

Expression of endoplasmic reticulum oxidoreductase I- α in cholangiocarcinoma tissues and its effects on the proliferation and migration of cholangiocarcinoma cells

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Wendi Yan,^{1,*} Xue Wang,^{1,*}
Tesi Liu,¹ Liyan Chen,²
Longzhe Han,³ Jing Xu,⁴
Guihua Jin,⁵ Kenichi
Harada,⁶ Zhenhua Lin,¹
Xiangshan Ren^{1,2}

¹Department of Pathology and Cancer Research Center, Yanbian University Medical College, Yanji 133002, People's Republic of China; ²Key Laboratory of Natural Resources of Changbai Mountain & Functional Molecules (Yanbian University), Ministry of Education, Yanji 133002, People's Republic of China; ³Department of Yanbian University Affiliated Hospital, Yanji 133002, People's Republic of China; ⁴Department of Shanxi Medical University Medical College, Taiyuan 030001, People's Republic of China; ⁵Department of Immunology and Pathogenic Biology, Yanbian University College of Medicine, Yanji 133002, People's Republic of China; ⁶Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan

*These authors contributed equally to this work

Abstract: Endoplasmic reticulum oxidoreductase 1- α (ERO1A) is a kind of hypoxia-induced endoplasmic reticulum oxidase that regulates translation and folding of oxidized proteins. This study aimed to explore the clinicopathological significance of ERO1A and the effect on the biological behavior of cholangiocarcinoma (CCA) cells.

Methods: Immunohistochemical staining was used to detect the expression of ERO1A, carcinoembryonic antigen (CEA), and carbohydrate antigen 19-9 (CA19-9) in cholangiocarcinoma. Immunofluorescence staining was performed to detect the subcellular localization of ERO1A in CCA cells. The expression of ERO1A in CCA cells after depletion or over-expression was verified by Western blot assay. Then, the effect of ERO1A on proliferation in CCA cells was verified by MTT assay and colony formation assay. Wound healing assays and migration assays were performed to detect the effect of ERO1A on cell migration ability. Finally, we explored the role of ERO1A in EMT and Akt/mTOR signaling pathway.

Results: In this study, our data demonstrated that ERO1A, CEA, and CA19-9 were expressed in cholangiocarcinoma tissues, and the positive rates were 95%, 95%, and 55%, respectively. The high expression of ERO1A is associated with clinical stage and pathological stage of CCA. In vitro data indicate that deletion of ERO1A can inhibit the proliferation and migration of CCA cells and vice versa. In addition, ERO1A has been shown to be closely related to EMT and Akt/mTOR pathways.

Conclusion: In summary, we found that high expression of ERO1A is associated with poor prognosis in patients, and ERO1A can promote the proliferation and migration of CCA cells. In conclusion, ERO1A can be used as an independent biomarker for predicting the prognosis of CCA.

Keywords: ERO1A, cholangiocarcinoma, survival, prognosis, EMT

Introduction

Cholangiocarcinoma (CCA) is a highly aggressive malignant tumor that represents approximately 10% of all hepatobiliary malignancies.¹ According to anatomical classification, it is roughly divided into intrahepatic cholangiocarcinoma (ICC) and extrahepatic bile duct carcinoma (ECCA). Treatment options for CCA are limited, and overall rates of survival are low.^{2,3} Surgical treatment is the only curative option for all subtypes.⁴ However, the early diagnosis rate of tumors is extremely low, and most tumors are diagnosed in advanced stages. High recurrence rates at least 50–60% remain

Correspondence: Xiangshan Ren
Department of Pathology and Cancer
Research Center, Yanbian University, 977
Gong Yuan Road, Yanji 133002, People's
Republic of China
Tel +86 433 243 5092
Email renxsh@ybu.edu.cn

even after surgical resection⁵⁻⁷ There is no effective diagnostic method to improve the survival rate of patients. Therefore, to find a new biomarker has become an urgent problem.

ERO1A is an oxidoreductase that is present in the endoplasmic reticulum, which regulates hypoxia-induced oxidative protein folding.⁸ This process is mediated by ERO1A and protein disulfide isomerase (PDI) catalysis, where ERO1A helps maintain the oxidative activity of PDI.^{9,10} ERO1A may be associated with apoptosis, infiltration, and endoplasmic reticulum stress (ERS)-induced metastasis.^{11,12} ERO1A is highly expressed in breast cancer, gastric cancer and is associated with the proliferation and migration of tumor cells.^{8,13} According to Kukita K, ERO1A is also highly expressed in colon cancer, and it can decrease tumorigenicity *in vivo*.¹⁴ Li G reported that ERO1A plays an important role in the induction of apoptosis.¹⁵ Therefore, ERO1A is highly expressed in various cancers and is closely related to tumor proliferation, migration, and apoptosis. Up to now, there is no relevant research reported on the interaction between ERO1A and cholangiocarcinoma.

In this study, we investigated the expression of ERO1A in cholangiocarcinoma, explored the relationship between ERO1A and clinicopathological features, and explored the effect of ERO1A expression on proliferation and migration of cholangiocarcinoma cells. This study can provide new ideas and effective molecular markers for the clinical treatment of cholangiocarcinoma.

Materials and methods

Tissue specimens

A total of 222 tissue microarrays and 20 cases of cholangiocarcinoma tissues of primary tumors in patients who were not treated before surgery, along with medical records, were collected from Outdo Biotech Co. Ltd. (Shanghai, China) between 2007 and 2016, including 124 cases of ICCA, 62 cases of ECCA, and 36 cases of adjacent tissue. There were 175 cases with survival information (including 104 cases of overall survival data and 71 cases of relapse-free survival data). Survival time was counted from the date of surgery to the follow-up deadline or date of relapse. The study complied with the principles of the Declaration of Helsinki and was approved by the Human Ethics Committee and research ethics committees of Yanbian University Medical College in China.

Cell lines and cell culture

Human cholangiocarcinoma cell line CCKS1 was provided by the Department of Human Pathology at Kanazawa University,¹⁶ and it was approved by Ethics Committee of Kanazawa University. HuCCT1 and TFK1 were obtained from the Health Science Research Resources Bank (Osaka, Japan). Human cholangiocarcinoma cell lines RBE, QBC939 and human normal bile duct epithelial cells HIBepiC were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). HuCCT1, TFK1, RBE, QBC939, and HIBepiC were all cultured in RPMI-1640 (GIBCO, Gaithersburg, MD). The CCKS1 was cultured in Dulbecco Modified Eagle Medium (GIBCO, Gaithersburg, MD), supplemented with 10% fetal bovine serum (GIBCO, Gaithersburg, MD) and 1% penicillin and streptomycin mixture at 37°C in a humidified incubator with 5% CO₂ atmosphere.

Immunohistochemistry staining

Immunohistochemistry (IHC) staining was routinely carried out using an immunohistochemistry kit (abcam, UK). The tissue sections were deparaffinized and rehydrated. The antigen was retrieved by sodium citrate buffer. Following endogenous peroxidase blocking and incubation in normal goat serum (Vector Lab, Burlingame, CA) for 20 mins, these sections were incubated with ERO1A (1:200, LSBio, USA), CEA (Use directly, zsbio, China), CA19-9 (Use directly, zsbio, China) overnight at 4°C and then with the peroxidase-conjugated secondary antibody (abcam, UK). Color development was performed with 3,3'-diaminobenzidine (DAB) and slides were counterstained with hematoxylin. Neutral resin sealing was performed. The results of immunohistochemical staining were observed under a microscope (Olympus Tokyo, Japan).

Immunohistochemistry score

Semi-quantitative IHC scores were interpreted according to a combination of staining intensity and rate of positive cancer cells. First, the scoring for staining intensity was: 0 point (negative), 1 point (weak), 2 points (moderate), and 3 points (intense). The percentage of positive tumor cells was assigned to five categories, which were 0, <5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; and 4, >75%. After multiplying the above two scores, the following scores 0–12 were obtained and further score was: 0 point, negative; 1–4 points, weakly positive; 6–8 points, moderately positive; and 9–12 points, strongly positive.¹⁷ Positive rate is the percentage of

positive cases with any positive staining score, with 0 point defined as negative. Three pathologists (Xiangshan Ren, Zhenhua Lin, and Tiefeng Jin) examined and scored all tissue specimens.

The depletion and overexpression of ERO1A

Synthetic siRNA for ERO1A (Table 1) and nonsilencing siRNA (negative control, NC) were purchased from RiboBio (Guangzhou, China). The cells were incubated with Opti-MEM (Reduced Serum Medium, Gibco) containing premixed siRNA (50 nM) and 5 μ L of Lipofectamine 3000 (Invitrogen, Carlsbad, CA), and were further incubated for 48 hrs. Lentiviral of overexpression ERO1A were constructed by VectorBuilder Inc (Table 1). The TFK1 cells were infected with 500 μ L of complete medium containing 5 μ g/mL of polybrene and 5 μ L of lentiviral vector (titer= 1×10^6 TU/mL). At 8 hrs after infection, fresh medium was replaced and maintained for a further 72 hrs. ERO1A-transduced cells were then incubated with 1 μ g/mL of puromycin in culture medium for 7 days to select stably transfected cells.

Western blot analysis

Total proteins were extracted from cultured cells using pierce T-PER protein extraction reagent (cwbiotech, China) and a sonicator (SONICS, USA). Protein quantification with BCA (Beyotime, Shanghai, China) Kit. Samples were denatured in sodium dodecyl sulfate (SDS) sample buffer. Total proteins were separated by loading 40 μ g of total cell lysate onto SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with primary antibodies against ERO1A (1:1,000, LSBio, USA), actin (1:3,000, Abcam, USA), p-Akt (1:1,000, CST, USA), Akt (1:1,000, CST, USA), p-4EBP1 (1:1,000, CST, USA), 4EBP1 (1:1,000, CST, USA), p-S6 (1:1,000,

CST, USA), S6 (1:1,000, CST, USA), E-cadherin (1:1,000, CST, USA), Vimentin (1:1,000, CST, USA), Snail (1:1,000, CST, USA), Slug (1:1,000, CST, USA), MMP-2 (1:1,000, CST, USA) and MMP-9 (1:1,000, CST, USA), and enzymatic signals were visualized using a chemiluminescence kit (Beyotime Biotechnology, China).

Immunofluorescence staining

The cells were seeded on 6-well culture plates at a density of 20,000 cells per well. The next day, the cells were fixed with 4% paraformaldehyde for 15 mins and permeabilized using 0.1% Triton X-100 for 10 mins (CWbio, China). After blocking, the cells were incubated with a primary antibody against ERO1A (1:100, LSBio, USA) for overnight at 4°C. Alexa-488 (1:500, GeneCopoeia USA) was used as a secondary antibody, and nuclei were stained with 4',6-diamino-2-phenylindole (Solarbio, China). The fluorescence signal was detected using a fluorescence microscope (Olympus Tokyo, Japan).

Proliferation assay

HuCC1, QBC939, and TFK1 cells were seeded on 96-well plate at a density of 5,000 cells per well (n=6). After incubation for 48 hrs, 72 hrs, and 96 hrs at 37°C, the cells were incubated with 100 μ L MTT (1 mg/mL, Amresco, USA) for 4 hrs. After adding 100 μ L of DMSO (Amresco, USA), the value of absorbance was measured at a wavelength of 490 nm using a full-wavelength microplate reader (TECAN, Switzerland).

Colony formation assay

After cell silencing and lentiviral vector overexpression of ERO1A, cells in logarithmic growth phase were seeded in 6-well plates at a density of 500 cells per well. After the cells were incubated for 15 days, cells were then fixed with 4% paraformaldehyde for 15 mins and stained with 1% crystal violet (Solarbio, China). Statistical analysis was performed using Image J software.

Wound healing assay

Cells were seeded on 6-well plates, and wounds were created by 200 μ L micropipette tip when cells were fused to 80%, replace the fresh medium. The effect of ERO1A on wound closure was observed by microscopy (Olympus, Japan). The results were analyzed by Image-J software.

Table 1 Interference sequences of ERO1A

| Gene | Interference sequence of ERO1A |
|---------------------------|---|
| siERO1A-1 | 5'-GGACTGTGCTGTCAAACCA-3' |
| siERO1A-2 | 5'-GACCAAGCATGATGATTCT-3' |
| siERO1A-3 | 5'-GCATTTGAGTGCAAGATAT-3' |
| VB160420-1011mqh (Vector) | https://en.vectorbuilder.com/vector/VB160420-1011mqh.html |
| VB181120-1445huv (ERO1A) | https://en.vectorbuilder.com/vector/VB181120-1445huv.html |

Cell migration assay

Cell migration analysis was carried out using transwell inserts (Costar, Corning Incorporated, USA). Cells with medium containing 1% FBS were seeded in upper chamber. HuCCT1 and QBC939 cells were inoculated with 50,000 cells per pore, TFK1 cells with 100,000 cells per pore. The medium containing 10% FBS was placed in the lower chamber. HuCCT1 cells were incubated for 24 hrs, and QBC939 and TFK1 cells were incubated for 96 hrs. The cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of migrated cells was counted using microscope (KX4, Olympus, Japan).

Statistical analysis

Statistical analyses were primarily performed using SPSS17.0 (SPSS, Chicago, IL), and graphical interpretations were generated with GraphPad Prism 6 (GraphPad Software, San Diego, CA). The chi-square test was used to

evaluate the correlation between ERO1A expression and clinicopathological features (statistics using percentages). The Kaplan–Meier method was used for the analysis of survival curves, and statistical significance was assessed using the Log-rank test. Cox proportional hazards regression model was used to examine univariate and multivariate hazard ratios for the study variables. Differences between groups were analyzed using a two-way ANOVA, and group comparisons for continuous data were done by a one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

The expression of ERO1A in CCA

IHC staining was performed to detect the expression of ERO1A in cholangiocarcinoma (Figure 1A and B). The results showed that the positive staining ratio of ERO1A in cholangiocarcinoma was 84.9% (158/186), but in the

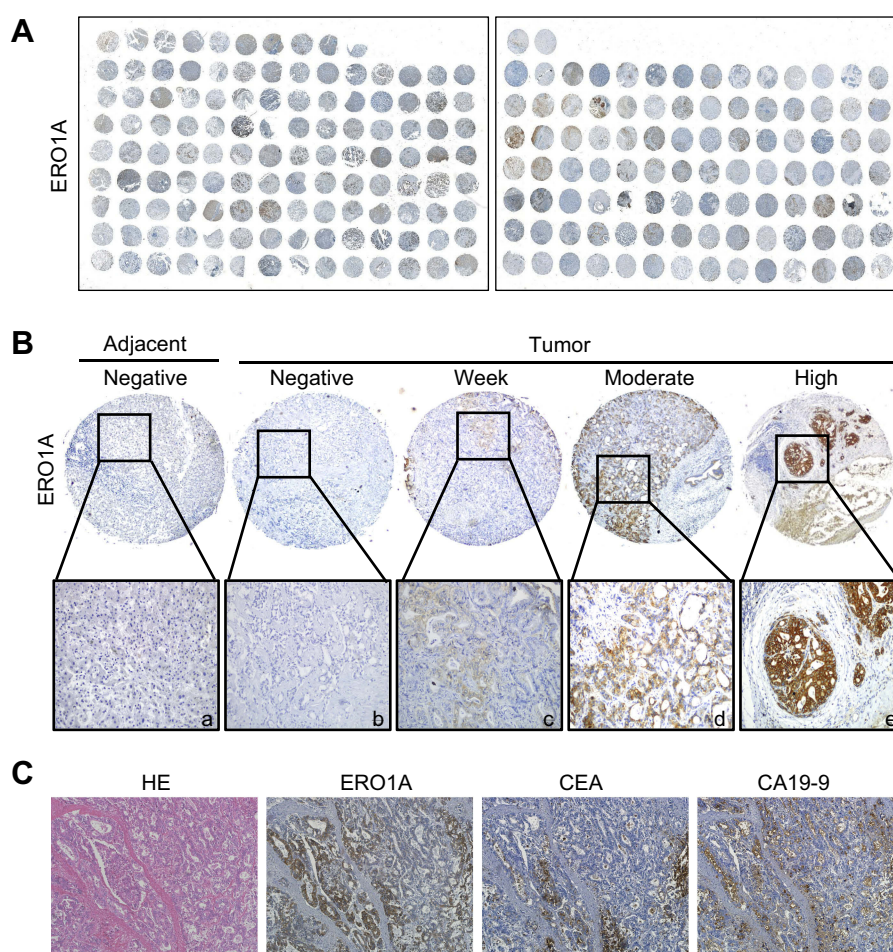


Figure 1 ERO1A protein expression in CCA tissues. **(A)** IHC staining for ERO1A protein expression in 222 cases of CCA tissue microarray. **(B)** a: Negative ERO1A staining in normal liver tissue; b: Negative ERO1A staining in CCA; c: weakly positive ERO1A staining in CCA; d: moderate positive ERO1A staining in CCA; e: strongly positive ERO1A staining in CCA (400×). **(C)** The expression of ERO1A, CEA, and CA19-9 in CCA tissue (200×).

Table 2 The ERO1A protein expression in cholangiocarcinoma

| Diagnosis | N | ERO1L expression | | | | Positive rate % |
|--------------------|-----|------------------|------|--------|---------|-----------------|
| | | Negative | Weak | Medium | Intense | |
| Cholangiocarcinoma | 186 | 28 | 36 | 59 | 63 | 84.90% |
| Normal tissues | 36 | 36 | 0 | 0 | 0 | 0.00% |
| ≤ 2 cm | 29 | 2 | 3 | 10 | 14 | 93% |
| > 2 cm | 149 | 23 | 32 | 46 | 48 | 84.60% |
| ICCA | 124 | 23 | 24 | 38 | 39 | 81.50% |
| ECCA | 62 | 3 | 12 | 21 | 26 | 95% |

Note: Positive rate is the percentage of positive cases with any positive staining score.

Table 3 The expression of ERO1A, CEA, and CA19-9 in cholangiocarcinoma

| Diagnosis | N | ERO1L expression | | | | Positive rate % |
|-----------|----|------------------|------|--------|---------|-----------------|
| | | Negative | Weak | Medium | Intense | |
| ERO1A | 20 | 1 | 4 | 6 | 9 | 95.00% |
| CEA | 20 | 1 | 5 | 6 | 8 | 95.00% |
| CA19-9 | 20 | 9 | 6 | 4 | 1 | 55.00% |

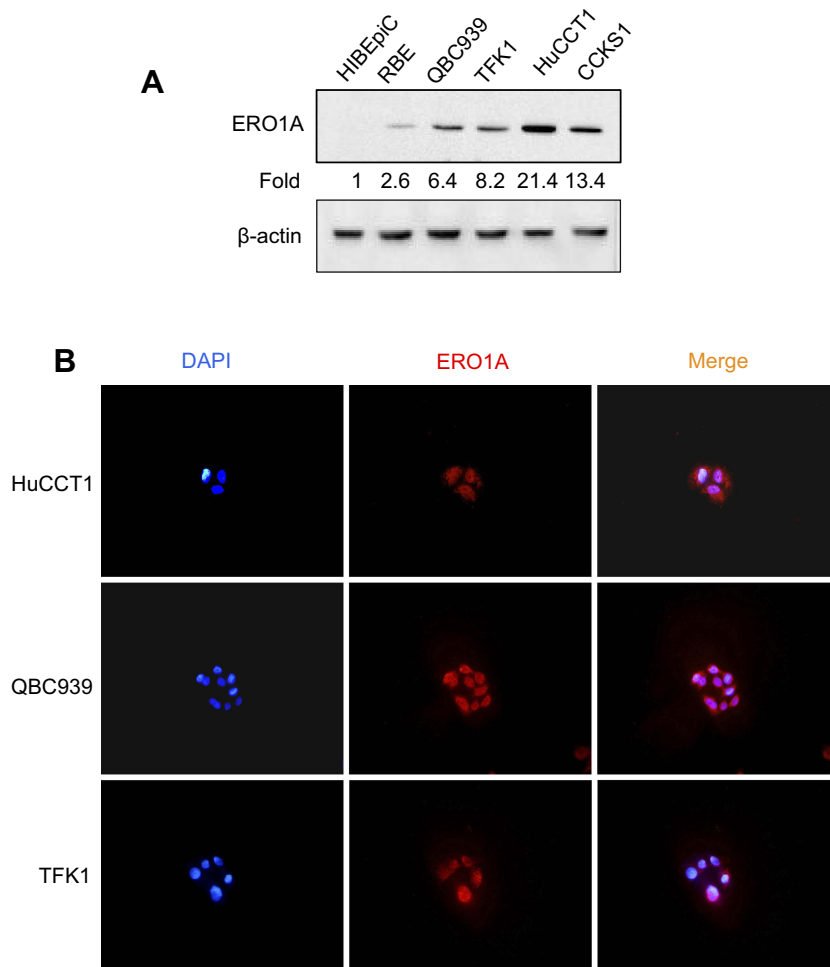


Figure 2 ERO1A protein expression in CCA cell lines. **(A)** Western blot analysis of ERO1A expression. **(B)** Localization of ERO1A was detected by immunofluorescence assay, and cytoplasmic staining was observed (400 \times).

adjacent non-tumor tissues was 0% (0/36) (Table 2). The expressions of ERO1A and CEA, CA19-9 were also compared, and the positive staining ratio was 95%, 95%, and 55% (Figure 1C, Table 3), respectively. Next, we examined the expression of ERO1A in cholangiocarcinoma cells. Western blot analysis revealed that protein levels of ERO1A were markedly upregulated in CCA cells (HuCCT1, CCKS1, QBC939, TFK1, and RBE) compared to normal bile duct epithelial cells (HIBepiC) (Figure 2A). Immunofluorescence staining showed that ERO1A was mainly expressed in cytoplasm (Figure 2B).

Correlations between ERO1A expression and clinicopathological features in CCA

After confirming the high expression of ERO1A in cholangiocarcinoma, we further explored the correlation between ERO1A and clinicopathological features of patients with cholangiocarcinoma. Chi-square test showed that high expression of ERO1A was closely related to clinical stage ($P<0.05$) and pathological stage ($P<0.05$), but there was no significant correlation with age, gender, or tumor size (Table 4).

High ERO1A expression was an independent biomarker for poor prognosis in CCA

To investigate the relationship between ERO1A expression and survival in patients with CCA, we analyzed overall survival (OS), recurrence-free survival (RFS), and median survival. The results showed that patients with high expression of ERO1A had a significantly shorter overall survival ($P<0.05$), indicating that ERO1A expression in CCA patients may have a significant impact on patient survival (Figure 3A–C). We further validated the above results by univariate and multivariate Cox regression analyses model, confirming the expression of ERO1A ($P<0.05$), tumor size ($P<0.05$), which is an independent risk factor for survival in patients with CCA and is significantly associated with poor prognosis in patients with CCA (Table 5).

The effect of ERO1A on the proliferation and migration ability of CCA cells

We further explored the effects of ERO1A on the biological behavior in CCA. Depleted or overexpressed ERO1A was verified by Western blotting (Figure 4A and B,

Table 4 Correlation between ERO1A expression and clinicopathological features of cholangiocarcinoma cancer patients (statistics using percentages)

| Clinical parameter | N | ERO1A expression | | P-value |
|-----------------------|-----|------------------|-----------|---------|
| | | Low (%) | High(%) | |
| Age (years) | | | | |
| <60 | 99 | 34(34.3) | 65(65.7) | 0.881 |
| ≥60 | 88 | 29(33) | 59(67) | |
| Sex | | | | |
| Male | 110 | 39(35.4) | 71(64.6) | 0.574 |
| Female | 77 | 24(31.2) | 53(68.9) | |
| Tumor size | | | | |
| >5 cm | 10 | 3(30) | 7(70) | 0.102 |
| ≤5 cm | 74 | 15(20.3) | 59(79.7) | |
| Lymphatic invasion | 45 | 14(31.1) | 31(68.9) | 0.879 |
| No lymphatic invasion | 90 | 29(32.2) | 61(67.8) | 0.508 |
| Present | 38 | 10(26.3) | 28(73.7) | |
| Absent | 50 | 11(22) | 39(78) | |
| T classification | | | | 0.009* |
| T1 | 7 | 3(42.9) | 4(57.1) | |
| T2 | 61 | 13(21.3) | 48(78.7) | |
| T3 | 17 | 5(29.4) | 12(70.6) | |
| T4 | 3 | 1(33.3) | 2(66.7) | |
| N classification | | | | 0.963 |
| N0 | 90 | 27(30) | 63(70) | |
| N1 | 44 | 13(29.5) | 31(70.5) | |
| M classification | | | | 0.000* |
| M0 | 86 | 19(22.1) | 67(77.9) | |
| M1 | 3 | 0(0) | 3(100) | |
| Clinical stage | | | | 0.022* |
| I | 8 | 3(37.5) | 5(62.5) | |
| II | 39 | 7(17.9) | 32(82.1) | |
| III | 27 | 8(29.6) | 19(70.37) | |
| IV | 14 | 4(28.5) | 10(71.4) | |
| Stage | | | | 0.010* |
| I | 16 | 8(50) | 8(50) | |
| II | 112 | 32(28.6) | 80(71.4) | |
| III/IV | 59 | 23(38.9) | 36(61.1) | |
| Relapse | | | | 0.256 |
| Present | 54 | 12(22.2) | 42(77.8) | |
| Absent | 34 | 10(29.4) | 24(70.6) | |

Note: * $P<0.05$.

Figure 6A). MTT assays (Figure 4C and D) and colony formation assays (Figure 4E and G) showed that ERO1A depletion can inhibit the proliferative capacity in HuCCT1 and QBC939 cells. As expected, the migration capacity of HuCCT1 and QBC939 was also reduced (Figure 5A–H).

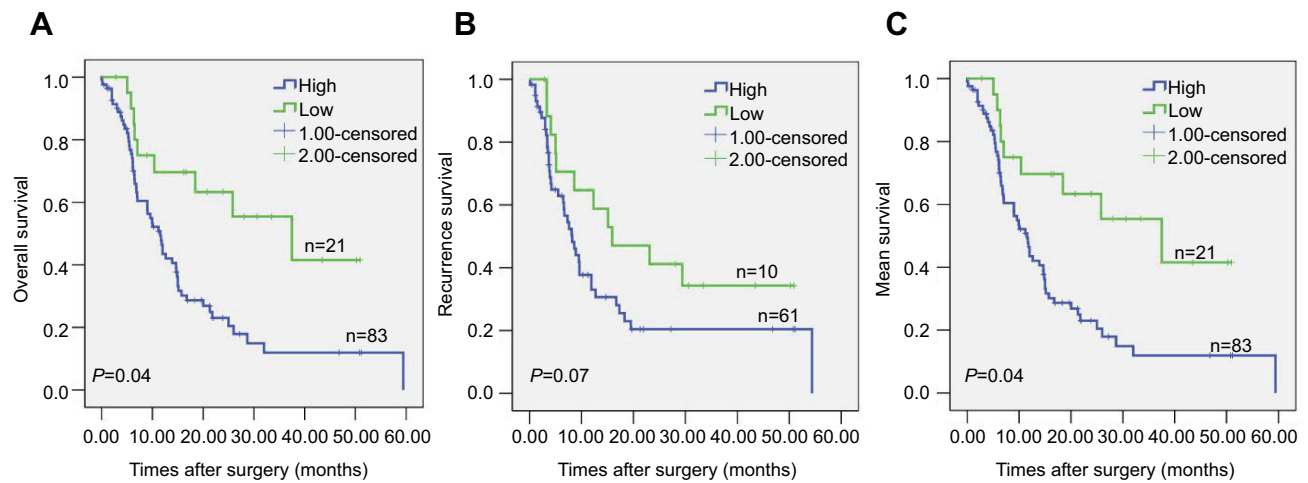


Figure 3 Kaplan–Meier survival curves illustrating the significance of ERO1A in CCA. **(A)** Overall survival of patients with high and low ERO1A expression. **(B)** Recurrence-free survival of patients with high and low ERO1A expression. **(C)** Mean survival of patients with high and low ERO1A expression.

Table 5 Univariate and multivariate analysis of prognosis in patients with cholangiocarcinoma

| | Variable | Hazard ratio | 95.0% CI for Exp(B) | P-value |
|-------------------|--------------------|--------------|---------------------|-------------|
| Univariate | Age | 0.935 | 0.608–1.437 | 0.759 |
| | Gender | 0.746 | 0.484–1.15 | 0.184 |
| | Tumor size | 2.53 | 1.408–4.545 | 0.002* |
| | Relapse | 1.716 | 1.083–2.72 | 0.022* |
| | Vascular invasion | 1.654 | 1.047–2.613 | 0.031* |
| | Stage | 1.487 | 0.927–2.385 | 0.1 |
| | Clinical stage | 1.262 | 0.979–1.628 | 0.072 |
| | Lymphatic invasion | 1.464 | 0.934–2.293 | 0.096 |
| | ERO1A expression | 1.662 | 1.029–2.684 | 0.038* |
| | Multivariate | Tumor size | 1.979 | 1.047–3.739 |
| Relapse | | 1.335 | 0.796–2.239 | 0.274 |
| Vascular invasion | | 1.376 | 0.805–2.352 | 0.244 |
| ERO1A expression | | 1.766 | 1.08–2.886 | 0.023* |

Note: * $P < 0.05$.

In contrast, the proliferation and migration abilities of TFK1 cells were significantly enhanced after overexpression of ERO1A (Figure 6B–H).

ERO1A regulates EMT progression in CCA

In view of the close relationship between EMT and tumor migration,^{18,19} and considering the effect of ERO1A on the metastasis of CCA cells, we examined the role of EMT in CCA by Western blot assays. The results showed that depletion of ERO1A caused a significant increase in E-cadherin, while Vimentin, Snail, and Slug were inhibited in HuCCT1 and QBC939 cells.

As we speculated, the results were reversed in TFK1 cells (Figure 7A). In summary, ERO1A is involved in the regulation of EMT progression in cholangiocarcinoma cells.

ERO1A regulates the Akt/mTOR signaling pathways in CCA cells

The Akt/mTOR signaling pathway plays an important role in cell growth, migration, and other processes. Disorders in signal transduction of this pathway may cause various diseases, including cancer.²⁰ To further explore the mechanism by which ERO1A affects CCA cell proliferation and migration, we examined the

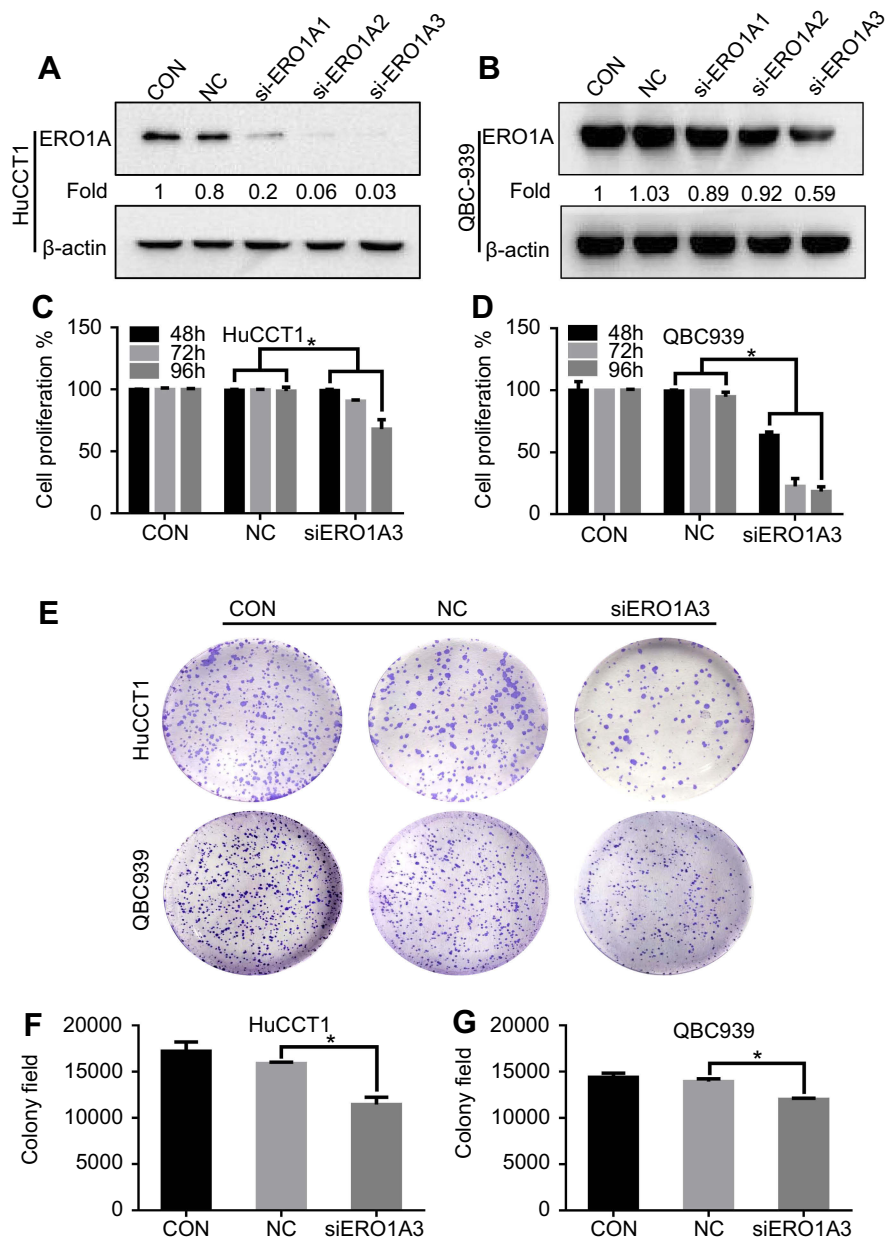


Figure 4 ERO1A depletion reduces CCA cell proliferation, colonization capacities. (A, B) Western blotting was used to detect expression of ERO1A after depletion. (C, D) The proliferation activity of ERO1A was determined by MTT assay in CCA cells. (E) Colony formation assays of HuCCCT1 and QBC939 cells. (F, G) The statistical chart of colony formation assays. * $P < 0.05$ vs NC.

Abbreviations: CON, control; NC, negative control.

expression of the Akt/mTOR signaling pathway. The results of Western blotting showed that the levels of p-Akt, p-4EBP1, and p-S6 were decreased after silencing ERO1A. Conversely, expression of p-Akt, p-4EBP1, and p-S6 was upregulated in TFK1 cells with ERO1A overexpression (Figure 8A). To further clarify this mechanism, we added the Akt signaling pathway inhibitor Akti-1/2, and the results showed that the change of ERO1A protein level was not obvious

(Figure 8B). These results confirm that ERO1A is located upstream of the Akt signaling pathway. However, the more specific molecular mechanism of this result still requires further study.

Discussion

Studies have shown that ERO1A is highly expressed in other cancers, such as gastric cancer, breast cancer, and pancreatic ductal adenocarcinoma, and is associated

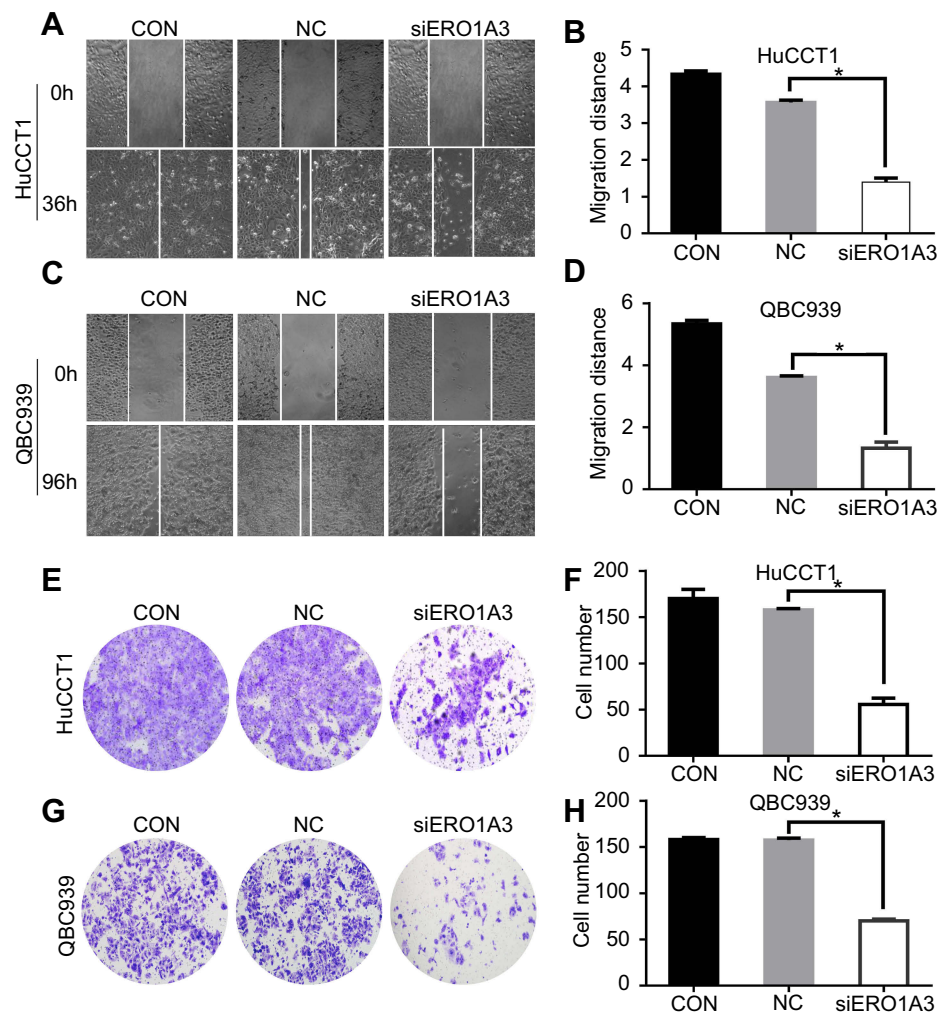


Figure 5 ERO1A depletion reduces CCA cell migration ability. **(A, C)** Wound healing assay was performed to detect the effect of ERO1A on the migration of CCA cells (40×). **(B, D)** The statistical chart of wound healing assay. **(E, G)** Transwell migration assay was performed to detect the effects of si-ERO1A on CCA cells migration ability (200×). **(F, H)** The statistical chart of migration test. * $P < 0.05$ vs NC. **Abbreviations:** CON, control; NC, negative control.

with poor prognosis.²¹ This is consistent with the results of our study. Our research showed that ERO1A was highly expressed in CCA tissues, with a positive rate of 84.9%. The overall survival time of patients with high ERO1A expression was significantly shortened, and ERO1A was closely related to pathological and clinical stages. These results suggest that ERO1A can be used as an independent biomarker for predicting the prognosis of CCA patients.

Due to the lack of early symptoms or reliable tumor biomarkers, most CCA patients have reached an advanced stage when diagnosed. Reliable marker for the diagnosis of CCA is crucial for treatment and prognosis. At present, CEA and CA19-9 are commonly used in clinical diagnosis of cholangiocarcinoma. However, these markers are not specific because CEA and

CA19-9 can be used as diagnostic markers for a variety of cancers and can also be found in some benign diseases such as cholangitis and intrahepatic bile duct stones.^{22,23} Loosen SH et al reported that CEA had more diagnostic values than CA19-9 in cholangiocarcinoma, which was consistent with the results of our study.²⁴ Our results showed that the positive staining rate of ERO1A and CEA in 20 cases of cholangiocarcinoma was 95%, while the positive staining rate of CA19-9 was only 55%. The number of cases we tested was only 20, which did not fully reflect the situation, but it also suggested that it could be an effective molecular marker in cholangiocarcinoma. The specific mechanism remains to be further studied. With the advent of new technologies such as intraductal ultrasound in clinical screening, early CCA testing has become feasible, but these invasive tests

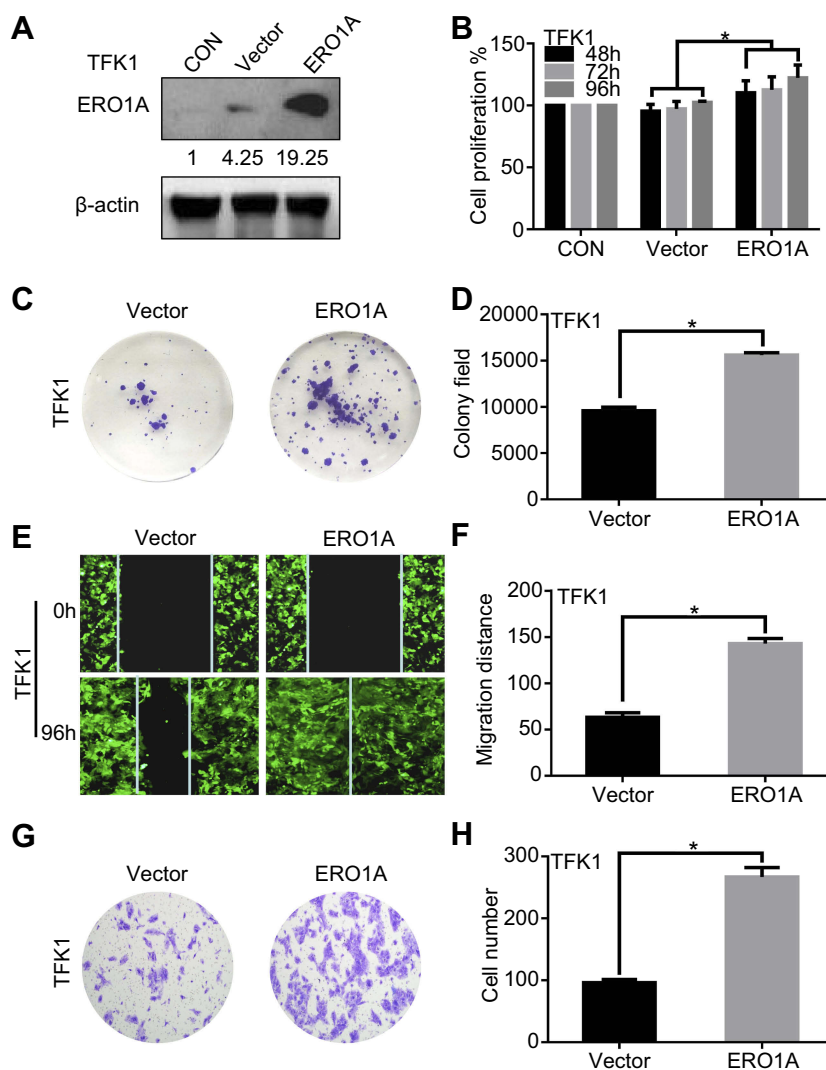


Figure 6 High expression of ERO1A enhances the proliferation and migration of CCA cells. (A) Western blotting was used to detect the expression of ERO1A after lentivirus-transfected in TFK1 cells. (B) MTT assay showed that the proliferation of TFK1 cells was enhanced. (C) Colony formation assays of TFK1 cells. (D) The statistical chart of colony formation assay. (E) Wound healing assay to detect the effects of overexpression ERO1A in CCA cells (40 \times). (F) The statistical chart of wound healing assay. (G) The effects of ERO1A on CCA cells migration ability were detected by migration test (200 \times). (H) The statistical chart of migration test. * $P < 0.05$ vs Vector.

Abbreviation: CON, control.

may cause pain in patients.²⁵ In recent years, tumor biomarkers have played an increasingly important role in diagnosis. According to an international multicenter study, very early cholangiocarcinoma is difficult to diagnose, especially when the tumor size ≤ 2 cm.²⁶ Therefore, we tested the positive rate of ERO1A was 93% when the tumor volume was ≤ 2 cm. However, whether ERO1A can be used clinically as an early diagnostic marker remains to be further studied.

According to the research by Zhang et al,²⁷ ERO1A can regulate the proliferation, migration, and tumorigenesis of cervical cancer. Our hypothesis that ERO1A is involved in the proliferation and migration of cholangiocarcinoma. As

we speculate, our results show that the proliferation and migration of cholangiocarcinoma cells were inhibited after ERO1A depleted, and the ability of proliferation and migration was enhanced when ERO1A was overexpressed.

Previous studies have reported that the expression of several tumor-associated factors is promoted by ERO1A, such as the master regulator for tumor metastasis.²⁸ Therefore, we speculate whether ERO1A is associated with tumor metastasis and EMT. EMT also plays an important role in the development of these tumor phenotypes.^{29,30} Several studies have shown that EMT can accelerate the invasiveness of hepatocellular carcinoma cells.^{31–33} Our results validate our hypothesis that

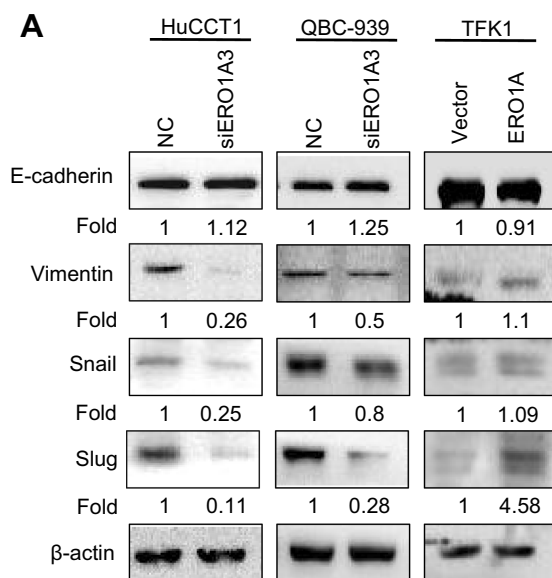


Figure 7 Correlation between ERO1A and EMT progression. (A) Relative expression level of E-cadherin was upregulated, While expression of Vimentin, Snail, Slug, was significantly downregulated in HuCCT1 and QBC939 cells. However, the results in TFK1 cells were the opposite. **Abbreviation:** NC, negative control.

ERO1A can suppress the progression of EMT after depleted, and when it is overexpressed, it can accelerate the EMT process. These results further indicate that ERO1A may affect the migration of cholangiocarcinoma cells by regulating EMT.

The Akt/mTOR pathway regulates many cellular processes including proliferation and survival.³⁴ Previous studies have shown that the proliferation of CCA cells was related to Akt/mTOR pathway.³⁵ In this study, depletion of ERO1A in HuCCT1 and QBC-939 cells can reduce the expression of p-Akt, p-4EBP1, and p-S6, whereas the result of overexpression of ERO1A is the opposite. We are curious about the regulatory mechanism of ERO1A on this pathway, so we did further research. The results showed that the change in ERO1A protein level was not significant after the Akt pathway inhibitor was used. Then, we hypothesize that ERO1A is located upstream of the Akt signaling pathway. However, the specific mechanism of action remains to be further studied.

In conclusion, we indicated that ERO1A is aberrantly overexpressed in patients with cholangiocarcinoma and is closely related to its poor prognosis. As a cancer-promoting gene, ERO1A can accelerate the proliferation and migration of tumor cells. Therefore, ERO1A can be a promising biomarker and a potential therapeutic target for judging the prognosis of cholangiocarcinoma.

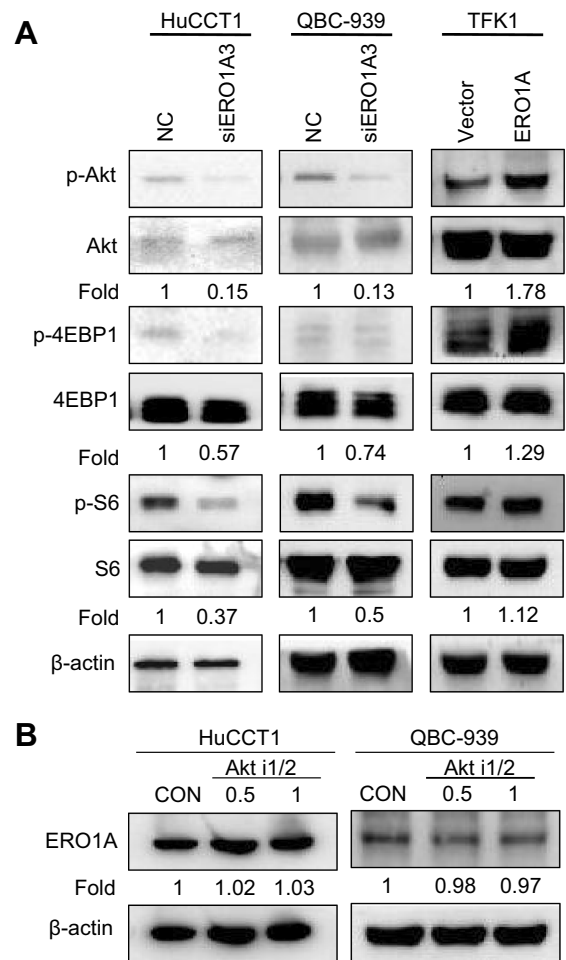


Figure 8 Correlation between ERO1A and Akt/mTOR signaling pathway. (A) Relative expression level of p-Akt, p-4EBP1 and p-S6 was significantly downregulated in HuCCT1 and QBC939 cells. However, in TFK1 cells, the results were reversed. (B) Relative protein expression level of ERO1A after Akt i1/2 was used. **Abbreviations:** CON, control; NC, negative control.

Highlights

- Endoplasmic reticulum oxidoreductase 1- α is overexpressed in cholangiocarcinoma and is a potential predictor of poor prognosis in patients with cholangiocarcinoma.
- Endoplasmic reticulum oxidoreductase 1- α is closely related to the proliferation and migration ability of cholangiocarcinoma cells.

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Disclosure

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