FOXK1 plays an oncogenic role in the progression of hilar cholangiocarcinoma

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Abstract. Hilar cholangiocarcinoma (HC) has a poor outcome in terms of survival. Forkhead box K1 (FOXK1) dysregulation is critical in solid tumors, which serves a pivotal role in the biological characteristics, such as invasion and migration, but its expression and functions in HC are unclear. The present study investigated the clinical significance and biological functions of FOXK1 in HC. Tumor microarrays and immunohistochemistry were used to evaluate FOXK1 in HC and its expression was modulated to determine its effects on chemoresistance and tumorigenesis. FOXK1 was highly expressed in HC and cell lines, which was associated with tumor invasion, regional lymph node metastasis, tumor recurrence and poor prognosis. Silencing FOXK1 in HC cells inhibited invasion and migration, upregulated E-cadherin, and downregulated vimentin, matrix metallopeptidase 9 and Twist in HC cells. Sensitivity to 5-fluorouracil and cisplatin was increased, and glutathione S-transferase π, multidrug resistance mutation 1 and P-glycoprotein expression levels were downregulated in RBE cells *in vitro* following FOXK1 knockdown. These results indicated that FOXK1 plays an oncogenic role in HC progression and can serve as a novel therapeutic target for HC.

Introduction

Hilar cholangiocarcinomas (HCs) are the most common type of cholangiocarcinoma and are highly prevalent in Southeast Asia, although uncommon in the USA (1). The only strategy for treating patients and prolonging overall survival (OS) is complete resection with negative surgical margins (2). However, in most patients, the tumors are unresectable due to locally advanced or metastatic disease at diagnosis (3). Recently, advances in revealing the genetic landscape of HC have led to the identification of several promising systemic therapeutic agents or strategies that could improve the outcome of patients with HC (4).

Forkhead box K1 (FOXK1) is a member of the FOX transcription factor family, which plays critical roles in embryonic development and organogenesis, and regulates a variety of physiological processes, including metabolism, cell signaling and cell proliferation (5-7). The dysregulation of FOXK1 expression and subcellular localization leads to the uncontrolled development and progression of human solid cancer types. For instance, increasing evidence has demonstrated that FOXK1 knockdown can inhibit cell proliferation, migration and invasion in prostate cancer (8), hepatocellular carcinoma (9) and esophageal cancer (10), whereas its enhanced expression facilitates cell proliferation and metastasis in ovarian (11) and esophageal cancer (10). These studies suggest that FOXK1 has a role in tumorigenesis. However, its expression pattern and function in HC are not well defined.

The present study investigated the clinical significance and biological functions of FOXK1 in HC. The results demonstrated that FOXK1 was upregulated in HC tissues and that its high expression was significantly associated with neural invasion and lymph node (LN) metastasis. In addition, the nuclear expression of FOXK1 was found to be associated with recurrence and poor outcome in patients with HC. FOXK1 knockdown *in vitro* inhibited the proliferation and migration of HC cells. To the best of the authors' knowledge, the present study was the first to reveal the expression profile of FOXK1 and its function in HC cell proliferation and metastasis, highlighting its potential as a therapeutic target for this disease.

Materials and methods

Patient samples and cell culture. Tissue microarrays (TMAs) of 48 resected HC specimens and 15 matched non-cancerous bile duct tissues (with >5-mm distance from the primary tumor's edge) obtained from the Eastern Hepatobiliary Hospital

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and Changhai Hospital (Shanghai, China) were constructed as previously described (12). Of the patients, 31 were men and 17 women, with a mean age of 62 years (range, 42-78 years). Patient characteristics are given in Table I. The median follow-up duration was 16 months (range, 1-59 months). The tissue sample experiments were approved by the Ethics Committee of The Affiliated Hospital of Qingdao University. Informed consent written from all participants (or their parent or legal guardian in the case of children under 16) was obtained to participate in the study or to use their tissues. Human cholangiocarcinoma cell lines (FRH-0201, QBC939 and RBE) were obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences and. The expression of FOXK1 in the four cell lines were detected by western blotting, and it was identified that RBE cells exhibited the highest expression, so the RBE cell line was selected as the experimental cells. These cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and cultured at 37˚C with 5% CO_2 (12).

Immunohistochemistry and evaluation of HC specimens. Sections (4 μm) of TMAs were prepared and processed for the immunohistochemical analysis of FOXK1 (1:100; cat. no. ab18196; Abcam), which was carried out according to a previous study (13). A streptavidin-biotin kit (cat. no. KIT-9720; Fuzhou Maixin Biotech Co., Ltd.) was used to visualize antibody binding in these sections. Immunostaining of FOXK1 was evaluated by two individuals (ZGB and YJF) and a semi-quantitative scoring system was used in the present study, as previously reported (12). A weighted score was generated for each case ranging from 0 (0% of cells stained) to 300 (100% of cells stained at >3 intensity) as previous study described, a score of <75 was defined as low expression and that of ≥75 was defined as high expression (12).

Cell transfection. FOXK1 short hairpin (sh)RNA (cat. no. PR6021) and scrambled shRNA plasmids (pLent-U6-GFP-Puro) were constructed by Shanghai GeneChem Co., Ltd. Next, 1x10^5 RBE cells were seeded in 6-well plates and transfected with FOXK1 shRNA (shFOXK1) or scrambled shRNA (shNC) at 5 ng shRNA plasmid per well using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. These cells were then subcultured and selected in the presence of puromycin (1 μg/ml) for 3 days between transfection and experimentation at 37˚C to generate stable NC and FOXK1-knockdown cells.

Cell Counting Kit-8 (CCK-8) analysis. After obtaining stable FOXK1-knockdown or NC RBE lines, 5,000 cells per well were seeded in 96-well plates. At the indicated times (24, 48 and 72 h), the CCK-8 assay (Dojindo Molecular Technologies, Inc.) was performed to assess the results. This experiment was performed in triplicate.

Cell viability assay. FOXK1-knockdown and NC RBE cells were seeded in 96-well plates and treated with different concentrations (0, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 μg/ml) of 5-FU and cisplatin (DDP) when the cell density reached 60-70%. Cell viability was assayed using an MTT assay (Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions.

Table I. Association between the expression of FOXK1 and clinicopathological parameters of hilar cholangiocarcinoma.

Clinicopathological parameters	N	FOXK1, n (%)	P-value
Tumor size, cm			0.135
≤3	17	8 (47.1)	
3	31	23 (74.2)	
Nerve invasion			0.838
Yes	25	17 (68.0)	
No	23	15 (65.2)	
T stage			0.005
T1-3	6	1 (16.7)	
T4	42	31 (73.8)	
N stage			0.008
N0	15	6 (40.0)	
N1-2	33	26 (78.8)	
Differentiation			0.480
High/moderate	36	23 (63.9)	
Low/undifferentiated	12	9 (75.0)	
TNM			0.095
I/II	19	10 (52.6)	
III/IV	29	22 (75.9)	

FOXK1, forkhead box K1; T, tumor; N, node; M, metastasis.

Transwell invasion assay. Transwell chambers (8 μm pore size; Corning, Inc.) were used to assess the effect of FOXK1 knockdown on cell invasion. After stable transfection with shFOXK1 or shNC, 10,000 cells were resuspended in serum-free DMEM and placed in the upper well of the chamber coated with 25 μg Matrigel (BD Biosciences) for the invasion assay. The lower well was filled with DMEM containing 10% FBS. The chambers were maintained at 37˚C for 24 h, and then removed. The cells on the upper surface of the chambers were removed using a cotton swab, while the cells on the lower surface were stained with 0.1% crystal violet for 30 min at room temperature and counted in five representative (magnification, x200) fields per insert under an Inversion Microscope (Zeiss AG) by two individuals (YJF and ZGB), who were blinded to the study.

Wound healing assay. The indicated cells were cultured in 6-well plates in monolayers and were pre-incubated with Mitomycin-C (10 μg/ml) for 1 h at 37˚C to suppress cell proliferation. Then, cells were plated in serum-starved medium. A sterile 200-μl pipette tip was used to create wounds and the areas of the wound fields were observed and images (magnification, x200) were taken at 0, 24, 48 and 72 h following wound creation using an inverted microscope (Carl Zeiss AG).

Western blotting. Total protein was extracted from the cells. Western blotting was performed as previously described (14).

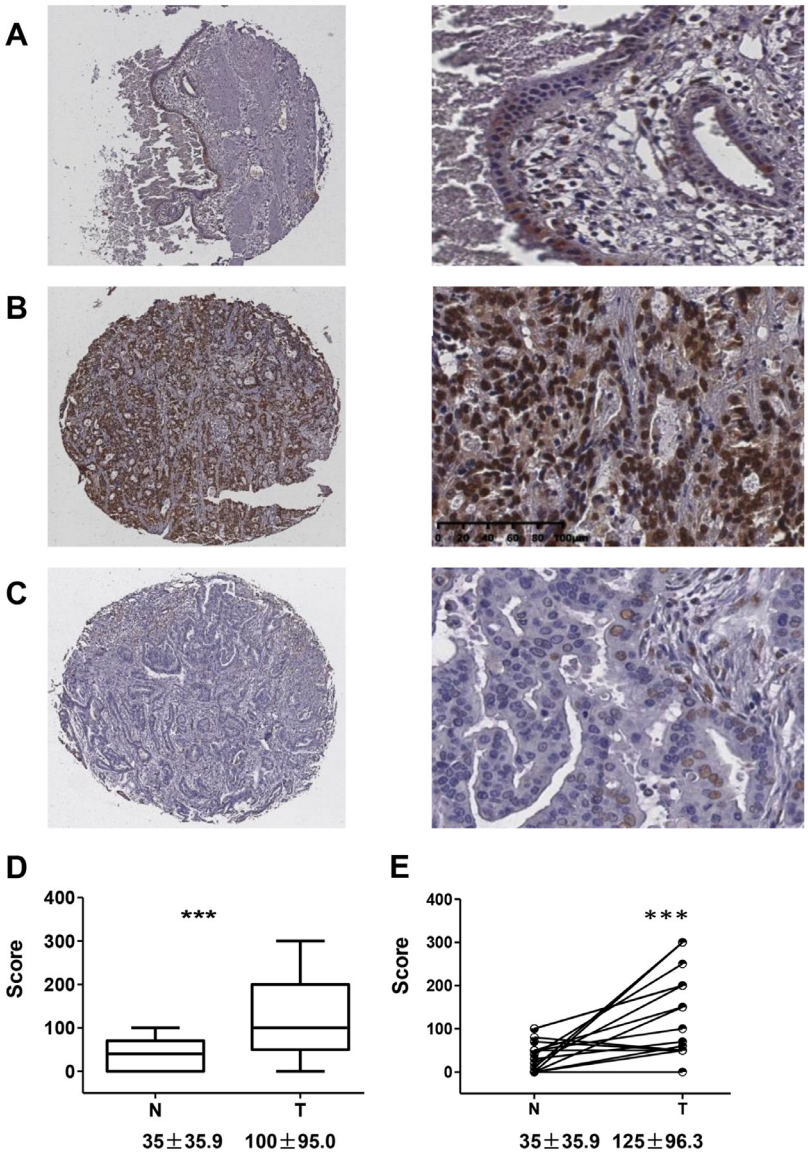


Figure 1. FOXK1 expression in tumor tissues and non-cancerous bile duct epithelium. (A) Low expression of FOXK1 in non-cancerous bile duct tissues; (B) high expression of FOXK1 in representative tumor tissues; (C) low expression of FOXK1 in representative tumor tissues. Left panel magnification, x40; right panel magnification, x200. (D) The positive average score of all cases of FOXK1 staining in the N group was lower than that of the T group. (E) For the 15 matched cases, the positive average score of FOXK1 staining in N was also lower than T. ***P<0.001 vs. T group. FOXK1, forkhead box K1; N, non-cancerous tissues; T, tumor samples.

All antibodies used are as follows: Anti-FOXK1 (1:1,000; cat. no. ab18196; Abcam), anti-matrix metallopeptidase (MMP)-9 (1:500; cat. no. ab228402; Abcam), anti-Twist (1:1,000; cat. no. ab50581; Abcam), anti-E-cadherin (1:1,000; cat. no. 3195; Cell Signaling Technology, Inc.), anti-vimentin (1:1,000; cat. no. 5741; Cell Signaling Technology, Inc.), anti-glutathione S-transferase (GST)-π (1:500; cat. no. 66001-2-Ig; ProteinTech Group, Inc.), anti-MDR-1 (1:200; cat. no. sc13131; anti-Santa Cruz Biotechnology, Inc.), anti-P-glycoprotein (P-gp; 1:500; cat. no. ab170904; Abcam), and anti-GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.). The signals were detected using horseradish peroxidase-based chemiluminescence analysis.

Statistical analyses. Statistical analyses were carried out using SPSS statistical software (version 16.0; SPSS, Inc.) and Prism software (version 5.0; GraphPad Software, Inc.). Data are presented as the mean ± standard error of the mean. The differences between two groups were evaluated with a Student's t-test. Categorical data were analyzed using χ^2 tests. The Kaplan-Meier log-rank method was used to estimate survival rates, and the Cox proportional hazards model for multivariate survival analysis was used to assess predictors related to recurrence or survival. P<0.05 was considered to indicate a statistically significant difference.

Results

High expression of FOXK1 in patients with HC. Immunostaining revealed that FOXK1 was primarily localized to the nucleus of the normal bile duct epithelium and cancer cells (Fig. 1). The epithelium in the adjacent non-cancerous tissues showed low expression of FOXK1, with an average score of 35±35.9 (Fig. 1A and D). Furthermore, 32 cases (66.7%) showed high

Table II. Univariate and multivariate analysis of variables associated with disease-free survival in patients with hilar cholangiocarcinoma.

Variables	N	DFS, months	P-value		HR	95% CI
			Univariate analysis	Multivariate analysis		
Tumor invasion			0.006	0.065	0.146	0.019-1.125
T1-T3	6	51.2				
T4	42	16.0				
Regional LN positive			0.05	0.423	0.690	0.279-1.710
No	15	37.0				
Yes	33	15.0				
FOXK1			<0.001	0.014	0.253	0.084-0.759
Low	16	42				
High	32	11				

HR, hazard ratio; CI, confidence interval; FOXK1, forkhead box K1; LN, lymph node.

Table III. Univariate and multivariate analysis of variables associated with OS in patients with hilar cholangiocarcinoma.

Variables	N	DFS, months	P-value		HR	95% CI
			Univariate analysis	Multivariate analysis		
Tumor invasion			0.014	0.114	0.297	0.066-1.340
T1-T3	6	50				
T4	42	16				
Regional LN positive			0.033	0.390	0.678	0.279-1.646
No	15	42				
Yes	33	14				
FOXK1			<0.001	0.037	0.357	0.136-0.940
Low	16	50				
High	32	14				

HR, hazard ratio; CI, confidence interval; FOXK1, forkhead box K1; OS, overall survival; LN, lymph node.

expression (Fig. 1B) and the other 16 cases presented low expression (Fig. 1C). The average score for FOXK1 in HC was 100±95.0, which was significantly higher than that in the non-cancerous bile duct epithelium (P=0.0003). Similarly, in the matched cases with both HC and adjacent non-cancerous epithelium, the score was 125±96.3 and 35±35.9, respectively (P=0.0006; Fig. 1E). In one intracholangial papillary tumor (Fig. 2), ~50% of the cells on the surface showed FOXK1 expression (Fig. 2A), whereas the tumor cells at the front edge of the invasive front showed 100% FOXK1 expression (Fig. 2B and C), indicating that FOXK1 might be involved in the progression of HC.

Association between high FOXK1 expression and clinicopathological variables of HC. Table I summarizes the association between high FOXK1 expression and clinicopathological variables of HC. A statistically significant association was observed between high FOXK1 expression and tumor invasion and regional LN metastasis. Furthermore, high FOXK1 expression occurred more frequently in highly invasive tumors (invasion level T4, 73.8%) than in less invasive tumors (levels T1-T3, 16.7%; P=0.005). With regard to the N stage, FOXK1 was highly expressed in HCs with regional LN metastasis (78.8%) compared with that in HCs without LN metastasis (40%) (P=0.008). This indicated that the expression of FOXK1 is associated with the prognosis of HC.

Relationship between high FOXK1 expression and HC tumor recurrence or outcome. For this assessment, the cohort consisted of 34 male (70.8%) and 14 female (29.2%) patients with a median age of 55 years (range, 31-79 years). The median disease-free survival (DFS) in patients with resected HC was 14 months. Patients with tumors exhibiting high FOXK1 expression had a significantly shorter DFS than that in patients with low FOXK1 expression (11 vs. 42 months; P<0.001; Fig. 3A). Additionally, it was noted that tumor invasion (P=0.006) and regional LN metastasis (P=0.05) significantly affected DFS based on the univariate analysis. In contrast to the univariate

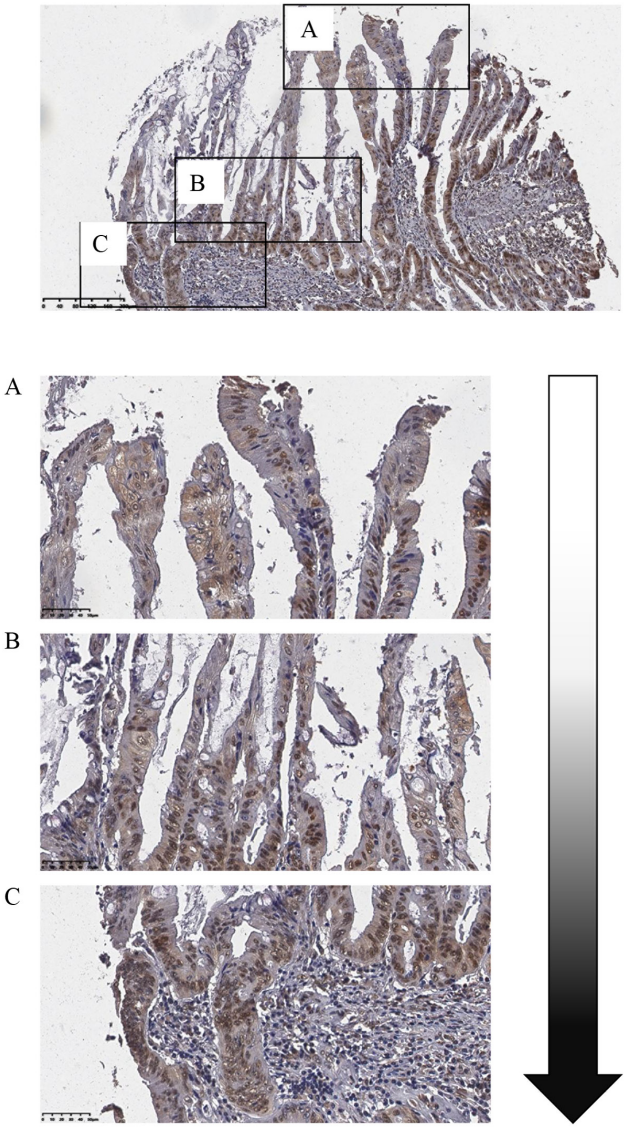


Figure 2. Heterogeneity of FOXK1 expression in one tumor. (A) Low expression of FOXK1 on the surface of the tumor. (B) High expression of FOXK1 at the bottom of the tumor. (C) High expression of FOXK1 in invasive tumor cells. Top panel magnification, x40; bottom panels magnification, x200. FOXK1, forkhead box K1.

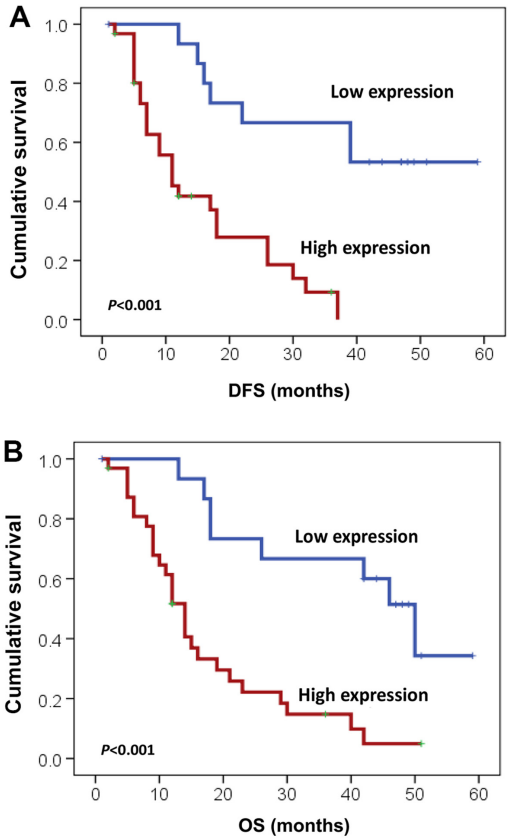


Figure 3. Kaplan-Meier curves of DFS and OS in patients with hilar cholangiocarcinoma. (A) DFS was significantly worse in patients with high FOXK1 expression in tumors than in those with low FOXK1 expression (11 vs. 42 months, P<0.001). (B) OS was significantly worse in patients with high FOXK1 expression compared with those with low FOXK1 expression (14 vs. 50 months, P<0.001). DFS, disease-free survival; OS, overall survival; FOXK1, forkhead box K1.

analysis, the multivariate analysis using the Cox proportional hazards model showed that high FOXK1 expression was an independent predictor of tumor recurrence (P=0.014; Table II).

With respect to OS, the median for patients with resected HC was 16 months. However, patients with high FOXK1 expression in tumors had a significantly worse OS than those with low FOXK1 expression (14 months vs. 50 months; P<0.001; Fig. 3B). Additionally, tumor invasion (P=0.014) and regional LN metastasis (P=0.033) also significantly influenced OS based on the univariate analysis (Table III). The multivariate analysis also showed that high FOXK1 expression was an independent prognostic factor (P=0.037; Table III).

FOXK1 knockdown attenuates cell invasion and migration in vitro. To provide direct evidence for the critical role of FOXK1 in HC, its expression was knocked down in HC cells *in vitro* using shRNA. HC cells exhibited high endogenous FOXK1 expression (Fig. 4A). Fig. 4B shows that the silencing of FOXK1 expression using shRNA was successful in RBE cells (Fig. 4B). Silencing endogenous FOXK1 did not affect cell proliferation (Fig. 4B), but it significantly inhibited cell invasion, as revealed by the Transwell assay (P=0.0013; Fig. 4C), and cell migration, as revealed by the wound healing assay (Fig. 4D). Western blotting revealed that FOXK1 knockdown resulted in the upregulation of E-cadherin and the downregulation of vimentin, MMP-9 and Twist (Fig. 4E).

FOXK1 knockdown reduces drug resistance in vitro. To explore chemoresistance in HC, FOXK1-knockdown RBE cells were treated with 5-FU and DDP. It was found that silencing endogenous FOXK1 expression in RBE cells increased their sensitivity to 5-FU and DDP (Fig. 5A). In addition, western blotting revealed that markers related to drug resistance, such as GST-π, MDR1 and P-gp, were downregulated in RBE cells *in vitro* when FOXK1 was suppressed (Fig. 5B).

Discussion

The present study determined the expression profile of FOXK1 in patients with HC and identified that it was considerably increased and significantly associated with tumor invasion and metastasis. However, it had no effect on the proliferation

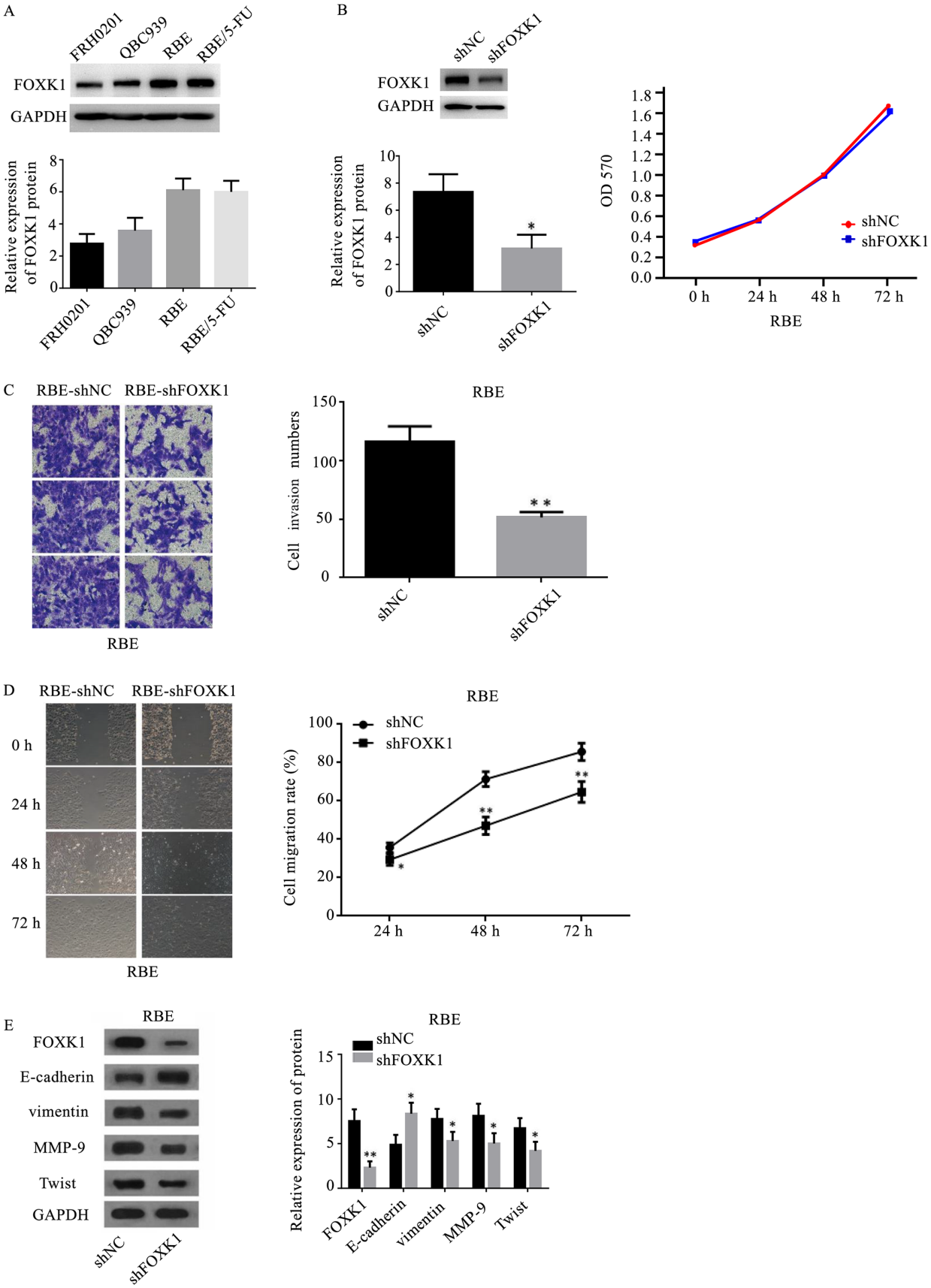
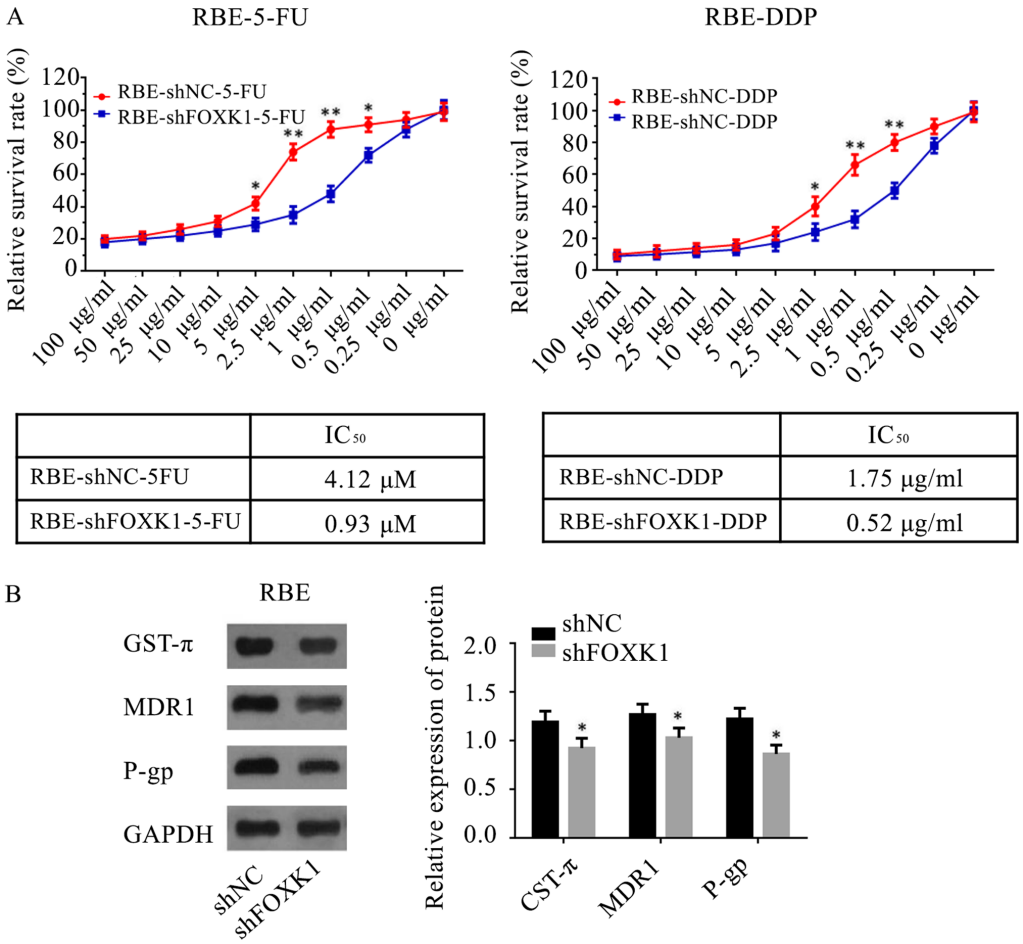


Figure 4. Inhibitory role of silencing FOXK1 on hilar cholangiocarcinoma cells. (A) Endogenous levels of FOXK1 in FRH0201, QBC939, RBE and RBE/5-FU cells. (B) RBE cells were infected with shFOXK1 or shNC and examined by western blotting. Cells infected with the shNC or shFOXK1 lentivirus were seeded into 96-well plates and cell growth was assessed by performing Cell Counting Kit-8 assays. (C) Transwell assay was performed to evaluate the effect of FOXK1 on cell invasion (magnification, x200) in RBE cells transfected with shFOXK1 compared with that in the sh-NC group. (D) Wound healing assay was used to evaluate the effect of FOXK1 on the migration (magnification, x200) of RBE cells transfected with shFOXK1 compared with the sh-NC group. (E) Levels of E-cadherin, vimentin, MMP-9 and Twist were measured by western blotting in FOXK1-silenced RBE cells. *P<0.05, **P<0.01 vs. shNC group. FOXK1, forkhead box K1; sh, short hairpin RNA; NC, negative control; MMP, metallopeptidase.



	IC_{50}
RBE-shNC-5FU	4.12 μM
RBE-shFOXK1-5-FU	0.93 μM

	IC_{50}
RBE-shNC-DDP	1.75 μg/ml
RBE-shFOXK1-DDP	0.52 μg/ml

Figure 5. Role of FOXK1 in chemotherapy resistance of hilar cholangiocarcinoma cells. (A) RBE cells transfected with shFOXK1 have a lower IC_{50} of 5-FU and DDP compared with the sh-NC group. (B) Levels of GST-π, MDR1 and P-gp were measured by western blotting in FOXK1-silenced RBE cells and the expression of GST-π, MDR1 and P-gp decreased in the shFOXK1 group compared with that in the sh-NC group. $^{*}P<0.05$, $^{**}P<0.01$ vs. shNC group. FOXK1, forkhead box K1; sh, short hairpin RNA; 5-FU, 5-fluorouracil; DDP, cisplatin; GST, glutathione S-transferase; MDR1, multidrug resistance mutation 1; P-gp, P-glycoprotein; NC, negative control.

of HC. The data of survival follow-up revealed that its high expression was an independent predictor of tumor recurrence and OS after HC resection. However, the migratory and invasive abilities of RBE cells were inhibited and the expression of several epithelial-mesenchymal transition (EMT)-associated proteins were influenced following knockdown of FOXK1, which provided a novel molecular basis for the key role of FOXK1 in HC development and progression.

FOXK1 plays an oncogenic role in a number of solid tumors, and it has been observed to be upregulated in hepatocellular carcinoma, and gastric, colorectal, prostate, esophageal and ovarian cancer (7-11,15-17). This high expression of FOXK1 is also found to correlate significantly with malignant behaviors, including poor differentiation of esophageal cancer (10), size and metastasis of ovarian cancer (9), and differentiation, LN metastasis and AJCC stage of colorectal cancer (18). Consistent with these studies, the present study confirmed that FOXK1 was highly expressed in HC tissues, using resected HC specimens and matched specimens. High expression was identified to be associated with tumor invasion and metastasis. All these data supported the role of FOXK1 as an oncogene and suggested that it might serve as a potential therapeutic target for patients with HC.

A number of clinicopathological variables have been identified as associated with tumor recurrence and OS for HC, including tumor invasion, nerve invasion, regional LN metastasis, curative resection and disease progression (19-21). In the small HC cohort of the present study, tumor invasion and LN metastasis significantly affected disease progression and OS. However, these factors did not independently predict tumor recurrence and patient outcome. Possible reasons for this discrepancy are the limited number of subjects in the cohort and the fact that this cohort involved a number of patients with HC with advanced-stage disease. A number of studies have introduced biological markers into the Cox regression model, including amino acid transporter A1, proline-rich protein 11, pyruvate kinase PKM and Annexin A1 (12,13,22-24). The survival analysis from the present study showed that high FOXK1 expression in tumors was associated with shorter PFS and worse outcome. In addition, FOXK1 was an independent predictor of tumor recurrence and OS in patients with HC. These findings suggested that this protein might be a potential biomarker to predict disease progression and outcome in patients with HC.

Locally advanced or metastatic HC at diagnosis makes patients ineligible for surgical resection and thus limits their

overall 5-year survival. Given the association between high FOXK1 expression and tumor invasion or LN metastasis, the present study aimed to detect the role of FOXK1 in cell migration by suppressing its expression and exploring a possible associated mechanism. The present study used shRNA interference transfection to construct an effective FOXK1-knockdown cell line. It was identified that silencing endogenous FOXK1 significantly inhibited HC cell migration and invasion, as revealed by the Transwell and wound healing assays. Overall, the data indicated that FOXK1 played a critical role in disease progression. Several previous studies have revealed that EMT confers properties that are critical for invasion and distant metastasis to neoplastic epithelial cells (25,26). It has been demonstrated that the knockdown of FOXK1 inhibits transforming growth factor β-induced EMT (27). Its overexpression induces this process by upregulating cysteine-rich angiogenic inducer 61 in colorectal cancer (7), whereas knockdown prevents an EMT phenotype through the upregulation of E-cadherin and downregulation of N-cadherin in prostate cancer cells (28). Therefore, the present study determined the expression of EMT-related proteins, such as E-cadherin, vimentin, MMP-9 and Twist, by western blotting in FOXK1-knockdown cell lines. The loss of E-cadherin and the upregulation of vimentin and Twist represent the EMT process, while MMP-9 is a key component that mediates cell adhesion. According to previous studies (7,25-28) and the results of the present study, FOXK1 could be a critical inducer of EMT and it is proposed that FOXK1 plays a critical oncogenic role by promoting the EMT process. Although further research is needed to explore the molecular mechanisms, the results of the present study provided a novel molecular basis for the key role of FOXK1 in HC development and progression.

In addition to the lack of effective biomarkers, chemoresistance is also an important factor in the high mortality of HC. Silencing FOXK1 increased sensitivity to 5-FU and DDP, and downregulated the expression of GST-π, MDR1 and P-gp, which are related to drug resistance, in RBE cells *in vitro*. Thus, FOXK1 may serve as a putative target for HC.

In summary, FOXK1 was highly expressed in HC and associated with tumor invasion and LN metastasis in Chinese patients with HC. Furthermore, high FOXK1 expression was an independent predictor of tumor recurrence and OS. Thus, these results indicated that FOXK1 can facilitate cancer metastasis by regulating EMT-associated proteins in HC and plays a role in chemoresistance.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZZ conceived and designed the study. YF and ZB acquired the data. YF, ZB and JS were responsible for data analysis and interpretation. YF and ZZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The tissue sample experiments were approved by the Ethics Committee of The Affiliated Hospital of Qingdao University. Informed consent written from all participants (or their parent or legal guardian in the case of children under 16) was obtained to participate in the study or to use their tissues.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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