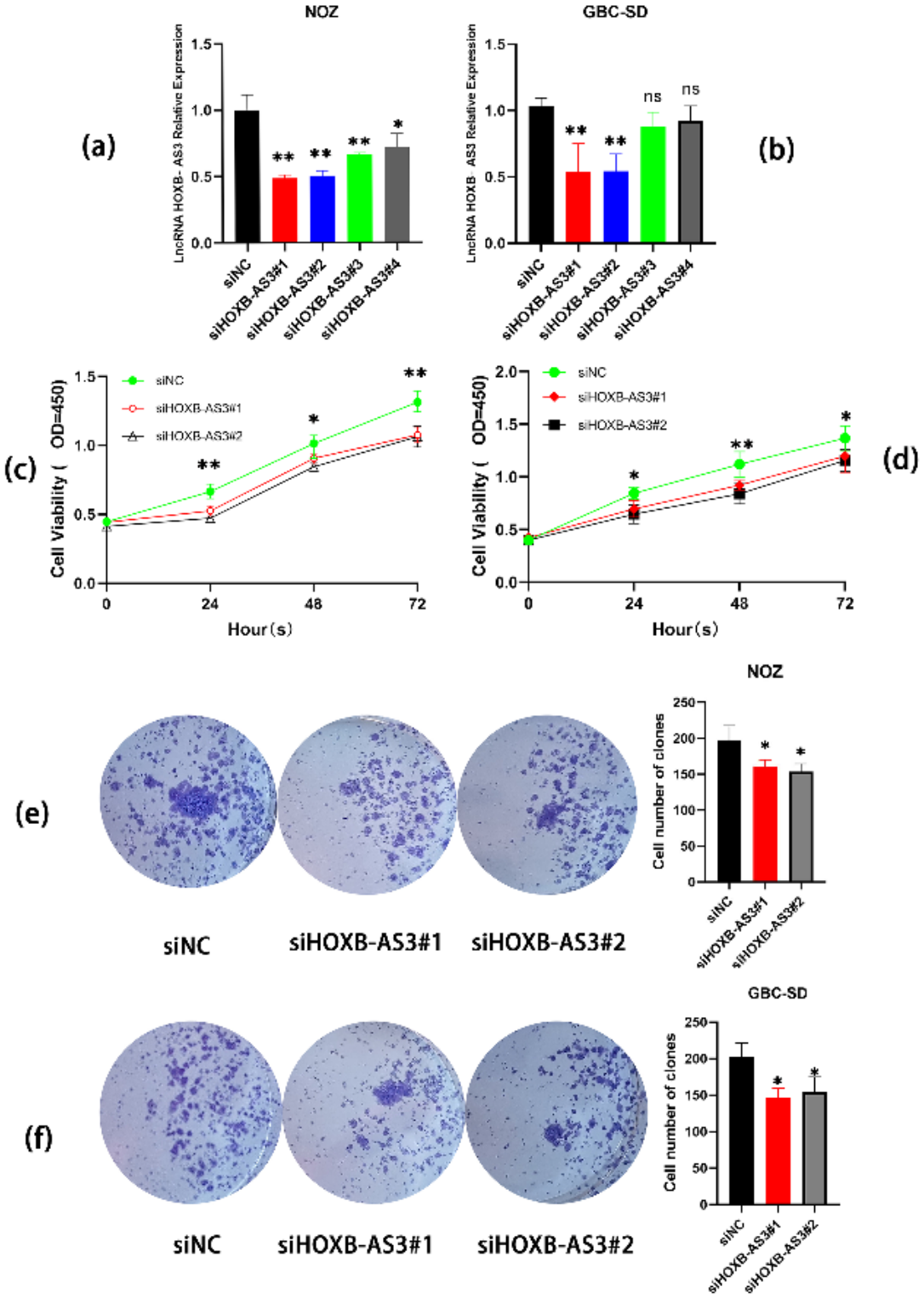

Figure 2

HOXB-AS3 promotes GBC cell proliferation (a,b) The HOXB-AS3 expression levels were reduced by small interfering RNA (siRNA) targeting HOXB-AS3 with qRT-PCR analysis. HOXB-AS3 expression levels were

normalized to GAPDH. Compared with the negative control group, the expression levels of HOXB-AS3 were significantly reduced by siRNA targeting HOXB-AS3 in NOZ and GBC-SD. However, there was no significant difference between siNC group and siHOXB-AS3#3 group or siHOXB-AS3#4 group in GBC-SD cell. (c,d) Cell growth rates were evaluated with CCK-8 assay in NOZ and GBC-SD cells following HOXB-AS3 interference, and the results showed that knockdown of HOXB-AS3 significantly inhibited the growth of GBC-SD cells. (e,f) The colony formation assay was used to analyze the colony formation capacity of GBC cells, and the results showed that HOXB-AS3 knockdown significantly inhibited the colony formation capacity of NOZ and GBC-SD cells. Three independent experiments were performed, and the data are presented as the mean ± SD. *$p < 0.05$, **$p < 0.01$, ns: not significant.

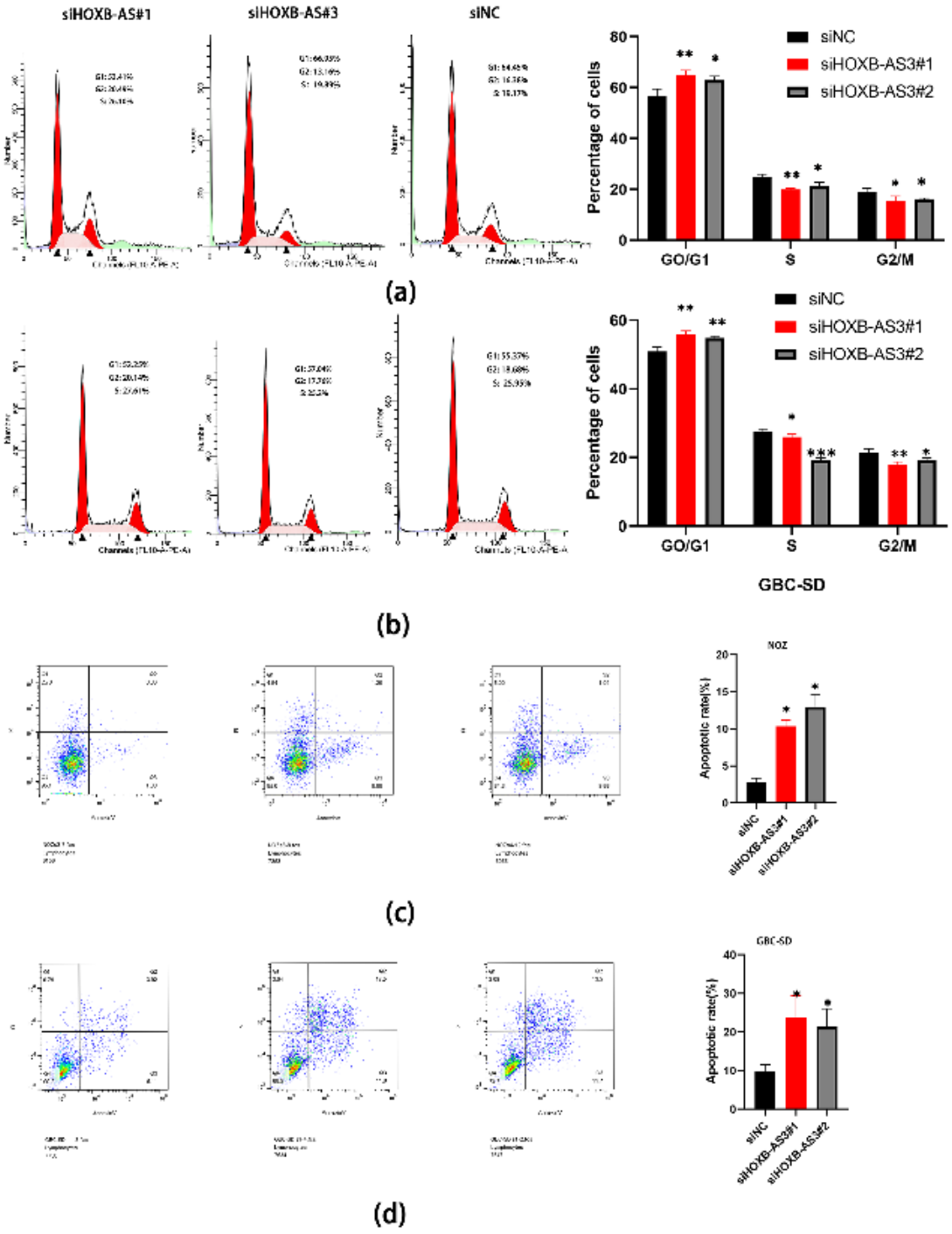

Figure 3

Flow cytometry analysis was performed to assess the effect of HOXB-AS3 on the cell cycle and cell apoptosis of GBC cells (a,b) Inhibition of HOXB-AS3 significantly increases G1 phase and decreases S phase in NOZ and GBC-SD cells compared with the negative control group. (c,d) Compared with the negative control group, inhibition of HOXB-AS3 significantly induces cell apoptosis in NOZ and GBC-SD

cells. Three independent experiments were performed, and the data are presented as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4

Down-regulation of HOXB-AS3 suppresses the migration, invasion, and EMT of human gallbladder cancer cells (a, b) Wound healing assay was performed to determine the cell migration in NOZ and GBC-SD cells. Compared with the negative control group, HOXB-AS3 knockdown significantly inhibited the migration ability of NOZ and GBC-SD cells. (c, d) Transwell invasion assay was performed to detect the cell invasive ability of GBC cells. The results showed that down-regulation of HOXB-AS3 significantly inhibited the invasive ability of NOZ and GBC-SD cells. (e, f) Differences among epithelial-mesenchymal transition marker expression in NOZ and GBC-SD cells following transfection with siRNA were detected by western blotting. Compared with the negative control group, down-regulation of HOXB-AS3 significantly increased the epithelial protein E-cadherin and decreased the mesenchymal protein N-cadherin and vimentin in NOZ and GBC-SD cells. Three independent experiments were performed, and the data are presented as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

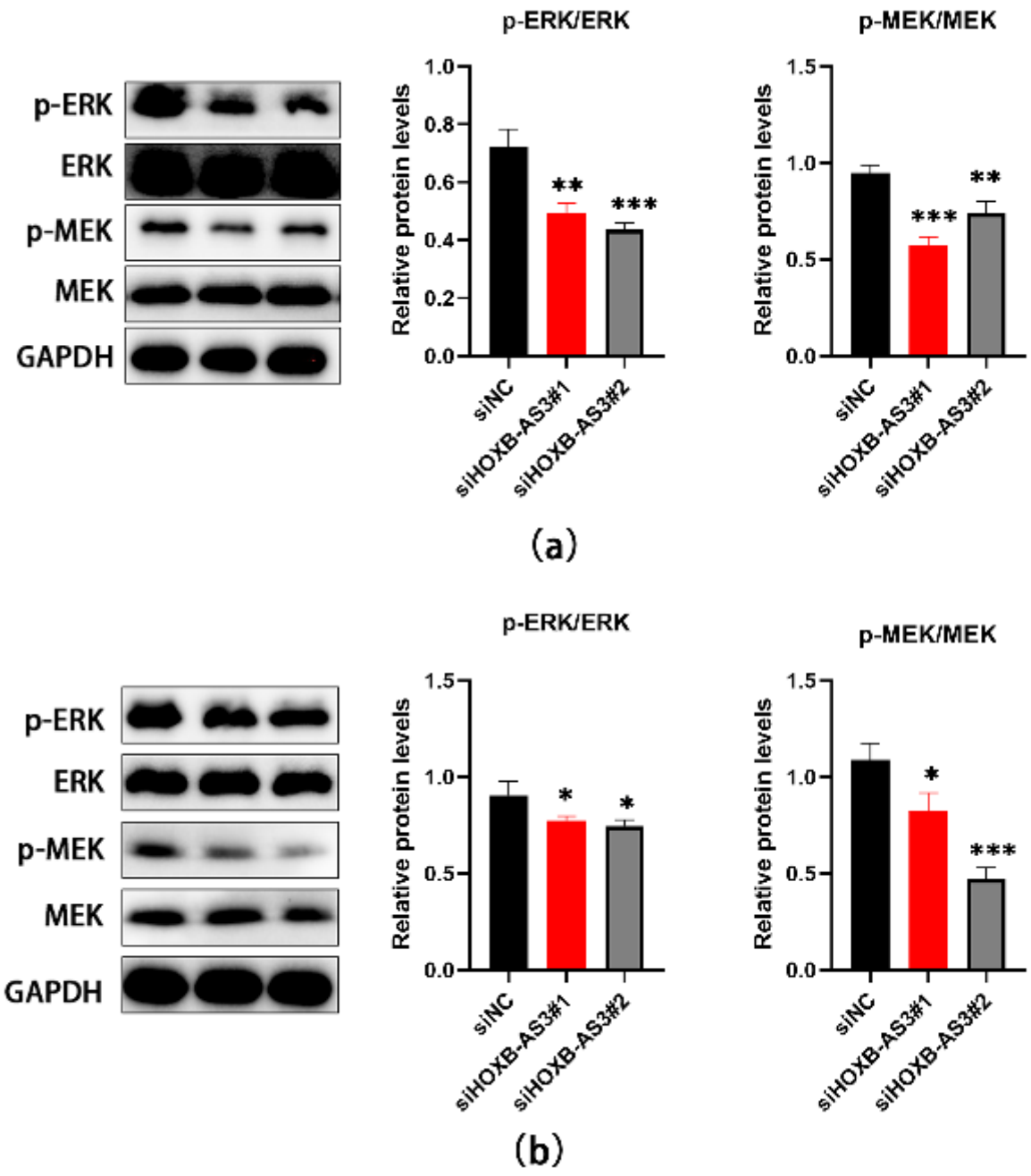

Figure 5

Effects of HOXB-AS3 on MEK/ERK pathway (a) p-ERK and p-MEK protein expression in NOZ cells.Inhibition of the expression of HOXB-AS reduced the levels of MEK and ERK phosphorylation, but had no effect on the expression of total MEK and ERK in NOZ cell. (b) Protein expression of p-ERK and p-MEK in GBC-SD cell. Inhibition of the expression of HOXB-AS reduced the levels of MEK and ERK phosphorylation, but had no effect on the expression of total MEK and ERK in GBC-SD cell. Three independent experiments were performed, and the data are presented as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.