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Fibroblast growth factor receptor 4 promotes progression and correlates to poor prognosis in cholangiocarcinoma

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ABSTRACT

Fibroblast growth factor receptor 4 (FGFR4) is related to poor prognosis of several cancers, but the correlation between FGFR4 expression and cholangiocarcinoma (CCA) has not been well elucidated. We investigated the expression of FGFR4 in 83 intrahepatic cholangiocarcinomas (IHCCs), 75 perihilar cholangiocarcinomas (PHCCs) and 41 distal cholangiocarcinomas (DCCs) by immunohistochemistry (IHC), and subsequently evaluated association of FGFR4 with clinicopathologic parameters and survival rate. The rate of FGFR4 higher expression was 61.4% (51/83) in IHCCs, 53.3% (40/75) in PHCCs and 56.1% (23/41) in DCCs. FGFR4 expression was significantly related to poor prognosis of IHCC ($P = 0.002$) and PHCC ($P = 0.019$) with univariate analysis, and also identified as an independent prognostic factor in IHCC ($P = 0.045$) and PHCC ($P = 0.049$) with multivariate analysis. Additionally, with functional assays in vitro, we found FGFR4 can induce proliferation, invasion and epithelial–mesenchymal transition (EMT) of CCA cell lines with FGF19 stimulation. Moreover, FGFR4 inhibitor AP24354 can suppress proliferation, invasion and induce apoptosis of CCA cells. In conclusion, FGFR4 expression can be identified as a significant independent prognostic biomarker of IHCC and PHCC. FGFR4 played a pivotal role in proliferation, invasion and EMT of CCA. FGFR4 inhibitor can suppress proliferation, invasion and induce apoptosis of CCA, indicating that FGFR4 may act as a potential therapeutic target.

1. Introduction

Cholangiocarcinoma is a relatively rare tumor but the most common malignancy of the biliary tract [1]. The incidence and mortality of CCAs are increasing worldwide [2]. According to the 7th edition of AJCC/UICC TNM staging classification [3], CCAs are classified to intrahepatic, perihilar and distal cholangiocarcinomas, which are identified to have different biological and neoplastic features [4]. PHCC is the most common type of cholangiocarcinoma, followed by the DCC and then IHCC [5]. Incidence of CCAs is low with approximately 9760 new cases diagnosed annually in the United States [6]. However, the mortality of CCAs is very high. Moreover, the radical resection and liver transplantation are the only known curative ways but more than 65% patients lose surgical indications when diagnosed because of early lymph node and distant metastases. In addition, the recurrence of CCAs is high and few data support that adjuvant approach can benefit survival outcomes [7,8]. Consequently, the prognosis of cholangiocarcinoma is very poor, with 5-year survival rate ranging from 5% to 15% [9,10]. In summary, CCAs are difficult to be treated successfully and have a poor prognosis as a result of difficulty of early diagnosis, early metastasis and easy recurrence [11]. Hence, there is an urgent need for the predictive, prognostic and therapeutic markers. Unfortunately, few studies on CCAs aimed at identifying prognostic factors have been reported and the researches on new biomarkers of CCAs make progress slowly because that the majority of patients present are usually at an unresectable stage which makes it difficult to obtain samples and perform a large randomized trial.

Abbreviations: FGFR4, fibroblast growth factor receptor 4; IHCC, intrahepatic cholangicolangcinoma; PHCC, perihilar cholangiocarcinoma; EMT, epithelial–mesenchymal transition; CCA, cholangiocarcinoma.

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Fibroblast growth factors (FGFs) are heparin-binding proteins involved in many cellular processes like proliferation, differentiation and angiogenesis. They have a large family consisting of 18 members in human, which are essential for signal transduction through interacting with cell-surface-associated heparan sulfate proteoglycans [12]. FGF signaling is induced by FGFs and transduced through FGF receptors. Fibroblast growth factor receptors (FGFRs) comprise of five members, including four tyrosine kinase receptors (FGFR1, FGFR2, FGFR3 and FGFR4) and a non-tyrosine kinase receptor (FGFR5, also known as FGFRL1) [13]. FGFRs overexpression has been reported to correlate with the progression and poor prognosis in a wide variety of tumors including prostate, breast, bladder, and gastric cancer [14]. Moreover, different types of FGFRs are overexpressed depending on the tumor types [15]. Among all the FGFRs, FGFR4 upregulation was observed in hepatocellular carcinoma, prostate, breast, pancreatic, gynecologic gastric cancers and rhabdomyosarcomas [16–18]. FGFR4 is highly expressed in hepatocytes and can regulate metabolism of bile acid and glucose [19]. In addition, FGF19, the specific FGFR4 ligand, is demonstrated to induce hepatocytes proliferation and hepatocellular carcinoma progression via stimulating FGFR4 [20,21].

To identify the role of FGFR4 in CCAs, we detected the expression of FGFR4 and VEGF in 83 samples of IHCC, 75 samples of PHCC and 41 samples of DCC by immunohistochemistry, subsequently evaluated the association between FGFR4 expression and clinicopathologic parameters with Chi-Square test. Furthermore, we investigated the correlation between FGFR4 and survival rate with univariate and multivariate analysis. To confirm the observation acquired from clinical investigation, FGFR4 function assays in CCA cells were evaluated subsequently, including proliferation, invasion and EMT. Moreover, significance of FGFR4 inhibitor AP24354 on proliferation, invasion and apoptosis of CCA cells were also detected. As a result, we inferred that FGFR4 expression correlated to poor prognosis through inducing cancer progression and could be a potential therapeutic drug target in CCAs.

2. Materials and methods

2.1. Patients and follow-up

All the 83 patients with IHCC, 75 patients with PHCC and 41 patients with DCC underwent tumor resection during the period from 2002 to 2010. The diagnosis was confirmed by the routine pathology. All samples were obtained from the Department of Pathology of Qilu Hospital and Central Hospital of Yishui, Shandong Province, with prior patient consents and the approval of the Institutional Clinical Ethics Review Board. Clinical data, including age, gender and other clinicopahtologic features were abstracted from the patients' medical records. Pathologic tumor-node-metastasis (pTNM) staging is based on the 7th staging classification of AJCC/UICC (2009).

In our study, 252 patients were diagnosed to have cholangiocarcinoma by routine pathology and 199 patients (83 patients with IHCC, 75 patients with PHCC and 41 patients with DCC) were enrolled in validation cohort following the criteria: (i) available formalin-fixed tumor tissues, (ii) available clinical follow-up data and complete medical records and (iii) no history of previous anticancer therapy and other malignancies. The information of primary and validation cohorts is provided in Supplemental Table S1.

2.2. Tissue microarrays

The tissue microarrays (TMA) were made using buffered formalin-fixed and paraffin-embedded tissue sections from all the 199 patients (83 IHCCs, 75 PHCCs and 41 DCCs). Before immunohistochemistry detection, hematoxylin and eosin staining were performed to confirm the histological characterization of all samples. Two 1 mm cylinders were used for representing each sample in the TMA slide (164 cores per slide) [22].

2.3. Immunohistochemistry and evaluation

The protocol of IHC staining was described before [23]. Detail for IHC can be seen in Supplemental Materials and methods. Immunohistochemical staining was evaluated independently by two senior pathologists unaware of the clinical information. Both the intensity of the color reaction and the percentage of stained cells were considered when evaluated. Positive (normal bile duct control) and negative controls (absence of primary or secondary antibody) were applied for quality control. Both FGFR4 and VEGF staining intensity were scored as negative (0), weak (1), moderate (2) and strong (3). Criteria percentage of FGFR4 positive tumor cells were defined as follows: (1) <10% of cells were positive, (2) 10–50% of cells were positive and (3) >50% of cells were positive. Both scores were multiplied and the mean resulting score was used to define the lower and higher expressions. The cut-off scores of FGFR4 in IHCCs, PHCCs and DCCs were 3.69, 3.71 and 3.34 respectively. Cut-off of VEGF in IHCCs, PHCCs and DCCs were 5.85, 5.51 and 5.49 respectively.

2.4. Cell culture and reagents

The IHCC cell line RBE, PHCC cell line QBC939 and FRH0201, HepG2 and HEK-293 cells were all purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). IHCC cell line HUCCT-1 was obtained from RIKEN Bioresourse Center (Koyadai, Japan). All CCA cells were cultured in the RPMI-1640 medium, while HepG2 and HEK-293 were cultured in DMEM medium, supplemented with 10% fetal bovine serum (Gibco, USA) and 1% ampicillin/streptomycin (HyClone, USA) in 5% CO_2 resuscitation. FGF19 was purchased from PeproTech Company. Inhibitor AP24534 was purchased from Selleck Chemicals. All other reagents were purchased from Sigma Company.

The following antibodies were used: anti-FGFR4, anti-VEGF, and anti-actin (Santa Cruz Biotechnology, USA), EMT antibody sampler kit (Cell Signaling Technology, USA) and anti-FLAG (Sigma, USA).

2.5. FGFR4 knockdown and transfection

Human FGFR4 (transcript variant 1) open-reading-frame plasmid was purchased from Sino Biological Incorporation (Beijing, China), then subcloned into the pFLAG-CMV-2 vector by double-enzyme digestion. FGFR4 knockdown is accomplished by small interfering RNA (siRNA) purchased from the Invitrogen Company. The siRNA sequences were designed as reported by Ye et al previously [21], with sense sequence 5′-GCCGACACAAGAACAUCAUTT-3′, and antisense sequence 5′-AUGAUGUUCUUGUGUCGGCTT-3′. As to the scramble oligo RNA, the sense sequence was 5′-UUCUCCGAACGUGUCACGUTT-3′, and the anti-sense sequence was 5′-ACGU GACACGUUCGGAGAATT-3′. Transfection of siRNA or vector was accomplished by Lipofectamine 2000 (Invitrogen, USA) according to the reverse transfection manual. Results of knockdown and overexpression were detected by Western blotting 48 h after transfection.

2.6. Matrigel invasion assay

Detailed in Supplementary material.

2.7. Cell proliferation assay

Detailed in Supplementary material.

2.8. Western blotting and analysis

Detailed in Supplementary material.

2.9. Apoptosis assay

Detailed in Supplementary material.

2.10. Statistical analysis

All the statistical analyses were performed by SPSS 17.0 software. The correlation between FGFR4 and clinicopathologic features was assessed by χ^2 test. The survival curve was evaluated in the Kaplan–Meier method, and the survival curve difference between high and low expression was calculated in a log-rank test. The independent prognostic factors were analyzed in Cox proportional hazards regression model. *P*-values <0.05 was considered to be significant. The statistical comparisons between control and test group were made with the student-*t* tests.

3. Results

3.1. Expression of FGFR4 and VEGF in cholangiocarcinoma

Characteristics of patients were displayed in Table 1. In all the 199 patients, FGFR4 expression was observed mainly in cytoplasm, or both cytoplasm and membrane (Fig. 1A and B). According to the criteria described previously, FGFR4 expression was divided into the higher and lower expression with average score as the cutoff. The higher FGFR expression percent was 61.4% (51/83) in IHCC, 53.3% (40/75) in PHCC, and 56.1% (23/41) in DCC (Table 1).

To examine the correlations of FGFR4 with recognized prognostic factor, VEGF expression was also detected. Consistent with previous study, VEGF was only observed in cytoplasm. The higher rate of VEGF in IHCC, PHCC and DCC was 54.2% (45/83), 60.0% (45/75) and 53.7% (22/41) respectively.

Table 1
Characteristics of patients with cholangiocarcinoma.

Factor	Category	IHCC			PHCC			DCC	
		n	%		*n*	%		*n*	%
Age (years)	<65	62	74.7		47	62.7		31	75.6
	⩾65	21	25.3		28	37.3		10	24.4
Gender	Male	44	53.0		55	73.3		26	63.4
	Female	39	47.0		20	26.7		15	36.6
Tumor size (cm)	<5	37	31.3	<3	41	54.7	<3	30	73.2
	⩾5	46	68.7	⩾3	34	45.3	⩾3	11	26.8
Differentiation	Well	19	22.9		34	45.3		22	53.7
	Moderately	39	47.0		29	38.7		12	29.3
	Poorly	25	30.1		12	16.0		7	17.1
T stage	T1	40	48.2		11	14.7		9	22.0
	T2	20	24.1		21	28.0		10	24.4
	T3	23	27.7		20	26.7		21	51.2
	T4	0	0.0		23	30.6		1	2.4
N stage	N0	57	68.7	N0	60	80.0		28	68.3
	N1	26	31.3	N1	15	20.0		13	31.7
			0.0	N2	0	0.0			0.0
M stage	M0	79	95.2		69	92.0		41	100.0
	M1	4	4.8		6	8.0		0	0.0
TNM stage	I	31	37.3	I	11	14.7	I A	7	17.1
	II	10	12.0	II	13	17.3	I B	8	19.5
	III	16	19.3	III A	16	21.3	II A	11	26.8
	IVA	22	26.6	III B	6	8.0	II B	14	34.1
	IVB	4	4.8	IVA	23	30.7	III	1	2.4
			0.0	IVB	6	8.0	IV	0	0.0
Satellites	N	63	75.9		65	86.7		41	100.0
	P	20	24.1		10	13.3		0	0.0
Macrovascular invasion	N	80	96.4		65	86.7		40	95.1
	P	3	3.6		10	13.3		1	4.9
Microvascular invasion	N	67	80.7		67	89.3		37	90.2
	P	16	19.3		8	10.7		4	9.8
HBV	N	73	88.0		75	100.0		41	100.0
	P	10	12.0		0	0.0		0	0.0
Cirrhosis	N	67	80.7		75	100.0		41	100.0
	P	16	19.3		0	0.0		0	0.0
FGFR4	Low	32	38.6		35	46.7		18	43.9
	High	51	61.4		40	53.3		23	56.1
VEGF	Low	38	45.8		30	40.0		19	46.3
	High	45	54.2		45	60.0		22	53.7

Abbreviations: IHCC, intrahepatic cholangiocarcinoma; PHCC, perihilar choangiocarcinoma; DCC, distal cholangiocarcinoma; FGFR4, fibroblast growth factor receptor 4; VEGF, vascular endothelial growth factor; N, negative; P, positive.

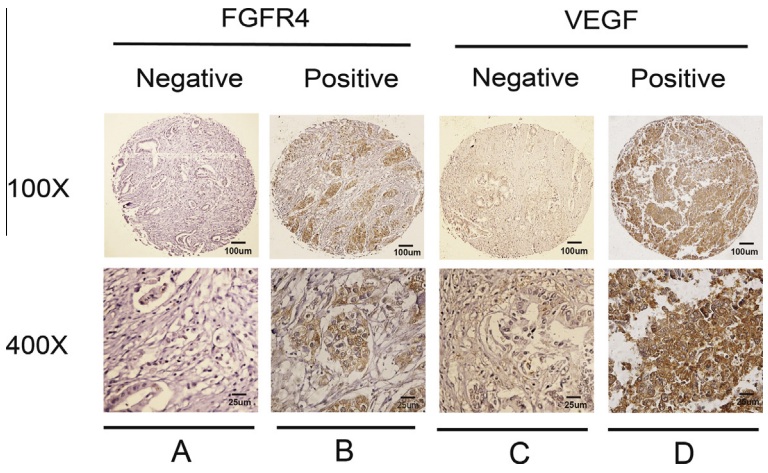

Fig. 1. Representative immunohistochemical staining of FGFR4 and VEGF. (A) FGFR4 negative. (B) FGFR4 positive. (C) VEGF negative. (D) VEGF positive. The bar represents 100 μm and 25 μm respectively.

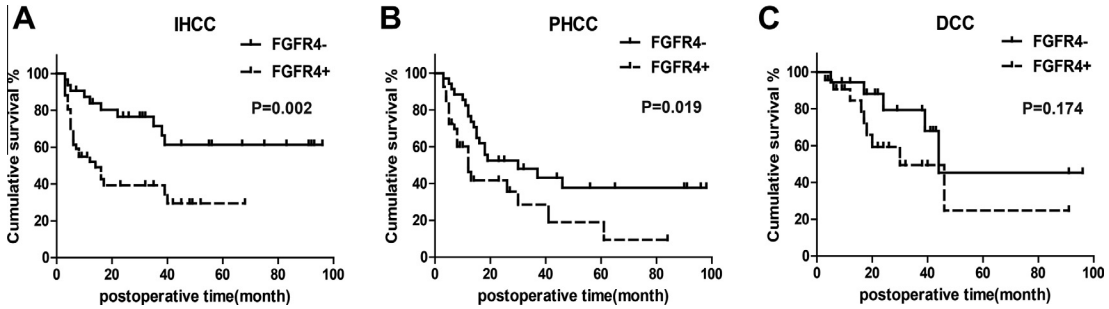

Fig. 2. Survival curves stratified by FGFR4 expression in (A) IHCC, (B) PHCC and (C) DCC (Kaplan–Meier method). Patients with higher FGFR4 expression had a significantly poorer overall survival rate than those with negative FGFR4 expression in IHCC (29.5% vs. 61.3%, $P = 0.002$), PHCC (19.1% vs. 37.8%, $P = 0.019$).

Table 2
Multivariate analysis of clinicopathologic features.

Category	IHCC			PHCC			DCC		
	HR	95%CI	P^*	HR	95%CI	P^*	HR	95%CI	P^*
Differentiation									
Well	1			1			1		
Moderately	0.69	0.24–2.00	0.507	1.03	0.48–2.24	0.938	1.04	0.28–3.86	0.954
Poorly	1.33	0.49–3.61	0.577	3.36	1.27–8.86	0.014	2.53	0.33–19.55	0.374
T stage									
T1 + T2	1			1			1		
T3 + T4	1.64	0.79–3.39	0.182	1.85	0.88–3.89	0.108	1.07	0.29–3.95	0.92
N stage									
N0	1			1			1		
N1(+N2)	2.53	0.99–6.50	0.053	1.57	0.63–3.92	0.336	0.29	0.06–1.50	0.286
M stage									
M0	1			1			–		
M1	1.8	0.54–5.97	0.335	2.94	0.93–9.29	0.066	–	–	–
Satellites									
Negative	1			1			–		–
Positive	1.01	0.43–2.36	0.987	0.87	0.26–2.95	0.820	–	–	
Macrovasular invasion									
Negative	1			1			1		
Positive	4.05	0.82–20.00	0.086	1.38	0.50–3.81	0.540	2.1	0.15–30.0	0.058
Microvascualr invasion									
Negative	1			1			1		
Positive	1.52	0.62–3.72	0.362	3.13	1.14–8.61	0.027	0.94	0.10–8.63	0.096
FGFR4									
Low	1			1			1		
High	2.51	1.02–6.16	0.045	2.09	1.00–4.33	0.049	2.9	0.86–9.773	0.085

Abbreviations: HR, hazard ratio; CI, confidence interval. FGFR4, fibroblast growth factor receptor 4; IHCC, intrahepatic cholangiocarcinoma; PHCC, perihilar choangiocarcinoma; DCC, distal cholangiocarcinoma.
* Cox proportional hazards regression.

3.2. Associations between FGFR4 expression and clinicopathologic parameters

The relationships between FGFR4 expression and clinicopathologic factors were analyzed with Chi-Square test and shown in Supplemental Table S2. In IHCC, FGFR4 expression had a significant relationship with N stage ($P = 0.045$), indicating FGFR4 may involve in CCA lymphatic invasion. In PHCC, FGFR4 was defined as a close relevant factor with T stage ($P = 0.036$) and TNM stage ($P = 0.018$), suggesting FGFR4 could promote tumor growth and infiltration in PHCC. No significant correlation with clinicopathologic factors was observed in DCC. As a known biomarker in CCA, the association between VEGF and FGFR4 was also analyzed. VEGF had a significant relationship to FGFR4 only in IHCC ($P = 0.049$), while VEGF expression had a tendency to correlate with FGFR4 expression but not to a significant degree in PHCC and DCC ($P = 0.058$ and 0.092).

3.3. Univariate and multivariate analysis

Kaplan–Meier survival curves were made to analyze overall 5-year cumulative survival rate differences. The univariate analysis indicated higher FGFR4 group had a significant poorer prognosis than lower FGFR4 in IHCC ($P = 0.002$, Fig. 2A) and PHCC ($P = 0.019$, Fig. 2B), whereas the tendency was not significant in DCC ($P = 0.174$, Fig. 2C). The correlations between other clinicopathologic parameters and survival rate were also analyzed (Supplemental Table S3). In IHCC, T stage, N stage, M stage and VEGF expression were closely related to poor prognosis ($P = 0.002$, 0.004, 0.004 and 0.029 respectively). In PHCC, the prognostic factors were differentiation ($P = 0.002$), N stage ($P = 0.012$) and expression ($P = 0.005$), while in DCC, no significant prognostic factors were observed.

Multivariate analysis was performed to further identify the independent prognostic factors. Tumor differentiation, T stage, N stage, M stage, macrovascular and microvascular invasion and FGFR4 expression were enrolled in a Cox regression model. FGFR4 was proved to be an independent prognostic factor in IHCC ($P = 0.045$, HR = 2.51, CI = 1.02–6.16) and PHCC ($P = 0.049$, HR = 2.09, CI = 1.00–4.33) (Table 2). Moreover, only poor differentiation ($P = 0.014$, HR = 3.36, CI = 1.27–8.86) and microvascular invasion ($P = 0.027$, HR = 3.13, CI = 1.14–8.61) in PHCC was defined as independent prognostic factors in addition to FGFR4 expression among all the other evaluated factors.

3.4. FGFR4 expression in CCA cell lines

FGFR4 expressions were detected by Western blotting in cell lines originated from hepatocellular carcinoma (HepG2), IHCC (RBE and HUCCT-1) and PHCC (QBC939 and FRH0201). The

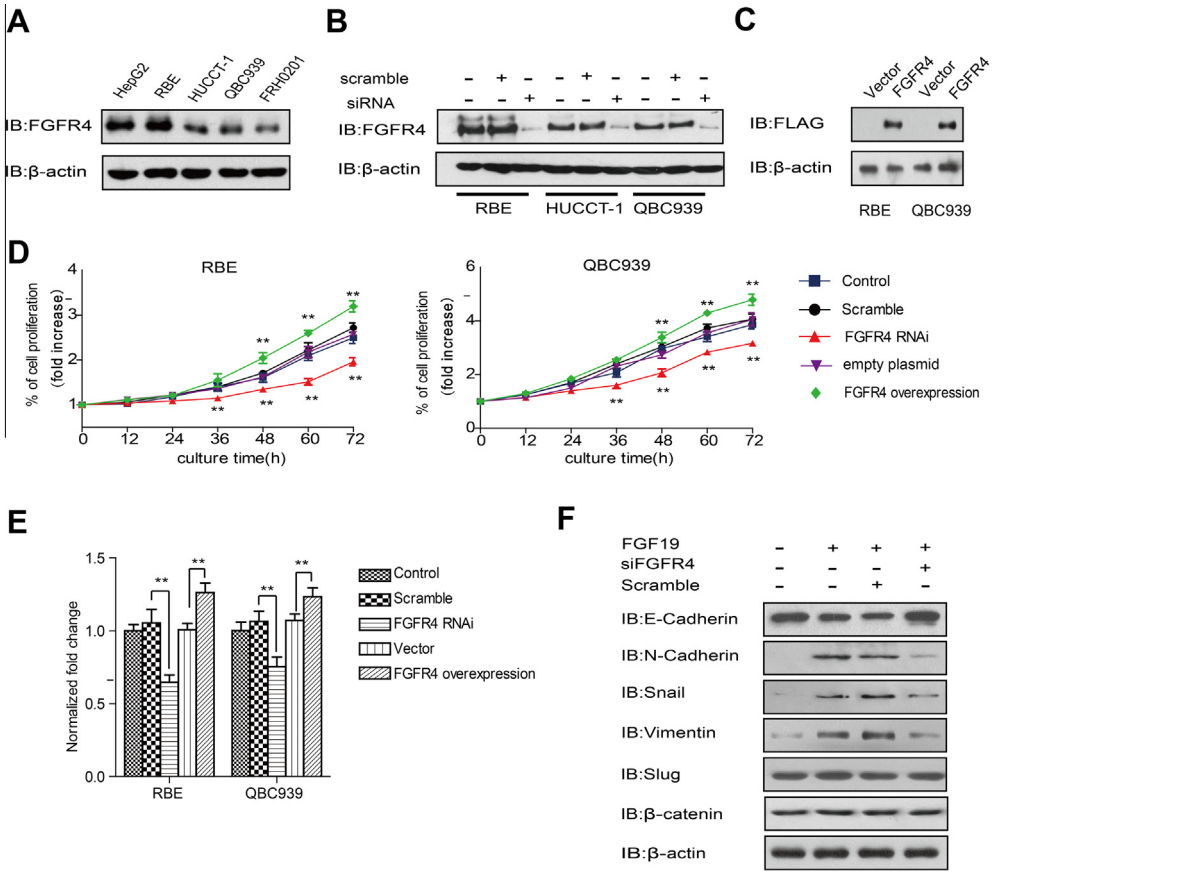

Fig. 3. FGFR4 promotes CCA cells proliferation, invasion and EMT. (A) FGFR4 expression in HepG2, RBE, HUCCT-1, QBC939 and FRH0201. (B) Validation of successful FGFR4 knockdown. Cells were transfected with FGFR4 siRNA or scramble RNA, cultured for 48 h and subsequently detected by Western blotting with primary anti-FGFR4 antibody. (C) Overexpression of FGFR4 was confirmed by Western blotting. RBE and QBC939 cells were transfected with empty pFLAG-CMV or FLAG-FGFR4 plasmid, cultured for 48 h and subsequently lysed for Western blotting. The FLAG-tag can be detected 48 h after transfection. (D) FGFR4 is required for FGF19-induced proliferation in RBE and QBC939. After transfection with FGFR4 siRNA, scramble siRNA, empty vector or pFLAG-FGFR4, cells were starved in serum-free medium and stimulated with 100 ng/ml FGF19 for 0–72 h. Cell proliferation was evaluated by MTT assay. OD490 of 0 h after FGF19 stimulation was set as a base line. Data are representative of three different experiments and analyzed by *t*-test. ** Means $P < 0.01$. (E) FGFR4 can promote invasion in RBE and QBC939. Invasive ability was detected by matrigel transwell assay. Cells were starved for 6h and stimulated with 100 ng/ml FGF19 for 12 h, then fixed and stained by gentian violet. Cell numbers were counted from ten random visions and normalized to the baseline, which was set as the number of control group without FGF19 stimulation. Data are analyzed by student *t*-test and presented by means of three different experiments ± SEM. ** Means $P < 0.01$. (F) FGFR4 knockdown decreases FGF19-induced EMT. RBE cells were transfected with FGFR4 siRNA or scramble siRNA, then starved in serum free medium for 12 h and incubated in 100 ng/ml FGF19 for 48 h. Whole cell lysates were detected by Western blotting with EMT antibody sampler kit.

expression of FGFR4 existed in all cell lines but differed. HepG2 and RBE had a higher expression than other cell lines (Fig. 3A). To evaluate the results of RNA knockdown or overexpression, cells were transfected with FGFR4 siRNA, scramble siRNA, empty pFLAG-CMV2 or FLAG-FGFR4 plasmid 48 h before detection by anti-FGFR4 or anti-FLAG antibody. Validation of successful FGFR4 knockdown and overexpression was displayed in Fig. 3B and C respectively.

3.5. FGFR4 promotes proliferation, invasion and EMT of CCA cells

After regulating FGFR4 expression by knockdown or overexpression, effect of FGFR4 on tumor progression was examined with activation of FGF19, which is a known specific ligand to FGFR4. From Fig. 3D, we demonstrated that FGFR4 knockdown could significantly decrease proliferation after 36 h of FGF19 stimulation, whereas FGFR4 overexpression obviously accelerated proliferation in RBE and QBC939 cells. The invasive ability of CCA cells was also detected because of the clinical observation that FGFR4 was significantly related to lymph invasion in IHCC. The invasive ability increased notably when FGFR4 was overexpressed and decreased significantly when FGFR4 was knocked down (Fig. 3E), suggesting that FGFR4 could induce invasion and may explain why patients with higher FGFR4 expression usually had more severe lymph and distant metastasis.

EMT is a crucial process in cancer progression that promotes cancer cells to migrate, escape from the primary focus and invade stromal tissues. To detect the importance of FGFR4 on EMT in CCA, we knocked down FGFR4, stimulated with 100 ng/ml FGF19 and detected proteins involved in EMT signaling pathway in RBE cells. Notably, E-cadherin expression had a lower expression, whereas N-cadherin, Snail and Vimentin had higher expressions under FGF19 stimulation, indicating that FGF19 can trigger the EMT process in CCA cells. Moreover, this tendency fade away when FGFR4 was knocked down (Fig. 3F), suggesting FGFR4 was required in this FGF19-induced EMT process.

3.6. FGFR4 inhibitor AP24534 can reduce proliferation, invasion and induce apoptosis

AP24534 (Ponatinib) was reported to be the most potent FGFR4 inhibitor [24], though it has a relatively broad inhibitory effect to other receptors [25]. To investigate the probability of AP24354 as a potential CCA drug, we first detected the AP24534 IC50 of proliferation in FGFR4-expressing HEK-293 cells. HEK-293 cells were transfected with FLAG-FGFR4 plasmid and then cultured in different dose of AP24354 from 0.01 μM to 2 μM. The proliferative ability was finally detected by MTT assay. AP24354 was proved to have significant inhibitory effect on proliferation of FGFR4-293 cells with IC50 at 0.2 μM (Fig. 4A). To investigate AP24534 influence on CCA, proliferative and invasive ability of RBE was evaluated with 100 ng/ml FGF19 or 0.2 μM AP24534 stimulation. AP24534 can significantly inhibit the FGF19-induced RBE proliferation (Fig. 4B) and invasion (Fig. 4C). To evaluate the value of FGFR4 as therapeutic target, apoptosis of RBE was detected after 48 h of AP24534 stimulation at 0 μM, 0.2 μM, 1 μM or 5 μM (Fig. 4D and E). AP24534 with concentration above 1.0 μM can markedly induce apoptosis of RBE, suggesting that it could be a potential chemical drug for CCA therapy.

4. Discussion

FGFR4 is distinctive among the other members of the FGFR family in genomic structure, ligand binding, and signal transductionl. One fascinating character of FGFR4 is that FGFR4 plays an important role in bile acid metabolism. Bile acids can activate FGF19/FGFR4 signaling pathway, which can inhibit bile acid synthesis as a feedback [26]. Biliary obstruction and cholestasis has been considered as risky factors of CCAs for many years and the reason remains controversy until now. Moreover, FGF19 was demonstrated to prove prostate cancer progression as endocrine hormone [27]. According to our study of FGFR4 role in CCAs, we boldly suspected

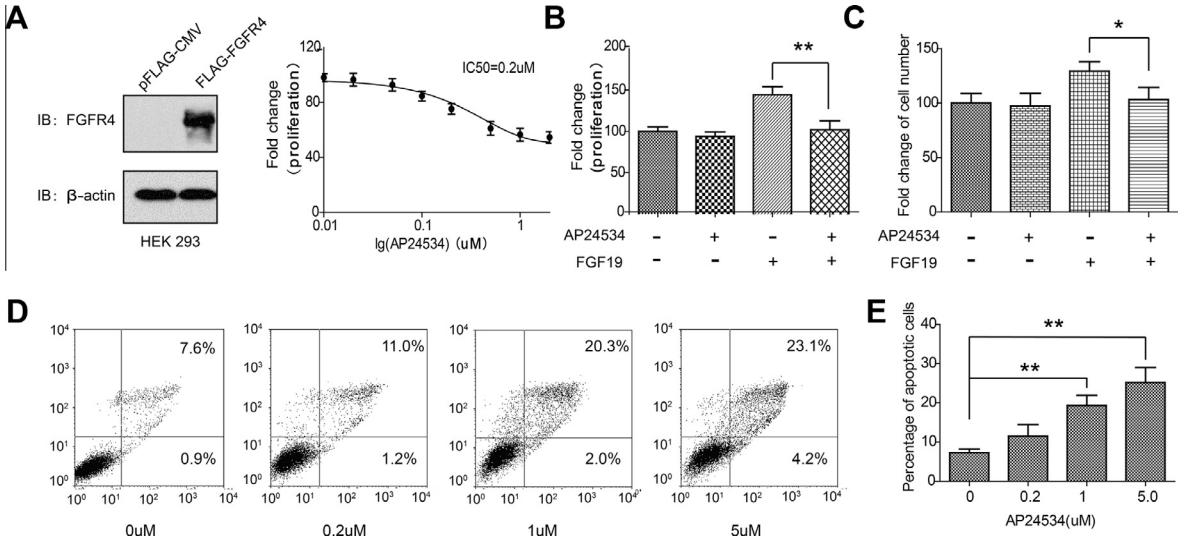

Fig. 4. FGFR4 inhibitor AP24534 suppresses proliferation, invasion and induces apoptosis of CCA cells. (A) AP24534 IC50 on the proliferation of FGFR4-expressing 293 cells. After transfection with FLAG-FGFR4 for 48 h and starvation in serum free medium overnight, 293 cells were cultured in medium with 10% FBS for 48 h, supplemented with indicated concentration of AP24534. MTT assay was used to measure the fold change of proliferation. OD of 0 μM AP24534 was set as baseline and fold change was calculated by ratio to the base line. (B) AP24534 can decrease the FGF19-induced proliferation of RBE cells. RBE cells were starved overnight and then incubated with 100 ng/ml FGF19 or 0.2 μM AP24354 for 48 h. OD of cells without FGF19 and AP24354 was set as baseline. Fold change was calculated by ratio to base line. Data are analyzed by *t*-test and displayed by means of three different experiments ± SEM and ** means $P < 0.01$. (C) AP24534 inhibits the FGF19-induced RBE invasion. RBE cells were cultured in upper transwell chamber in 100 ng/ml FGF19 or 0.2 μM AP24354 for 24 h after 6 h starvation in SFM. Then cells were fixed and stained. Cell numbers of 10 random fields were counted under 100× magnification microscope. Represented data was analyzed from 3 independent experiments ± SEM. * Means $P < 0.05$. (D) AP24534 at different dose can induce apoptosis of RBE cells. AP24534 at concentration of 0 μM, 0.2 μM, 1 μM or 5 μM was used to incubate RBE cells for 48 h and then apoptosis was detected by flow cytometry with Annexin V-FITC and PI staining. (E) Percentage of both early and late apoptotic cells were quantified and displayed in the chart. Results are shown as average of 3 independent experiments ± SEM. ** Means $P < 0.01$.

that FGFR4 signaling pathway prolonged activation, induced by bile acid imbalance of homeostasis, might result in carcinogenesis and progression in CCAs. In addition, FGF19, secreted by CCA or other organs, may promote the CCA progression by endocrine, paracrine or even autocrine pathway. Another distinctive feature of FGFR4 is the single nucleotide polymorphism (SNP) on codon 388. Previous study proved that Gly388 and Arg388 alleles of FGFR4 have different impact on many malignances [28]. Unfortunately, the data of Gly388 and Arg388 alleles were not provided in our experiments because they cannot be distinguished by immunohistochemistry. Further studies on FGFR4 gene polymorphism function in CCAs should be performed.

The exciting discovery of ErbB2 association with prognosis led to the drug Herceptin, which dramatically improved survival time of patients with breast cancer. We expect there is a breakthrough in CCAs just like Herceptin. Unfortunately, there is no available FGFR4 specific inhibitor until now. The broad-spectrum FGFR family inhibitors such as Nintedanib and Dovitinib, have strong inhibitory effect on FGFR1, 2 and 3, but hardly any inhibitory effect on FGFR4. There is a vital need for FGFR4 specific inhibitor or monoclonal antibody since importance of FGFR4 in tumor progression was gradually discovered. We hope our finding of FGFR4 role on CCAs can introduce a new insight into drug development and help find an attractive therapy.

In conclusion, we investigated the expressions of a series of predicted markers in 199 CCA patients by IHC and explored their association with survival rate, finally demonstrated that FGFR4 was an independent prognostic factor in IHCC and PHCC with univariate and multivariate analysis. Moreover, we demonstrated that FGFR4 played a pivotal role in CCA proliferation, invasion and EMT. FGFR4 inhibitor AP24534 can decrease proliferation, invasion and induce apoptosis of CCA, suggesting that FGFR4 may be a potential drug target in CCAs.

Disclosure statement

The authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.050.

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