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Inhibition of KLHL21 prevents cholangiocarcinoma progression through regulating cell proliferation and motility, arresting cell cycle and reducing Erk activation

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ABSTRACT

Kelch-like family member 21 (KLHL21) is involved in cell mitosis and motility. Nevertheless, the clinical significance and biological function of KLHL21 in cholangiocarcinoma (CCA) are elusive. This is the first study to describe a pivotal role for KLHL21 in the progression of CCA. The expression of KLHL21 was elevated in CCA tissues compared with paired normal bile duct tissues. In addition, immunohistochemical and statistical analyses demonstrated that the expression of KLHL21 correlated inversely with tumor histological grade ($p < 0.05$) and the overall survival of patients ($p < 0.01$). In CCA cells, we found that the inhibition of KLHL21 significantly reduced proliferation, migration and invasion. Further results indicated that inhibition of KLHL21 triggered G0/G1 cell cycle arrest, leading to the increased expression of P21 and P27 and decreased expression of Cyclin E1, which eventually resulted in proliferation suppression in CCA cells. Furthermore, KLHL21 knockdown alleviated the activation of the Erk signaling pathway via decreasing the expression of phospho-Erk1/2. Our data demonstrated that KLHL21 plays an essential role in the tumorigenesis and progression of CCA, implying that it might serve as a potential therapeutic target for CCA treatment.

1. Introduction

For over the past four decades, cholangiocarcinoma (CCA) has been one of the most common hepatic malignancies worldwide [1–3]. Only 10% of patients with CCA are surgically resectable due to tumor recurrence and metastasis, which leads to a poor prognosis [4]. Recently, with the rise of molecular targeted therapy, biomarker-driven trials gradually become indispensable for cholangiocarcinoma treatment [5,6]. Therefore, novel potential targets need to be explored in CCA prevention and treatment.

KLHL21 is a member of the kelch-like (KLHL) gene family [7]. It has been reported that members of this family are involved in numerous cellular and molecular processes such as tumorigenesis, inflammatory responses, oxidative stress responses, embryonic development and lymphogenesis [8–12]. A previous study has demonstrated that KLHL21 is necessary for successful cytokinesis by mediating the chromosomal passenger complex to translocate from chromosomes to the spindle midzone during anaphase [13]. In addition, KLHL21 is also involved in cell migration and has been

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identified as a regulator of cell motility by spatially and temporally regulating microtubule dynamics at the cell cortex and focal adhesions [14]. However, the expression features, clinical relevance and biological function of KLHL21 in CCA remain elusive.

In the present study, we identified the expression of KLHL21 in CCA and its associations with clinicopathology variables and the survival of patients. We also explored the function of KLHL21 in carcinogenesis of CCA and the underlying mechanisms related to functional roles of KLHL21 in CCA. Our results demonstrate that KLHL21 might be a novel target for the treatment of patients with CCA.

2. Materials and methods

2.1. Patients and tissue specimens

The Servicebio 1.5 mm CCA Tissue Microarray (TMAs) (59 cases, Servicebio, Wuhan, China) containing 59 pairs of cholangiocarcinoma and adjacent normal tissues were utilized to detect the expression of KLHL21. These tissues were collected from cholangiocarcinoma patients who underwent surgical resection without adjuvant therapy between 2010 and 2015 at the Department of Hepatobiliary Surgery, First Affiliated Hospital of Zhejiang University (Zhejiang China). The study was approved by the First Affiliated Hospital of Zhejiang University Ethics Committee, and signed informed consent was obtained from every patient.

2.2. Cell lines and cell culture

Two human CCA cell lines, RBE and CCLP1, were utilized in this study. RBE was obtained from the Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China) and CCLP1 was sourced from the Japanese Cancer Research Resources Bank (JCRB, Japan). CCA cells were cultured in RPMI1640 supplemented with 10% FBS and maintained in an atmosphere containing 5% CO_2 at 37 °C.

2.3. Cell proliferation assay

Proliferation of RBE and CCLP1 were measured using a Cell Counting Kit-8 (Dojindo, Japan). Specifically, cells were seeded in 96-well plates after transfection and cultured in a 37 °C incubator for 6, 24, 48, 72 and 96 h after seeding. The absorbance at 450 nm was detected by Varioskan Flash (Thermo Scientific, America).

2.4. Colony formation assay

After transfection, 1×10^3 CCA cells per well were seeded in 6-well plates and cultured at 37 °C in an incubator for 10 days. Then, cells were stained with crystal violet, counted and photographed.

2.5. Analysis of migration and invasion

The migration and invasion of RBE and CCLP1 cells were evaluated using Transwell chambers assays. Filters were covered with Matrigel (BD Biosciences) for invasion assays. Two-hundred μL of serum-free medium containing 4×10^4 transfected cells were added into the upper chamber and 800 μL of cell culture medium containing 10% FBS were added into the lower compartment. The migrating or invading cells were stained with crystal violet and photographed.

2.6. Cell cycle analysis

For the cell cycle analysis, CCA cells were harvested, fixed in 75% ethanol and stored at 4 °C overnight. Then, the cells were stained with DNA Prep (Beckman Coulter, Brea, CA, USA), and flow cytometry was performed to detect the percentage of cells in different phases according to their DNA content.

2.7. Cell apoptosis analysis

To analyze cell apoptosis, CCA cells were harvested after transfection in 6-well plates. Then, cells were washed with PBS and stained with Annexin V-FITC and propidium iodide according to the manufacturer's protocols. An FACS Calibur flow cytometer (BD Biosciences, America) was utilized to determine CCA cell apoptosis.

2.8. KLHL21 small interfering RNA (siRNA) transfection

Two CCA cell lines, RBE and CCLP1, were transfected with KLHL21 siRNA using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol. The targeting sequences were: 5′-CGUCCAUGAAUCAGGUACATT-3′ for KLHL21-siRNA1 and 5′-GCAUCUUCCGCCAGUUCAUTT-3′ for KLHL21-siRNA2. The protein expression of KLHL21 in RBE and CCLP1 was detected using the western blot technique after transfection for 72 h.

2.9. RNA extraction and quantitative real-time polymerase chain reactions (qRT-PCR)

The total RNA from cells was extracted using TRIZOL (Thermo Fisher Scientific) and cDNA was synthesized using PrimeScriptTM RT Master Mix (TAKARA). Then, qRT-PCR was performed using 7900HT Fast Real-Time PCR System (ABI America). The primers for PCR were: GAPDH, sense 5′-GAGCCAAAAGGGTCATCATCT-3′ and antisense 5′-TTCCACGATACCAAAGTTGTCA-3'; KLHL21, sense 5′-GACTGCGAC- TCTAAACGGACTC-3′ and antisense 5′-ATG-TATTGTCGTATCCCCCAGA-3'.

2.10. Western blot analysis

Tissues and cells were homogenized in RIPA buffer (Thermo Fisher Scientific) to extract the proteins. BCA Protein Assay Kit (Pierce) was utilized to detect the concentration of extracting proteins. The proteins were separated by electrophoresis, transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) and then blocked for 1 h. Different primary antibodies and relevant secondary antibodies were used to incubate with the membranes. The primary antibodies used in this study were GAPDH (#ab8245), KLHL21 (#ab111604), P21 (#2947S), P27 (#3686S), Cyclin E1 (#4129S), Erk1/2 (#9102S) and p-Erk1/2 (#9101S). An EZ-ECL chemiluminescence detection kit (Biological Industries) was used to detect the signals. GAPDH was utilized as the loading control.

2.11. Immunohistochemistry

The CCA TMAs containing 59 pairs of cholangiocarcinoma and adjacent normal bile duct tissues were used for immunohistochemical staining to detect the expression of KLHL21. The TMAs were deparaffinized and dehydrated before antigen retrieval. After cooling, the TMAs were exposed to anti-KLHL21 antibody (diluted 1:200, #ab111604) overnight at 4 °C. Then, the TMAs were rinsed and washed in PBS and incubated with HRP-conjugated secondary antibody for 30 min at 37 °C. Staining intensity for KLHL21 scores were: 0 (no color reaction), 1 (mild reaction), 2 (moderate reaction) and 3 (intense reaction). The extent of staining was scored as: 0 (0),

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