Research Article

Overexpression of 14-3-3σ Modulates Cholangiocarcinoma Cell Survival by PI3K/Akt Signaling

Qiao Wu, Hua Fan, Ren Lang, Xianliang Li, Xingmao Zhang, Shaocheng Lv, and Qiang He

Department of Hepatobiliary Surgery, Beijing Chao Yang Hospital, Capital Medical University, Beijing 100020, China

Correspondence should be addressed to Qiang He; heqianggandan@163.com

Received 4 April 2020; Revised 22 May 2020; Accepted 4 June 2020; Published 23 June 2020

Guest Editor: Jialiang Yang

Copyright © 2020 Qiao Wu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The protein 14-3-3σ is involved in numerous cellular processes through its ability to bind phosphorylated serine/threonine residues. It is a key regulator of the cell cycle involving in G2 arrest by p53. Deregulation of 14-3-3σ expression has been associated with a large variety of human cancers. However, its physiological function and therapeutic significance have rarely been investigated in cholangiocarcinoma. Using immunohistochemistry (IHC), we evaluated 14-3-3σ expression in 65 human extrahepatic cholangiocarcinomas. As a result, we found that 14-3-3σ is expressed in the tissue of 56 patients (86.2%), and its expression is positively correlated with tumor size, lymph node metastasis, and tumor stage. We also explored the significance of 14-3-3σ and found that 14-3-3σ exerts cell type-dependent effects on cell proliferation through PI3K/Akt signaling in both in vitro and in vivo xenograft models. These results suggest that 14-3-3σ assumes a constitutive role in tumorigenesis rather than acting as a cell cycle regulator in cholangiocarcinoma, which makes 14-3-3σ a new potential target for therapeutic intervention.

1. Introduction

Cholangiocarcinoma (CCA) is a rare malignancy with devastating outcomes. Tumors can arise from the lining of the bile duct epithelium anywhere [1, 2]. Depending on their anatomical location, CCAs are categorized as intrahepatic or extrahepatic CCA; the individual categories differ in terms of biological behavior, clinical presentation, and management [3]. While numerous risk factors for CCA are known, the pathogenesis of this disease is still obscure [1, 4–6]. In clinical practice, extrahepatic CCAs are usually asymptomatic at early stages and are often diagnosed at an advanced stage of the disease. For the last four decades, the worldwide incidence of CCA progressively increased [7]. Surgical excision is considered to be the most effective therapeutic route for CCA; however, the 5-year survival rate remains around 20–30% [8, 9]. Therefore, it is paramount to identify novel tumor markers for this disease that will allow early detection, as well as new potential targets for therapeutic intervention [10].

14-3-3σ proteins are highly conserved. In mammals, seven distinct isoforms are known (β, σ, γ, θ, δ, ε, and η). The 14-3-3 proteins are crucial regulators of intracellular signaling pathways. The physiological function of these proteins is still unclear. However, through binding to diverse target proteins, 14-3-3 proteins appear to be involved in a wide variety of cellular processes, such as cell proliferation, cell cycle progression, apoptosis, signal transduction, and malignant transformation [11, 12].

14-3-3σ, also called stratifin, belongs to the 14-3-3 family; it was first identified as an epithelial-specific marker (HME1). This protein is mostly expressed in human epithelial cells and is involved in various processes such as signal transduction and growth regulation. Accumulating data indicate that 14-3-3σ can bind to other important proteins associated with tumor initiation and development through its ligand-binding domain. Silencing of 14-3-3σ due to hypermethylation of the CpG island has been observed in breast cancer [13–15], lung cancer [16], ovarian cancer [17], hepatocellular carcinoma...
[18], and certain squamous cell carcinomas [19]. In contrast, 14-3-3-σ appears to be upregulated in gastric cancer [20], colon carcinoma [21], pancreatic cancers [22, 23], and head and neck squamous cell carcinoma [24].

To date, there is no data on the role of the 14-3-3-σ on CCA initiation and development. Here, we assess the expression of 14-3-3-σ in CCA as well as its potential role as a regulator of the essential signaling proteins involved in relaying the inputs from multiple upstream survival pathways in CCA.

2. Materials and Methods

2.1. Tumor Samples. Sixty-five surgically resected samples were collected from patients diagnosed with extrahepatic cholangiocarcinoma according to radiologic findings with no preceding therapy at the Department of Hepatobiliary Surgery, Beijing Chao Yang Hospital, Capital Medical University, from January 2010 to September 2013. Clinicopathological data including age, sex, tumor size, pathologic differentiation, lymphatic permeation, and tumor stage are reported in Table 1. Depending on their degree of papillary or tubular formation, tumors were distributed into 3 groups (30 well-differentiated, 29 moderately differentiated, and 6 poorly differentiated). If more than one type was observed, the tumor was classified according to the most prominent type. The tumor histologic stage was defined from stage I to stage IVB based on histologic examination according to the pTNM classification issued by the International Union Against Cancer. The prognosis was collected by reviewing patient case records. Specimens were fixed with 10% formalin and embedded in paraffin wax.

2.2. Tissue Preparation and Antibodies. The histologic sections of extrahepatic cholangiocarcinoma fixed in paraffin were rehydrated and dewaxed. Antigen retrieval was carried out after endogenous peroxidase activity has been blocked through incubation with 3% hydrogen peroxide in absolute methanol for 10 minutes. The histologic sections were incubated for 1 h at 37°C with the primary antibody directed toward 14-3-3-σ isomeric (Santa Cruz, CA); the primary antibody was diluted to 1:200 ratio. The slides were then incubated for 50 minutes at ambient temperature with a secondary antibody (Dako). The histologic sections were visualized with 3,3-diaminobenzidine (DAB) and counterstained with Mayer’s hematoxylin. The methods we used in this part are the same as what we did previously [25].

2.3. Scoring of Immunoreactivity. The intensity of IHC staining in tumor cells was scored independently by two pathologists that were blinded to the clinicopathological data following the semiquantitative IRS (immunoreactive score) scale. The final score was defined as the average from the two referees. 14-3-3-σ staining intensity (14-3-3-σ-IRS), percentage of 14-3-3-σ-positive tumor cells (14-3-3-σ-PP), and a resulting 14-3-3-σ immunoreactivity score (14-3-3-σ-IRS) were all determined following a slightly modified version of the protocol published elsewhere [26]. Briefly, the immunoreactivity score (14-3-3-σ-IRS: negative 0, weak 1–3, moderate 4–6, and strong 8–12) was obtained by multiplying the values for 14-3-3-σ staining intensity (14-3-3-σ-IRS: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining) and the values for percentage of 14-3-3-σ-positive tumor cells (14-3-3-σ-PP: score 1, 0-10%; score 2, 11-25%; score 3, 26-50%; and score 4, >51%).

2.4. Cell Lines and Cell Culture. The TFK-1 cell line was obtained from the Department of Biochemistry (Chinese Academy of Medical Sciences and Peking Union Medical College, China) and grown in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U penicillin, and 100 mg/mL streptomycin. All cells were grown at 37°C with 5% CO2 in a humidified incubator. The human CCA cell line TFK-1 underwent lentiviral transduction to stably silence 14-3-3-σ.

2.5. Transient Transfection. A specific 14-3-3-σ small interfering RNA (siRNA) was designed according to the cDNA sequence of 14-3-3-σ (sequence: 5′-UGAAGAUGAAGGU GACUATT-3′). The negative control is a scrambled non-target siRNA. The siRNAs were synthesized by Invitrogen Company (Shanghai, China). Lipofectamine 2000 (Invitrogen) was used for siRNA transfection in TFK-1 cells following the manufacturer’s instructions. 24 h after transfection, TFK-1 cells were used in CCK8, flow cytometry assay, and Transwell migration assays. The expression level of 14-3-3-σ was monitored by western blot.

2.6. Western Blot Analysis. The rabbit anti-14-3-3-σ was from Santa Cruz (Santa Cruz, CA). Antibodies against p-Bad (cat # 4366S), p21 (cat # 2946S), cyclin D1 (cat # 2926S), p-GSK-3α/β (cat # 9331S), PTEN (cat # 9559S), p-P3K (cat # 4228S), p-AKT (cat # 4605S), p27 (cat # 2559S), Bax (cat # 2772S), Bad (cat # 9292S), and β-actin (cat # 4970S)
were purchased from Cell Signaling Technologies (Danvers, MA). Cells were harvested through trypsinization followed by centrifugation. Pelleted cells were lysed in lysis buffer supplemented with protease inhibitors and processed for western blotting.

2.7. Cell Proliferation Assay. Cell proliferation was measured with the cell count kit (CCK-8, Domino, Shanghai, China). In short, cells were seeded in 96-well plates at $5 \times 10^3$ cells/well and transfected with 14-3-3σ siRNA or a negative control. 48 hours after transfection, the CCK-8 assay was performed. Transfected cells were incubated with culture medium containing the CCK-8 reagent for 4 hours; the absorbance was read at 450 nm using a microplate enzyme-linked immunosorbent assay reader (Labsystems Dragon, Wellscan).

2.8. Flow Cytometry Assay of Cell Apoptosis. Cells were harvested by trypsinization, collected by centrifugation, and washed once with phosphate-buffered saline (PBS). Cells

Table 2: Clinical and histopathological characteristics according to 14-3-3σ status in 65 extrahepatic cholangiocarcinomas.

|                | 14-3-3σ expression | Total (n = 65) | Negative (n = 9) | Positive (n = 56) | P value |
|----------------|--------------------|---------------|-----------------|-------------------|---------|
| Gender (%)     |                    |               |                 |                   |         |
| Male           | 37 (56.9)          | 5 (55.6)      | 32 (57.1)       | NS                |         |
| Female         | 28 (43.1)          | 4 (34.4)      | 24 (42.9)       |                   |         |
| Age (%)        |                    |               |                 |                   |         |
| ≥65 years      | 22 (33.8)          | 4 (34.4)      | 18 (32.1)       | NS                |         |
| <65 years      | 43 (66.2)          | 5 (55.6)      | 38 (67.9)       |                   |         |
| Tumor size (%) |                    |               |                 |                   |         |
| >2.5 cm        | 45 (69.2)          | 3 (33.3)      | 42 (75.0)       | 0.020             |         |
| ≤2.5 cm        | 20 (30.8)          | 6 (66.7)      | 14 (25.0)       |                   |         |
| Pathologic differentiation (%) | | | | | |
| Well           | 30 (46.2)          | 2 (22.2)      | 28 (50.0)       |                   |         |
| Moderate       | 29 (44.6)          | 4 (44.4)      | 25 (44.6)       | NS                |         |
| Poor           | 6 (9.2)            | 3 (33.4)      | 3 (5.4)         |                   |         |
| Lymphatic permeation (%) | | | | | |
| No             | 27 (41.5)          | 7 (77.8)      | 20 (35.7)       | 0.027             |         |
| Yes            | 38 (58.5)          | 2 (22.2)      | 36 (64.3)       |                   |         |
| Tumor stage (%)|                    |               |                 |                   |         |
| I+II           | 25 (38.5)          | 8 (66.7)      | 17 (32.1)       | 0.001             |         |
| III+IV         | 40 (61.5)          | 1 (33.3)      | 39 (67.9)       |                   |         |

Figure 1: Immunoreactivity of 14-3-3σ in extrahepatic cholangiocarcinoma specimens. Typical cases evaluated as negative (a) or positive (b) are shown.

Figure 2: Overall survival curves of 65 extrahepatic cholangiocarcinomas according to 14-3-3σ expression were generated using the Kaplan-Meier method. The statistical significance of differences in the survival analyses was calculated using the log-rank test. Significance was set as $P < 0.014$. 2.7. Cell Proliferation Assay. Cell proliferation was measured with the cell count kit (CCK-8, Domino, Shanghai, China). In short, cells were seeded in 96-well plates at $5 \times 10^3$ cells/well and transfected with 14-3-3σ siRNA or a negative control. 48 hours after transfection, the CCK-8 assay was performed. Transfected cells were incubated with culture medium containing the CCK-8 reagent for 4 hours; the absorbance was read at 450 nm using a microplate enzyme-linked immunosorbent assay reader (Labsystems Dragon, Wellscan). 2.8. Flow Cytometry Assay of Cell Apoptosis. Cells were harvested by trypsinization, collected by centrifugation, and washed once with phosphate-buffered saline (PBS). Cells
were then fixed at 4°C in 1 mL of 75% ethanol. Cells were washed once more with PBS before a 30 min incubation at room temperature in 1 mL PBS supplemented with 30 μg/mL propidium iodide and 0.25 mg/mL RNase A. Cells were analyzed for DNA content by flow cytometry using FACS (fluorescence-activated cell sorter) Cytomics FC 500 (Beckman). The data were treated with Multicycle AV for Windows (Beckman). Apoptosis was induced by 10 μg/mL of gemcitabine.

2.9. In Vitro Migration Assays. The cell migratory abilities were determined by Transwell assays. Cell culture inserts with 8 μm microporous filters without extracellular matrix coating (Becton Dickinson Labware, Bedford, MA) were seeded with 5.0 × 10⁴ TFK-1 cells in 200 μL serum-free DMEM. The bottom chambers were loaded with 500 mL DMEM containing 10% FBS. Following a 24 h incubation period, noninvasive cells were gently removed with a cotton swab. Migrating cells located on the lower surface of the chamber were stained with the Diff-Quick Staining Set (Dade) and counted under a microscope.

2.10. Stable Transduction. The 14-3-3σ miRNA-interfering (14-3-3σ-miR) and normal control (control-miR) cell lines were generated through lentiviral transduction. 14-3-3σ-miR was transduced with lentivirus containing an oligo antisense sequence of 14-3-3σ. Lentiviral particles were obtained by cotransfection of Packaging Mix with Opti-MEM and

---

Table 3: Univariate and multivariate analyses of overall survival in 65 extrahepatic cholangiocarcinomas.

|                          | Univariate | Multivariate |
|--------------------------|------------|--------------|
|                          | P value    | Relative risk (95% CI) |
| Gender                   | 0.602      | 2.51 (0.818–8.103)    |
| Age                      | 0.473      |               |
| Tumor size               | 0.020      | 2.29 (1.152–12.845)  |
| Pathologic differentiation| 0.074      |               |
| Lymphatic permeation      | 0.027      | 5.77 (0.449–11.017)  |
| Tumor stage              | 0.001      |               |
| 14-3-3σ level            | 0.034      | 9.28 (2.416–19.577)  |

1Data were considered significant by univariate analyses and examined by multivariate analyses.
Lipofectamine 2000 (Life Technologies). The supernatants containing the virus were concentrated using ultracentrifugation at 50,000 g for 2 h. TFK-1 cells grown in 12-well plates were infected in the presence of 8 μg/mL Polybrene (Sigma-Aldrich). The transduced cells were identified using blastidin (0.5 mg/mL-10 mg/mL).

2.11. Xenograft Studies. BALB/c nu/nu female mice were raised in an SPF room. TFK-1 cells were injected subcutaneously into the right axillary area of the 4-week-old mice. Mice were sacrificed 30 days later, and the tumor size and weight were measured.

2.12. Statistics. Statistical analyses were made with the SPSS 13.0 software (SPSS, Chicago, IL). Continuous data were presented as the mean ± standard deviation (SD) calculated from at least 3 individual duplicated experiments. Statistical significance was calculated by Student’s t-test. A value of *P* < 0.05 was considered statistically significant.

3. Results

3.1. Immunohistochemistry. In order to evaluate the expression of 14-3-3σ in extrahepatic cholangiocarcinoma, we first examined the presence of the protein in tissue samples from 65 patients by immunochemistry (patient details are summarized in Table 1). Positive cells showed an intense staining in the cytoplasm (see Figure 1 for cases typically evaluated as positive or negative). 56 cases were evaluated as 14-3-3σ-positive (86.2%) against 9 evaluated as negative (13.8%). 19 cases (out of 56: 35.8%) had 14-3-3σ-positive cancer tissue adjacent to morphologically normal glands. Immunoreactivity toward 14-3-3σ is significantly different between extrahepatic cholangiocarcinoma tissue and adjacent normal bile ducts.

3.2. Correlation between 14-3-3σ Expression and Clinicopathological Parameters. Since 14-3-3σ seems to be expressed in 86% of extrahepatic cholangiocarcinoma, we explored the connection between 14-3-3σ and clinicopathological factors. As can be seen in Table 2, expression of 14-3-3σ significantly correlates with tumor size (*P* = 0.02), lymph node metastasis (*P* = 0.027), and tumor stage (*P* = 0.01).

There was, however, no significant relationship between 14-3-3σ expression and patient sex, age, pathologic differentiation, and serum tumor markers (CEA, CA-242, or CA19-9).

3.3. Correlation between 14-3-3σ Expression and Clinical Outcome. The overall survival curve of 65 extrahepatic cholangiocarcinomas following the Kaplan-Meier method is shown in Figure 2. The 1-, 3-, and 5-year survival rates were 100%, 77.8%, and 44.4% for the 14-3-3σ-negative group compared to 73.2%, 21.4%, and 1.8% for the 14-3-3σ-positive group. 14-3-3σ-positive cases showed significantly worse prognosis than 14-3-3σ-negative cases (*P* = 0.014).

The univariate analysis by Cox (Table 3) reveals that tumor size (*P* = 0.02), lymph node metastasis (*P* = 0.027), tumor stage (*P* = 0.001), and 14-3-3σ level (*P* = 0.034) are
14-3-3 cell lines were engineered to express either a siRNA targeting (Figure 3(e)) while the proportion of cells in S phase increased (Figure 3(d)), suggesting that 14-3-3σ suppression can induce slight G1 arrest (Supplementary Fig 1).

3.5. Effects of 14-3-3σ Suppression in TFK-1 Cells on Gemcitabine-Induced Apoptosis. To determine whether silencing of 14-3-3σ would make CCA cells more sensitive to gemcitabine, we quantified cell apoptosis using flow cytometry. In response to 1 μM gemcitabine, cells transfected with 14-3-3σ siRNA exhibited increased apoptosis compared to cells transfected with scrambled siRNA (Figures 4(a) and 4(b)).

3.6. 14-3-3σ Is Not Required for the Regulation of Metastatic Potential in TFK-1 Cells. The impact of 14-3-3σ on metastatic potential was assessed by Transwell assays. For TFK-1 cells, no significant difference in motility could be measured between cells transfected with 14-3-3σ siRNA and cell transfected with the scrambled siRNA (data not shown).

3.7. 14-3-3σ Regulates PI3K/Akt Pathway and p53 in TFK-1 Cells. Since knocking down 14-3-3σ in TFK-1 cells modified their proliferative and survival properties, we assessed the expression levels of related proteins by western blot in order to identify the molecular network underlying these observations (Figure 5).

Silencing of 14-3-3σ seems to be associated with a down-regulation of p-Akt but does not lead to any significant change in the levels of total Akt and its upregulation by p-PI3K. Cyclin D1 is downregulated, whereas p53, p27, and p21 are upregulated. Proapoptotic proteins (Bim and Bax) are dramatically upregulated. On the other hand, the antiapoptotic marker p-Bad is not significantly altered in response to 14-3-3σ suppression. Interestingly, the invasion suppressor E-cadherin does not seem to be significantly affected by downregulation of 14-3-3σ in TFK-1 cells.

3.8. Silencing of 14-3-3σ Inhibits Tumor Growth in BALB/C Nude Mice. To further investigate whether silencing of 14-3-3σ suppresses CCA tumor growth in vivo, stably transduced TFK-1 CCA cell lines were injected into the subaxillary regions of BALB/c nude mice (n = 8 in each group) (Supplementary Fig 2). The efficiency of stable transduction with lentivirus to extrahepatic cholangiocarcinoma cell line TFK-1 is about 78%-90% (Supplementary Fig 3). Tumor volume and weight were monitored in order to assess the proliferation speed of each group. Mice injected with 14-3-3σ-miR cells show significantly smaller tumor volume and weight compared to mice injected with control-miR cells (Figure 6). These observations suggest that silencing of 14-3-3σ in vivo can suppress CCA tumor growth.

Taken together, our results suggest that (i) 14-3-3σ is associated with a higher cell proliferation rate and a higher proportion of cells in S phase, (ii) 14-3-3σ plays a role in the chemoresistance of CCA toward gemcitabine, and (iii) 14-3-3σ may not be required for the regulation of the metastatic potential of CCA.

4. Discussion

14-3-3σ was first identified as a mammary epithelium marker. Only later was 14-3-3σ found to play a major role in the

![Figure 5: Western blotting for molecules critical for cell proliferation and apoptosis.](Image)
regulation of cell cycle checkpoints. In response to DNA damage, 14-3-3σ expressions are induced under the control of p53. Expression of 14-3-3σ leads to the sequestration of cyclin B1 to the cytoplasm, precluding it from entering the nucleus, consequently preventing the initiation of mitosis [13, 27]. Therefore, 14-3-3σ appears to act as a suppressor of cell cycle progression and is associated with cancer. However, the role of 14-3-3σ as a tumor suppressor is questionable as 14-3-3σ can also be upregulated in some cancers. Indeed, in this study, we observe overexpression of 14-3-3σ in the tissue of 56 out of 65 CCA patients. We could also show that 14-3-3σ is related to CCA progression since overexpression of 14-3-3σ expression is an independent prognostic factor for CCA, which suggests that 14-3-3σ harbors a role in tumorigenesis and/or progression of CCA.

Based on this observation, we propose that 14-3-3σ stimulates CCA progression. In order to identify the molecular mechanisms at hand, we silenced the expression of 14-3-3σ in human CCA cell lines by transient and stable transfection. Using this experimental setup in vitro and in vivo xenograft models, we could show that CCA cell growth and apoptosis are influenced by 14-3-3σ. Upregulation of 14-3-3σ leads to increased proliferation as well as resistance to apoptosis. These results indicate that 14-3-3σ shows an oncogenic potential as its expression is correlated with the expression of proteins involved in cancer initiation and progression. 14-3-3σ suppression seems to lead to G1 cell cycle arrest, increased gemcitabine-induced apoptosis, and reduced cell proliferation.

Next, we focused on the role of 14-3-3σ and its potential interplay with other key payers of cell cycle regulation as well as cell proliferation and invasion. We found that 14-3-3σ affects the PI3K/Akt pathways, by which dysregulation can lead to human cancers [28, 29]. The PI3K/Akt signaling pathways are known to be activated in CCA, regulating tumor growth and metastasis. Our western blot results revealed that downregulation of 14-3-3σ correlates with the downregulation of p-Akt but does not affect the levels of total Akt and its upregulation by p-PI3K. These observations suggest that 14-3-3σ could interfere with the phosphorylation of Akt and could maintain the proliferative abilities of the cell by activating the PI3K/Akt signaling cascade.

In conclusion, 14-3-3σ may generate an increase in cell proliferation, an increase in the proportion of cells in S phase, and a contribution to the chemoresistance of CCA toward gemcitabine.
enhances sensitivity toward gemcitabine in CCA cells and leads to significant cell apoptosis. We also observed that proapoptotic molecules (Bim and Bax) were dramatically upregulated following 14-3-3σ knockdown.

14-3-3σ has been proposed to be involved in metastasis and cancer invasion; increased 14-3-3σ expression appears to significantly correlate with large tumor size and higher invasion in some cancers [19, 32]. However, we did not find a significant correlation between 14-3-3σ expression and metastatic potential in CCA cells, similarly to what was observed in T3M4 cells [33].

In summary, we provided new evidence exploring the functional roles of 14-3-3σ protein in bile duct tumorigenesis. We anticipate that the interaction between 14-3-3σ and upstream factors of the PI3K/Akt pathway and p53 network could raise consideration for 14-3-3σ inhibitors as a new avenue to treat CCA.

Data Availability
The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval
This project was approved by the ethical committees of the hospital.

Consent
Informed consent was obtained from the participating patients.

Conflicts of Interest
The authors declare no conflict of interest.

Authors’ Contributions
QW designed the study, collected and organized the data, and wrote the manuscript. RL, HF, XL, XZ, and SL collected and organized the data. QH designed the study and wrote and reviewed the manuscript.

Acknowledgments
We thank all the patients who participated in this study. This work was supported by the Science and Technology Program of Beijing Education Commission (KM201510025016).

Supplementary Materials
Supplementary Fig 1: 14-3-3-σ suppression reduces the S phase cell ratio and induces slight G1 arrest in vitro. *P < 0.05. Supplementary Fig 2: silencing of 14-3-3σ (Group B) suppresses CCA tumor growth in BALB/c nude mice compared with control (Group A). Gross appearance of tumors from BALB/c nude mice in two groups. *P < 0.05. Supplementary Fig 3: the efficiency of stable transduction with lentivirus to extrahepatic cholangiocarcinoma cell line TFK-1 is about 78%-90%. (Supplementary Materials)

References
[1] B. Blechacz, Y. A. Ghouri, and I. Mian, "Cancer review: cholangiocarcinoma," Journal of Carcinogenesis, vol. 14, no. 1, p. 1, 2015.
[2] M. C. Bragazzi, L. Ridola, S. Safarikia et al., "New insights into cholangiocarcinoma: multiple stems and related cell lineages of origin," Annals of Gastroenterology, vol. 31, no. 1, pp. 42–55, 2018.
[3] J. M. Banales, V. Cardinale, G. Carpino et al., "Cholangiocarcinoma: current knowledge and future perspectives consensus statement from the European Network for the Study of Cholangiocarcinoma (ENS-CCA)," Nature Reviews. Gastroenterology & Hepatology, vol. 13, no. 5, pp. 261–280, 2016.
[4] R. R. Plentz and N. P. Malek, "Clinical presentation, risk factors and staging systems of cholangiocarcinoma," Best Practice & Research. Clinical Gastroenterology, vol. 29, no. 2, pp. 245–252, 2015.
[5] V. Cardinale, R. Semeraro, A. Torrice et al., "Intra-hepatic and extra-hepatic cholangiocarcinoma: new insight into epidemiology and risk factors," World Journal of Gastrointestinal Oncology, vol. 2, no. 11, pp. 407–416, 2010.
[6] P. L. Labib, G. Goodchild, and S. P. Pereira, "Molecular pathogenesis of cholangiocarcinoma," BMC Cancer, vol. 19, no. 1, p. 185, 2019.
[7] S. K. Saha, A. X. Zhu, C. S. Fuchs, and G. A. Brooks, "Forty-year trends in cholangiocarcinoma incidence in the U.S.: intrahepatic disease on the rise," The Oncologist, vol. 21, no. 5, pp. 594–599, 2016.
[8] M. Ghidini, C. Pizzo, A. Botticelli et al., "Biliary tract cancer: current challenges and future prospects," Cancer Management and Research, vol. 11, pp. 379–388, 2019.
[9] M. Aljiffry, A. Abdudealah, M. Walsh, K. Peltekian, I. Alwayn, and M. Molinari, "Evidence-based approach to cholangiocarcinoma: a systematic review of the current literature," Journal of the American College of Surgeons, vol. 208, no. 1, pp. 134–147, 2009.
[10] S. Rizvi, S. A. Khan, C. L. Hallemeier, R. K. Kelley, and G. J. Gores, "Cholangiocarcinoma – evolving concepts and therapeutic strategies," Nature Reviews. Clinical Oncology, vol. 15, no. 2, pp. 95–111, 2018.
[11] G. W. Porter, F. R. Khuri, and H. Fu, "Dynamic 14-3-3/client protein interactions integrate survival and apoptotic pathways," Seminars in Cancer Biology, vol. 16, no. 3, pp. 193–202, 2006.
[12] D. K. Morrison, "The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development," Trends in Cell Biology, vol. 19, no. 1, pp. 16–23, 2009.
[13] C. Laronga, H. Y. Yang, C. Neal, and M. H. Lee, "Association of the cyclin-dependent kinases and 14-3-3 σ negativity regulates cell cycle progression," The Journal of Biological Chemistry, vol. 275, no. 30, pp. 23106–23112, 2000.
[14] J. Luo, J. Feng, J. Lu et al., "Aberrant methylation profile of 14-3-3 σ and its reduced transcription/expression levels in Chinese sporadic female breast carcinogenesis," Medical Oncology, vol. 27, no. 3, pp. 791–797, 2010.
[15] M. Ye, T. Huang, Y. Ying et al., "Detection of 14-3-3 σ promoter methylation as a noninvasive biomarker using blood samples for breast cancer diagnosis," Oncotarget, vol. 8, no. 6, pp. 9230–9242, 2017.
[16] N. Sun, Y. Wu, B. Huang et al., “Decreased expression of 14-3-3σ, an early event of malignant transformation of respiratory epithelium, also facilitates progression of squamous cell lung cancer,” *Thoracic Cancer*, vol. 6, no. 6, pp. 715–721, 2015.

[17] D. Ravi, Y. Chen, B. Karia et al., “14-3-3σ expression effects G2/M response to oxygen and correlates with ovarian cancer metastasis,” *PLoS One*, vol. 6, no. 1, article e15864, 2011.

[18] N. Iwata, H. Yamamoto, S. Sasaki et al., “Frequent hypermethylation of CpG islands and loss of expression of the 14-3-3σ gene in human hepatocellular carcinoma,” *Oncogene*, vol. 19, no. 46, pp. 5298–5302, 2000.

[19] Z. Wang, C. G. Tropè, Z. Suo et al., “The clinicopathological and prognostic impact of 14-3-3 sigma expression on vulvar squamous cell carcinomas,” *BMC Cancer*, vol. 8, no. 1, p. 308, 2008.

[20] G. Mühlmann, D. Öfner, M. Zitt et al., “14-3-3 sigma and p53 expression in gastric cancer and its clinical applications,” *Disease Markers*, vol. 29, no. 1, pp. 21–29, 2010.

[21] D. O’Dwyer, L. D. Ralph, A. O’Shea, and G. I. Murray, “The proteomics of colorectal cancer: identification of a protein signature associated with prognosis,” *PLoS One*, vol. 6, no. 11, article e27718, 2011.

[22] C. A. Iacobuzio-Donahue, A. Maitra, M. Olsen et al., “Exploration of global gene expression patterns in pancreatic adenocarcinoma using cDNA microarrays,” *The American Journal of Pathology*, vol. 162, no. 4, pp. 1151–1162, 2003.

[23] D. Cao, A. Maitra, J. A. Saavedra, D. S. Klimstra, N. V. Adsay, and R. H. Hruban, “Expression of novel markers of pancreatic ductal adenocarcinoma in pancreatic nonductal neoplasms: additional evidence of different genetic pathways,” *Modern Pathology*, vol. 18, no. 6, pp. 752–761, 2005.

[24] B. M. Erovic, M. Pelzmann, M. C. Grasl et al., “Mcl-1, vascular endothelial growth factor-R2, and 14-3-3σ expression might predict primary response against radiotherapy and chemotherapy in patients with locally advanced squamous cell carcinomas of the head and neck,” *Clinical Cancer Research*, vol. 11, no. 24, pp. 8632–8636, 2005.

[25] Q. Wu, C. Z. Liu, L. Y. Tao et al., “The clinicopathological and prognostic impact of 14-3-3 protein isoforms expression in human cholangiocarcinoma by immunohistochemistry,” *Asian Pacific Journal of Cancer Prevention*, vol. 13, no. 4, pp. 1253–1259, 2012.

[26] R. Vijayashree, P. Aruthra, and K. R. Rao, "A comparison of manual and automated methods of quantitation of oestrogen/progesterone receptor expression in breast carcinoma," *Journal of Clinical and Diagnostic Research*, vol. 9, no. 3, pp. EC01–EC05, 2015.

[27] W. R. Taylor and G. R. Stark, “Regulation of the G2/M transition by p53," *Oncogene*, vol. 20, no. 15, pp. 1803–1815, 2001.

[28] M. L. Sokolosky, K. M. Stadelman, W. H. Chappell et al., “Involvement of Akt-1 and mTOR in sensitivity of breast cancer to targeted therapy,” *Oncotarget*, vol. 2, no. 7, pp. 538–550, 2011.

[29] J. K. Altman, A. Sassano, and L. C. Platanias, “Targeting mTOR for the treatment of AML. New agents and new directions," *Oncotarget*, vol. 2, no. 6, pp. 510–517, 2011.

[30] B. Blechacz, “Cholangiocarcinoma: current knowledge and new developments," *Gut and Liver*, vol. 11, no. 1, pp. 13–26, 2017.

[31] M. Tsukagoshi, K. Araki, T. Yokobori et al., "Overexpression of karyopherin-α2 in cholangiocarcinoma correlates with poor prognosis and gemcitabine sensitivity via nuclear translocation of DNA repair proteins," *Oncotarget*, vol. 8, no. 26, pp. 42159–42172, 2017.

[32] K. Raychaudhuri, N. Chaudhary, M. Gurjar et al., “14-3-3σ gene loss leads to activation of the epithelial to mesenchymal transition due to the stabilization of c-Jun protein," *The Journal of Biological Chemistry*, vol. 291, no. 31, pp. 16068–16081, 2016.

[33] D. Neupane and M. Korc, “14-3-3sigma modulates pancreatic cancer cell survival and invasiveness," *Clinical Cancer Research*, vol. 14, no. 23, pp. 7614–7623, 2008.