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Enhanced expression of lncRNA TP73-AS1 predicts adverse phenotypes for cholangiocarcinoma and exerts oncogenic properties *in vitro* and *in vivo*

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ABSTRACT

Cholangiocarcinoma (CCA) is one of the most aggressive malignancies with increasing incidence worldwide. Various evidence documents that abnormally expressed long non-coding RNAs (lncRNAs) play important roles in tumorigenesis and progression. TP73-AS1 is a novel cancer-related lncRNA that contributes to the development of several malignancies. However, its clinical value and potential effects on CCA remains unknown. RT-qPCR was used to measure the expression levels of TP73-AS1 in CCA tissues and paired non-tumor tissues and the association between TP73-AS1 expression and clinicopathological characteristics was analyzed. In addition, the functional roles of TP73-AS1 in CCA were detected both *in vitro* and *in vivo*. The results illustrated that TP73-AS1 transcription is enhanced in both CCA tissue samples and cell lines, and this upregulation is closely associated with larger tumor size ($p = 0.008$) and advanced TNM stage ($p = 0.026$) in patients with CCA. For the part of functional assays, silencing of TP73-AS1 could attenuate CCA cell growth both *in vitro* and *in vivo*. Additionally, silencing of TP73-AS1 facilitates apoptosis *via* activating caspase-3 and caspase-9. Importantly, TP73-AS1 expression did not affect HIBEC cell growth and apoptosis. Moreover, TP73-AS1 could also facilitate migration and invasion potential of CCA cells. Collectively, these findings may help to develop a potential therapeutic target for the patients with CCA.

1. Introduction

Cholangiocarcinoma (CCA) is a malignancy originated from neoplastic transformation of bile duct epithelial cells located in the intrahepatic or extrahepatic biliary tract. It is the second most common malignancy of primary hepatobiliary neoplasms according to public data [1]. According to its anatomic location, CCA is generally classified into three types, intrahepatic cholangiocarcinoma (ICC), hilar cholangiocarcinoma (HCC) and extrahepatic cholangiocarcinoma (ECC) [2]. The high mortality of patients diagnosed with CCA is partly due to most of the cases being diagnosed at too late stages to allow radical surgery, which is the most powerful therapy to cure CCA and leads to long-term survival [3]. Unfortunately, even surgical resection is correlated with a high rate of recurrence that results in a 5-year overall survival of 10% [4]. What is worse, this malignancy is resistant to common chemotherapy or radiotherapy [5]. Therefore, novel effective therapeutic targets and prognostic biomarkers for CCA patients are crucial and imperative.

Recent advances of whole-genome sequencing technology have made great contribution to the discovery of noncoding RNAs world [6,7]. Long non-coding RNAs (lncRNAs) are transcripts > 200 nucleotides in length that lack protein-coding potential [8,9]. They are extensively existed in mammalian cells and involved in numerous biological processes. Recently, lncRNAs have been demonstrated sensitive in the diagnosis and prognosis prediction for several malignancies [10–12]. Several evidence also ulteriorly documented that they functioned as an oncogene or tumor suppressor *via* sponging miRNAs [13]. For example, lncRNA TUG1 promotes cell proliferation and metastasis by negatively regulating miR-300 in gallbladder carcinoma [14]. However, the involvement of lncRNAs in CCA is not well established.

LncRNA TP73-AS1, which is located at chromosome 1p36, is reportedly associated with cell growth and tumor progression in glioma, esophageal squamous cell carcinoma, and breast cancer [15–17]. Zou et al. reported that TP73-AS1/miR-200a/ZEB1 axis could promote

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breast cancer cell migration and invasion [17]. Although TP73-AS1 has been regarded as a pivotal regulator in cancer progression, the clinical values and exact functions of TP73-AS1 in CCA are still unknown. Thus, we designed this study to investigate the expression level and clinical roles of TP73-AS1 in CCA patients. In addition, cell proliferation, apoptosis, migration and invasion altered by TP73-AS1 were evaluated after TP73-AS1 silenced. In summary, our findings illuminate the important roles of TP73-AS1 in tumorigenesis and progression of CCA and shed new light on the lncRNA-directed therapeutics in CCA.

2. Materials and methods

2.1. Patients and tissue collection

All the tissue samples were obtained from patients who underwent surgical resection at the Second Affiliated Hospital of Harbin Medical University between 2015 and 2017. The study was carried out with the approval of the Ethics Review Committees of Harbin Medical University. All the patients voluntarily joined this study with informed consents and didn't receive preoperative radiation or chemotherapy. All the specimens were examined by pathologists before preserved in liquid nitrogen. Normal bile duct tissues adjacent to the tumors were used as controls. Complete clinic-pathological follow-up data for the patients were then collected.

2.2. Cell lines and culture

Human CCA cell lines (HCCC-9810 and RBE) used in the study were commercially obtained from the Cell Bank of Type Culture of Chinese Academy of Sciences (Shanghai, China). The other CCA cell lines (QBC939, Huh-28, HuCCT1, KMBC and CCLP-1) as well as human intrahepatic biliary epithelial cell (HIBEC) were preserved in our laboratory. The cells were cultured in RPMI-1640 supplemented with 10% FBS (Invitrogen Life Technologies, Carlsbad, CA, USA) in a humidified incubator with 5% CO_2 at 37 °C.

2.3. RT-qPCR analysis and cell transfection

Total RNA from tissues or cells were isolated by using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity number (RIN) analysis was performed using an Agilent 2100 Bioanalyser and RNA 6000 LabChip kit with Agilent 2100 Expert software (Agilent Technologies). cDNA was then synthesized by using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) followed by the manufacturer's directions. FastStart Universal SYBR Green Master Kit (Roche, Germany) and a BIO-RAD C1000 Thermal Cycler was used to perform RT-qPCR. All primer sequences are summarized below: TP73-AS1, Forward: 5′- CCGGTTTTCCAGTTCTTGCAC -3′; Reverse: 5′- GCC TCACAGGGAAACTTCATGC -3′. GAPDH, Forward: 5′- GGGAGCCAAA AGGGTCAT -3′; Reverse: 5′- GAGTCCTTCCACGATACCAA -3′. The siRNA/shRNA specifically targeting TP73-AS1 and si-NC/shNC were constructed by GenePharma (Shanghai, China). Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, USA) was used for transfection according to the protocol recommended by the manufacturer. After 48 h transfection, the cells were harvested for further assays.

2.4. Cell counting Kit-8 (CCK-8) assays

CCK-8 assays were used to measure CCA cell viability altered by TP73-AS1. Transfected QBC939 and Huh-28 cells were seeded in 96-well plates at 1500 cells/well. 10 μl of CCK-8 solution (Dojindo, Japan) was added into each well before incubated at 37 °C atmosphere for 2 h. A microplate reader (Tecan, Männedorf, Switzerland) was used to quantify and record the absorbance at 450 nm.

2.5. Colony formation assays

Colony-forming assays were performed to evaluate the clonogenic ability of transfected CCA cells. In brief, cells were digested by tyrisin and suspended into single cells before seeded into six-well plates and cultured for about two weeks. Then, the visible colonies were fixed by 4% paraformaldehyde and stained with crystal violet solution (Beyotime, Beijing, China). At last, the colonies of each well were photographed and counted.

2.6. Flow cytometric analysis for cell apoptosis

Flow cytometric analysis for cell apoptosis was carried out to measure cell apoptosis influenced by TP73-AS1. Transfected cells were trypsinized before suspended with Annexin V-FITC binding buffer. Then, 5 μL of Annexin V and 10 μL of propidium iodide (PI, Beyotime, Beijing, China) were added into each group. The cells were incubated for 15 min in the dark. Afterwards, the stained cells were analyzed by flow cytometry immediately (FACScan, BD Biosciences).

2.7. Caspase-3/-9 activity determination

A caspase-3/9 activity kit (Solarbio, Beijing, China) was used for determination of caspase-3/9 activity in cell lysates following the manufacturer's protocol. In brief, cell proteins were isolated and added into 96-well plates, and then incubated with provided reaction buffer and caspase substrate. Subsequently, reaction mixtures were detected by a microplate reader (Tecan, Switzerland) at a wavelength of 405 nm before incubated at 37 °C for 4 h.

2.8. Transwell assays

The migration and invasion assays were performed using Transwell chambers with 8 μm polycarbonate nucleopore filters (Corning, NY, USA). Transwell assays were performed as previously described [13,18].

2.9. Tumor formation and immunohistochemistry (IHC) assays

BALB/c nude mice (Vital River, Beijing, China) were used for the *in vivo* study. The animal experiments were approved and reviewed by the Animal Care and Use Committee of Harbin Medical University. QBC939 cells were transfected with shTP73-AS1-1, shTP73-AS1-2 or the scrambled control. 1×10^7 cells were injected subcutaneously into either side of the posterior flank of female BALB/c nude mice (6 weeks of age, n = 6 per group). Tumor growth was measured every 2 days and tumor volumes were calculated using the equation: $V = 0.5 \times D \times d^2$ (V, volume; D, longitudinal diameter; d, latitudinal diameter). 12 days later, the mice were sacrificed by cervical dislocation, and the tumors were removed and weighed. IHC study was performed on the paraffin-embedded tumor tissues from nude mice. The location and relative expression of Ki67 (Abcam, Cambridge, MA, USA) was measured by the avidin-biotin-peroxidase method. In addition, total RNA was extracted from the tumors to measure the expression levels of TP73-AS1.

2.10. Statistical analysis

Statistical analyses were performed by using GraphPad Prism 5.01 software (GraphPad software, La Jolla, CA, USA). The clinical significance between TP73-AS1 expression and clinicopathologic variables was calculated by Fisher's exact tests. The results are presented as mean ± standard deviation (SD) from at least three repeats. To compare the significance of two groups, Student's *t*-test was performed. The level of significance was set at $p < 0.05$.