Flotillin-2 predicts poor prognosis and promotes tumor invasion in intrahepatic cholangiocarcinoma

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Abstract. Intrahepatic cholangiocarcinoma (iCCA) is a highly malignant neoplasm arising from the intrahepatic bile ducts. As a scaffold protein of lipid rafts, flotillin-2 is upregulated in several types of cancer and promotes tumor progression and metastasis. To the best of our knowledge, the present study was the first to detect the upregulation of flotillin-2 in iCCA tissues compared with matched adjacent non-tumor tissues. In addition, immunohistochemistry was used to investigate the expression of flotillin-2 in a microarray consisting of 92 iCCA tissues. A total of 59 samples (64.1%) exhibited high flotillin-2 expression, which was significantly related to lymph node metastasis (P=0.029) and tumor-node-metastasis stage (P=0.016). Further *in vitro* study demonstrated that knockdown of flotillin-2 inhibited the invasive capability of iCCA cell lines, further supporting the participation of flotillin-2 in cancer invasion and metastasis. Moreover, Kaplan-Meier analysis showed patients with high flotillin-2 expression had worse overall survival outcomes. The multivariate Cox proportional hazards model further revealed that high flotillin-2 expression was an independent indicator (P=0.005) of poor prognosis for patients with iCCA. Collectively, the present study revealed that as a promoter of invasion and an independent marker of poor prognosis, flotillin-2 may serve as a potential target for the development of novel therapeutic agents for iCCA.

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Introduction

Intrahepatic cholangiocarcinoma (iCCA) is a highly malignant neoplasm arising from the intrahepatic bile ducts, which accounts for 5-15% of primary hepatic malignancies, 3% of gastrointestinal malignancies, and 10% of biliary tract malignancies (1). Although iCCA is relatively rare, there is an increasing morbidity and mortality rate worldwide, at least in part, due to late diagnosis at advanced stages when surgery is no longer possible. Furthermore, despite the use of systemic chemotherapy and radiotherapy, the median survival time of patients with advanced stages still remains short (7-12 months) (2). Surgical resection is considered to be the optimal therapeutic intervention to increase patient survival time; however, only one third of patients with iCCA are eligible for surgery following diagnosis (3). Furthermore, the recurrence rate is reported to be greater than 60% with the 5-year survival rate ranging from 20 to 40% following curative surgery (4). Considering the overall poor prognosis of patients with iCCA, there is a requirement for the identification of novel diagnostic tumor biomarkers and the development of molecular targeted therapies.

As important marker proteins of lipid microdomains, flotillin-1 and flotillin-2 are widely distributed in mammals, plants, bacteria and fungi (5). The interactions between flotillins and various proteins, which lead to extensive effects on signaling molecules, enable flotillins to participate in various cellular processes, including endocytosis, proliferation and adhesion (6-8). In mammals, flotillin-1 is highly expressed in the brain, heart, placenta and hematopoietic cells, while flotillin-2 shows ubiquitous expression in numerous different tissues (9-11). However, in addition to participating in physiological functions, flotillins serve important roles in cancer progression. Flotillin-2 has been used as a candidate marker for predicting poor prognosis and as a useful therapeutic target for cancer (12). Furthermore, flotillin-2 is an important regulator of lung metastasis in breast cancer (13). However, the expression level and the clinical significance of flotillin-2 in iCCA have not yet been reported.

To the best of our knowledge, the present study was the first to investigate the expression of flotillin-2 in human iCCA tissues and cell lines. The expression of flotillin-2 in frozen resected samples was detected using western blotting, which demonstrated that flotillin-2 was upregulated in iCCA

tissues compared with matched adjacent non-tumor tissues. Furthermore, immunohistochemistry was used to detect flotillin-2 expression in 92 iCCA samples. Moreover, flotillin-2 knockdown decreased the invasion of iCCA cell lines *in vitro*, further supporting the notion that flotillin-2 serves important roles in iCCA invasion and malignant progression. The present study may provide the basis for the development of targeted therapy to reduce iCCA progression.

Materials and methods

Patients and surgical specimens. For the preliminary conduction of western blotting, 12 paired fresh iCCA tissues and adjacent non-tumor tissues were collected from patients who underwent radical surgery between September 2016 and July 2018 at the Affiliated Yantai Yuhuangding Hospital of Qingdao University. In addition, a cohort of 92 patients with iCCA with complete clinical information who had received surgery were recruited between March 2008 and February 2017 according to the following criteria: Radical resection alone, no preoperative adjuvant chemotherapy or radiation therapy, and no severe perioperative complications that may influence the survival time. The intact clinical information of the patients is summarized in Table I. The follow-up period ranged between 5 and 116 months, with a median follow-up period of 30 months. Clinicopathological classification and pathologic tumor-node-metastasis (pTNM) staging was determined according to the 8th edition AJCC/UICC staging system (2017). The experimental protocols were approved by the Ethics Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University, and written informed consent was obtained from all patients.

Tissue microarray and immunohistochemistry. Representative areas of tumor cores in the paraffin embedded tissue blocks were selected according to hematoxylin-eosin (H&E) staining, and then transferred into a recipient master. Antigen retrieval was performed by immersing the sections in citrate buffer (pH 6.0; cat. no. AR0024; Boster Biological Technology) and heating in a microwave for 5 min. The sections were subsequently treated with 3% hydrogen peroxide for 30 min at room temperature to quench endogenous peroxidase activity, followed by 10% normal goat serum for 30 min at 37˚C. Sections were incubated with mouse monoclonal antibody against human flotillin-2 (cat. no. sc-28320; 1:200; Santa Cruz Biotechnology, Inc.) overnight at 4˚C followed by incubation with anti-mouse secondary antibody for 30 min at 37˚C. The sections were subsequently stained with DAB (Beyotime), and the staining was evaluated by two independent observers.

Cell culture and small interfering RNA (siRNA) transfection. The human iCCA cell lines RBE and HuCCT1 were both purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and tested for mycoplasma. Cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS). Both RBE and HuCCT1 cells were transfected with siRNA targeting flotillin-2 using Lipofectamine 3000 (cat. no. L3000015; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The sequence of flotillin-2 siRNA was 5'-GACCTTGAAATCCATGACG-3', with the AllStars siRNA (cat. no. 1027281; Qiagen) as the negative control.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from tumor cells using an RNeasy Plus Mini Kit (cat. no. 74104; Qiagen) and reverse-transcribed into cDNA using the SuperScript First Strand cDNA system (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. qPCR was performed using the SYBR-Green and a LightCycler 480 PCR system (Roche Applied Science). The following primer pairs were used for qPCR: Flotillin-2 forward, 5'-TTGCTGACTCTAAGCGAG CC-3' and reverse, 5'-TCCACGGCAATCTGTTTCTTG-3'; and β-actin forward, 5'-CATGTACGTTGCTATCCAGGC-3' and reverse, 5'-CTCCTTAATGTCACGCACGAT-3'. The following thermocycling conditions were used for qPCR: 95˚C for 10 min followed by 40 cycles of two-step PCR (95˚C for 15 sec and 60˚C for 30 sec). mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method (14) and normalized to the internal reference gene β-actin. Every experiment was carried out in triplicate and repeated three times.

Assessment of flotillin-2 immunostaining. Flotillin-2 immunostaining was determined according to the intensity and proportion of immunohistochemical staining by a semi-quantitative method as previously described (15). Briefly, the intensity was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining) or 3 (strong staining), and the proportion was scored as 1 (0-10%), 2 (11-50%), 3 (51-75%) or 4 (76-100%). The product of the intensity and proportion was used as the final immunohistochemistry score. The optimal cutoff point of flotillin-2 immunostaining was determined according to the log-rank test with respect to overall survival (OS), with the ultimate immunostaining score of 5 to define flotillin-2 expression as low or high.

Western blotting. Cells and tissues were lysed in RIPA buffer containing protease inhibitor cocktail. After determination of protein concentration by a Bio-Rad BCA protein assay, equal amounts of proteins were separated on SDS-PAGE and were transferred to PVDF membranes, which were blocked in 5% non-fat milk in TBST buffer. The membranes were subsequently incubated with specific mouse monoclonal antibodies against human flotillin-2 (cat. no. sc-28320; 1:100; Santa Cruz Biotechnology, Inc.) and human β-actin (cat. no. sc-8432; 1:1,000; Santa Cruz Biotechnology, Inc.). Following washing with TBST at room temperature, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibodies for 1 h and subsequently developed through ECL detection reagent (Thermo Fisher Scientific).

Cell invasion assay. The invasion ability of tumor cells was assessed using Boyden chambers pre-coated with Matrigel (8-μm pore size; Thermo Fisher Scientific, Inc.). After transfection of flotillin-2 siRNA, $1x10^5$ transfected RBE or HuCCT1 cells were seeded on the upper chamber of the insert in serum-free medium. Medium supplemented with 10% FBS was added to the lower chamber. After 24 h, the cells on the upper side of the membrane were removed by cotton swab. The invading cells on the lower surface were fixed and stained.

Table I. Associations between the expression of flotillin-2 and the clinicopathological features of patients with intrahepatic cholangiocarcinoma.

Clinicopathological features	n	Flotillin-2 Low	Flotillin-2 High	P-value
Sex				0.930
Male	58	21	37	
Female	34	12	22	
Age, years				0.438
<60	48	19	29	
≥60	44	14	30	
Liver cirrhosis status				0.912
No	62	22	40	
Yes	30	11	19	
Differentiation				0.335
Well	19	9	10	
Moderately	45	13	32	
Poorly	28	11	17	
Tumor size, cm				0.827
<5	32	11	21	
≥5	60	22	38	
Tumor nodule				0.920
Solitary	84	30	54	
Multiple	8	3	5	
Hepatolith				0.653
No	80	28	52	
Yes	12	5	7	
Tumor stage				0.335
T1+T2	70	27	43	
T3+T4	22	6	16	
Lymph node metastasis				0.029[a]
No	56	25	31	
Yes	36	8	28	
M stage				0.651
M0	87	32	55	
M1	5	1	4	
TNM stage				0.016[a]
I	32	18	14	
II	14	5	9	
III	15	4	11	
IV	31	6	25	

[a]P<0.05.

Stained cells were counted in four-randomly selected fields (magnification, x400).

Statistical analyses. Statistical analyses were performed with SPSS 18.0 software. The χ^2 or Fisher's exact tests were used to compare qualitative variables and to determine the association between flotillin-2 expression and clinicopathological variables. Quantitative variables were compared through two-tailed Student's t-test. The OS was analyzed by the Kaplan-Meier method and the survival curves were compared by the log-rank test. The significant variables in the univariate analyses were further evaluated through multivariate analyses. P<0.05 was considered to indicate a statistically significant difference.

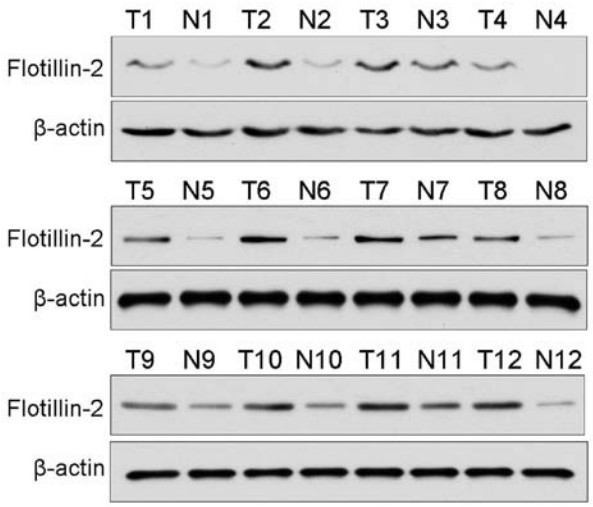

Figure 1. Flotillin-2 expression was significantly upregulated in iCCA tissues. Western blotting of 12 pairs of iCCA and matched adjacent non-tumor bile duct tissues. iCCA, intrahepatic cholangiocarcinoma; T, tumor; N, matched adjacent non-tumor tissue.

Results

Expression pattern of flotillin-2 in iCCA tissues. Western blotting was used to investigate the protein levels of flotillin-2 in paired frozen iCCA and matched adjacent tissues. As shown in Fig. 1, flotillin-2 protein levels were significantly upregulated in iCCA tissues compared with matched non-tumor bile duct tissues. The expression levels of flotillin-2 in a tissue microarray consisting of human iCCA samples were subsequently investigated using immunohistochemistry (Figs. 2 and S1). Flotillin-2 expression was observed predominantly in the cytoplasm and cell membranes of tumor cells, which were confirmed by H&E staining showing the tumor islands. Out of the 92 carcinoma specimens, 59 samples (64.1%) and 33 samples (35.9%) exhibited high and low flotillin-2 expression, respectively.

Associations between flotillin-2 expression and clinicopathological characteristics in patients with iCCA. The role of flotillin-2 in iCCA was further investigated by evaluating its potential association with clinicopathological parameters. The association between flotillin-2 upregulation and clinicopathological characteristics are presented in Table I. The upregulation of flotillin-2 was associated with lymph node metastasis (P=0.029) and TNM stage (P=0.016). However, there was no association between flotillin-2 expression and factors such as age, gender, tumor differentiation, tumor size or tumor nodule.

Prognostic indicator of flotillin-2 upregulation in iCCA. Kaplan-Meier analysis was performed to investigate whether

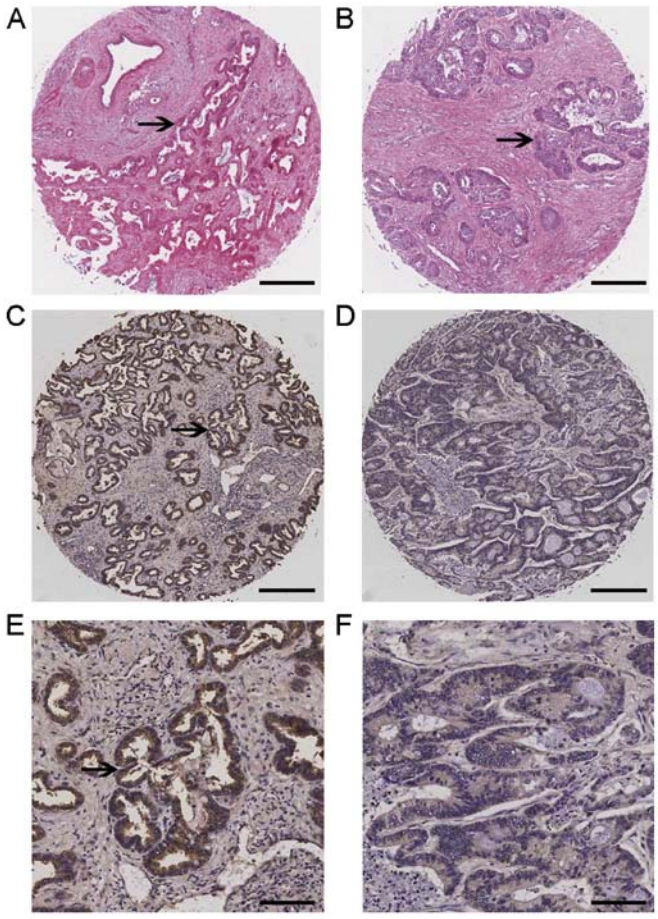

Figure 2. H&E staining and representative images of flotillin-2 immunohistochemical staining in the human iCCA tissue microarray. (A) H&E staining, and (C and E) high flotillin-2 expression of iCCA tissue. (B) H&E staining, and (D and F) low flotillin-2 expression of iCCA tissue. (A and B) Arrows indicate the representative tumor area. (C and E) Arrows indicate high flotillin-2 expression. (A-D) Scale bar, 300 μm. (E and F) Scale bar, 100 μm. H&E, hematoxylin and eosin; iCCA, intrahepatic cholangiocarcinoma.

there was an association between flotillin-2 upregulation and patient OS. As demonstrated in Fig. 3, patients with high flotillin-2 expression showed a worse OS outcome compared with those with low expression (P=0.003). In addition, univariate analysis revealed that tumor differentiation, lymph node metastasis, M stage, TNM stage as well as flotillin-2 upregulation were significantly associated with decreased OS but not with the other parameters (Table II). Furthermore, multivariate analyses revealed that high flotillin-2 expression (P=0.005), lymph node metastasis (P=0.003) and TNM stage (P=0.012) were independent markers for poor prognosis, which further demonstrated high flotillin-2 expression as an indicator for poor OS in patients with iCCA after curative resection (Table III).

Role of flotillin-2 in cell invasion. Flotillin-2 siRNA was used to knockdown the mRNA as well as protein expression levels of flotillin-2 in RBE and HuCCT1 cells (Fig. 4A and B). The effect of flotillin-2 knockdown on the invasion ability of the cells was subsequently determined using an invasion assay, which demonstrated that flotillin-2 knockdown significantly inhibited the invasion ability of RBE and HuCCT1 cells (Fig. 4C and D).

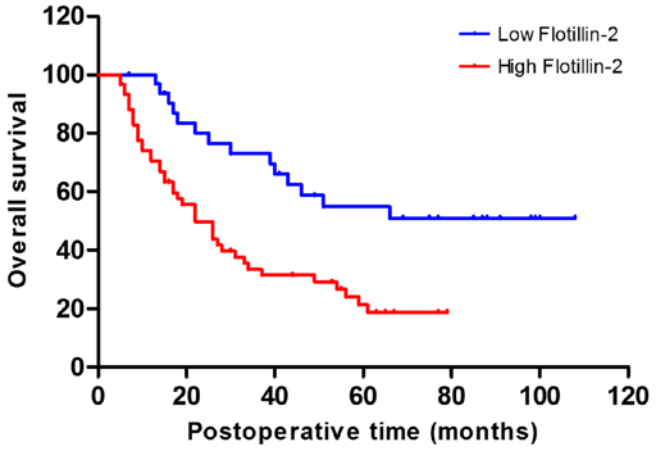

Figure 3. OS curves of patients with intrahepatic cholangiocarcinoma in regard to different flotillin-2 expression. Patients with high flotillin-2 expression exhibited a worse OS compared with patients with low expression. P=0.003. OS, overall survival.

Discussion

As highly conserved membrane proteins that assemble cholesterol- and sphingolipid-rich membrane microdomains, flotillins are not only considered to be scaffold proteins of lipid rafts (16), but are also associated with cellular trafficking, signal transduction, cytoskeleton remodeling and adhesion (17). In addition, flotillins serve important roles in tumor development. The dysregulation of flotillin-2 has been reported in a variety of tumors. The present study revealed that there was significantly increased flotillin-2 expression in iCCA tissues compared with the matched adjacent non-tumor tissues. Flotillin-2 expression was subsequently detected in an iCCA tissue microarray to investigate its potential association with clinicopathological parameters and patient OS. Immumohistochemistry revealed that high flotillin-2 expression was seen in 59 of the totally 92 iCCA samples (64.1%). Even though the mechanism underlying the upregaultion of flotillin-2 in iCCA remains still unclear, microRNAs have been shown to regulate the expression of flotillin-2. Previous studies found that miR-485 and miR-133 could target flotillin-2, resulting in inhibition of metastasis or epithelial-mesenchymal transition (EMT) in lung adenocarcinoma (18,19). Furthermore, flotillin-2 was targeted by miR-449 in glioma (20). In addition, miR-802 and miR-34a have also been reported to target flotillin-2 in prostate cancer and melanoma respectively (21,22). Therefore, the dysregulation of microRNAs may account for the upregulation of flotillin-2, leading to the progression of iCCA.

The present study revealed that high flotillin-2 expression was closely associated with lymph node metastasis and TNM stage. Further *in vitro* study demonstrated that knockdown of flotillin-2 inhibited the invasion ability of iCCA cell lines, suggesting that flotillin-2 may serve a role in tumor invasion and metastasis. This is consistent with several other reports, which revealed that flotillin-2 is upregulated and significantly associated with advanced TNM stage and metastasis in a variety of tumors (23). Flotillin-2 could act as a biomarker for lymphatic and distant metastasis, and promote cell metastasis in nasopharyngeal carcinoma (24). Moreover, flotillin-2 is associated with lymphovascular invasion in gastric cancer (25), as

Table II. Univariate analysis of the clinicopathological features for overall survival of the 92 patients with intrahepatic cholangiocarcinoma.

Characteristics	n	Survival rate, %	P-value
Sex			0.422
Male	58	37.9	
Female	34	41.2	
Age, years			0.583
<60	48	41.7	
≥60	44	36.4	
Liver cirrhosis status			0.330
No	62	41.9	
Yes	30	33.3	
Differentiation			0.029[a]
Well	19	57.9	
Moderately	45	42.8	
Poorly	28	21.4	
Tumor size, cm			0.695
<5	32	43.8	
≥5	60	36.7	
Tumor nodule			0.228
Solitary	84	39.2	
Multiple	8	37.5	
Hepatolith			0.736
No	80	38.8	
Yes	12	41.6	
Tumor stage			0.115
T1+T2	70	41.4	
T3+T4	22	31.8	
Lymph node metastasis			0.016[a]
No	56	51.8	
Yes	36	19.4	
M stage			0.021[a]
M0	87	41.4	
M1	5	0	
TNM stage			0.001[a]
I	32	65.6	
II	14	35.7	
III	15	33.3	
IV	31	16.1	
Flotillin-2 expression			0.003[b]
Low	33	57.6	
High	59	28.8	

[a]P<0.05; [b]P<0.01.

well as depth of invasion in colorectal cancer (15), supporting the participation of flotillin-2 in tumor invasion. Additionally, flotillin-2 is associated with differentiation in breast, gastric and cervical cancer (25-27), but not in colorectal cancer (15). In the present study, flotillin-2 had no association with differentiation, which suggested that this might be tumor type specific since flotillin-2 was also associated with tumor size in gastric cancer (25), but not in others. The consensus among the majority of studies is that flotillin-2 is related to invasion and metastasis in several types of cancer.

Metastasis marks tumor progression from local tumorigenesis to an incurable status as well as an increase in tumor aggressiveness, and significantly affects OS. The present study revealed a significantly poorer OS outcome in patients with high flotillin-2 expression compared to those with low flotillin-2 expression. More importantly, the multivariate Cox proportional hazards model revealed that high flotillin-2 expression was an independent indicator for poor prognosis. Flotillin-2 has been shown to be promising as new biomarkers to predict poor prognosis of patients with a few different kinds of tumors (28), such as non-small-cell lung cancer, esophageal squamous cell carcinoma and renal cell carcinoma (29). A link between flotillin-2 and tumor progression has been established, however, the mechanisms underlying the roles of flotillin-2 in cancer malignancy have not been completely elucidated. Flotillin-2 is involved in drug-resistance of colorectal cancer cells, potentially by mediating the PI3K/Akt signaling pathway (30). Moreover, flotillin-2 plays a pro-neoplastic role in nasopharyngeal carcinoma and promotes metastasis through both PI3K/AKT3 and NF-κB signaling pathways (31). In breast cancer, flotillin-2 induces tumor proliferation through modulation of AKT/FOXO signaling pathway (32). Furthermore, flotillin-2 modulated the cell cycle and induced EMT via the upregulation of twist as a result of ERK1/2 pathway activation in hepatocellular carcinoma (33).

In the multivariate analysis of our study, lymph node metastasis and advanced TNM stage, act as independent indicators for poor prognosis while some known clinicopathological parameters like tumor differentiation and M stage do not. There are several potential reasons for this. The sample size as well as the criteria utilized to enroll the sample, could affect independent prognostic factors associated with OS. Meanwhile, this may have been attributed to the interaction of TNM stage with T, N and M stage (34). In addition, the study design, for example, whether a study is prospective or multi-center, may also affect the results. Huang *et al* (35) showed that lymphatic metastasis, rather than TNM stage and tumor differentiation, was an independent risk factor for OS. By contrast, Yamaoka *et al* (36) revealed that tumor differentiation and lymph node metastasis were not independent pronostic factors. A large-scale study with multicenter analysis showed that lymph node metastasis was a significant factor affecting OS, while tumor differentiation was not (37). Therefore, additional multi-center studies with large sample numbers are required to validate the results obtained in the present study.

The present study had a number of limitations. Firstly, only patients that received radical surgery, without any pre-operative treatment, were enrolled. It should be noted that the majority of patients with iCCA had received chemotherapy instead of radical surgery due to late diagnosis, and were therefore not enrolled. Meanwhile, 9 patients diagnosed with TNM stage IV in the present study received chemotherapy following radical surgery. Therefore, the influence of adjuvant chemotherapy on clinical outcome is not clear, and whether there is

Table III. Multivariate analysis of the clinicopathological features for overall survival of the 92 patients with intrahepatic cholangiocarcinoma.

Factors	P-value	HR	95% CI
Flotillin-2 expression, low, high	0.005[b]	2.974	1.112-4.386
Tumor differentiation, well, moderately, poorly	0.461	0.852	0.510-2.839
Lymph node metastasis, no, yes	0.003[b]	1.105	0.882-3.456
M stage, M0, M1	0.114	0.806	0.410-3.447
TNM stage, I, II, III, IV	0.012[a]	0.584	0.213-1.795

[a]P<0.05; [b]P<0.01. HR, hazard ratio.

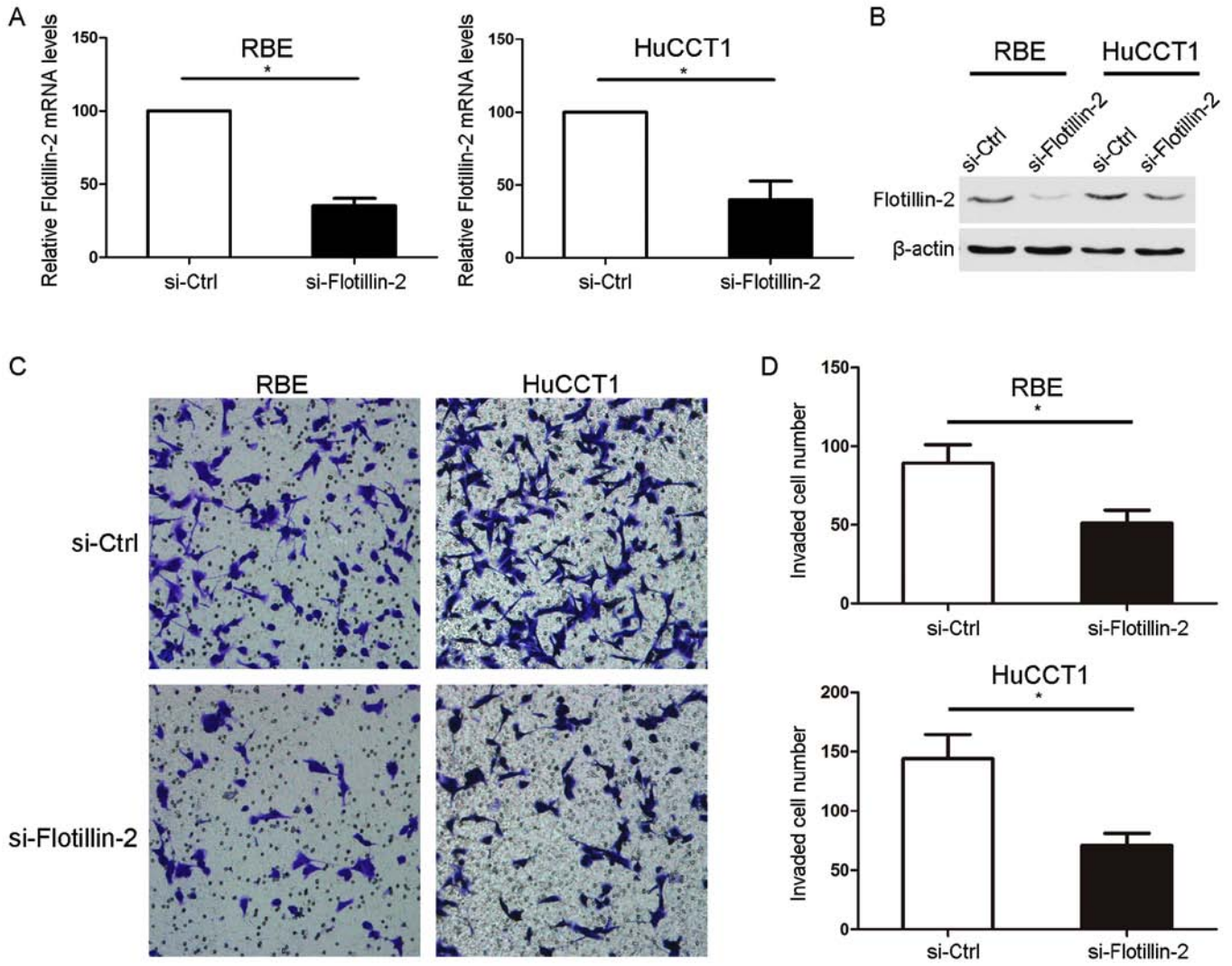

Figure 4. Role of flotillin-2 in the invasion of intrahepatic cholangiocarcinoma cells. (A and B) siRNA-mediated knockdown of flotillin-2 in RBE and HuCCT1 cell lines was validated by (A) reverse transcription-quantitative PCR and (B) western blotting. β-actin was used as a loading control. (C and D) Effect on the invasion of flotillin-2 knockdown in RBE and HuCCT1 cell lines. Magnification, x400. Data are presented as the mean ± SEM of three independent experiments. *P<0.05. si, small interfering; Ctrl, control.

an association between flotillin-2 expression and chemoresistance in iCCA requires further investigation. Furthermore, the limited sample size of this study requires future studies with a larger sample size.

In summary, the present study suggested that upregulation of flotillin-2 was associated with unfavorable clinicopathological factors, and more importantly, with decreased OS in patients with iCCA. Furthermore, flotillin-2 expression served as an independent marker of poor prognosis in patients with iCCA and promoted invasion of iCCA cells *in vitro*. Therefore, targeting flotillin-2 may provide a potential direction for the development of novel therapeutic agents for iCCA.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZX and TW were involved in the study concept and design. ZX acquired the samples, and conducted the western blotting and immunohistochemistry analysis. TW conducted the *in vitro* study. ZX and TW performed the analysis and interpretation of data. HS and XJ were responsible for the design of the study, and the writing, review and revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University approved the study. All patients received an explanation of the aims of the study and provided written informed consent.

Patient consent for publication

The study participants provided consent for the data to be published.

Competing interests

The authors declare that they have no competing interests.

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