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LncRNA ASAP1-IT1 positively modulates the development of cholangiocarcinoma via hedgehog signaling pathway

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ABSTRACT

Over the past decades, lncRNAs have attracted more and more attentions of researchers. It has been verified that lncRNAs can modulate multiple biological behaviors in various human cancers. LncRNA ASAP1-IT1 has been certified to be a tumor facilitator in several malignant tumors. This study aims to investigate the effects of dysregulated ASAP1-IT1 on biological processes of Cholangiocarcinoma. The high expression level of ASAP1-IT1 was tested in Cholangiocarcinoma tissues and cells with qRT-PCR. Upregulation of ASAP1-IT predicted the unfavorable prognosis for Cholangiocarcinoma patients. Next, ASAP1-IT1 was knocked down in cancerous cells for loss-of function assay. MTT, colony formation and transwell and western bot assays were performed to demonstrate the specific impacts of ASAP1-IT1 on proliferation, migration and EMT progression of Cholangiocarcinoma. Cells. As a results, the Cholangiocarcinoma progression was inhibited. Hedgehog signaling pathway has been discovered to be a treatment target in Cholangiocarcinoma. In this study, the interaction between ASAP1-IT1 and hedgehog pathway was specifically investigated. Smo and Gli1, two hedgehog-related proteins were examined in Cholangiocarcinoma cells. The results of qRT-PCR and western blot assay suggested that ASAP1-IT1 could positively modulate Smo and Gli1 in Cholangiocarcinoma. Finally, rescue assays were carried out to prove that ASAP1-IT1 could improve Cholangiocarcinoma progression and development via hedgehog signaling pathway.

1. Introduction

Cholangiocarcinoma (CCA) has threatened health and happiness of people all over the world, especially the Asia people [1,2]. CCA was acknowledged to be one of the commonest primary hepatic malignancy. The incidence of CCA only is only second to hepatocellular carcinoma [3,4]. According to the different tumor locations, CCA can be divided into intrahepatic cholangiocarcinoma (ICC) and extrahepatic cholangiocarcinoma (ECC) [5]. The major therapeutic methods for CCA are still limited to curative surgery and liver transplantation [6,7]. Although a lot of progress have been made in surgery and chemotherapy, the prognosis of CCA patients remains unfavorable [8]. Therefore, it is quite necessary to find more therapeutic targets for CCA.

As crucial modulators of cellular transcription, long noncoding RNAs (lncRNAs) are longer than 200 nt [9]. According to the previous reports, lncRNAs can act as tumor suppressors or tumor facilitators in human cancers [10–12]. The most significant function of lncRNAs in cancers is to modulate gene expression [13,14]. Additionally, dysregulated lncRNAs affects disease procession [15]. Some lncRNAs have been reported in hepatic carcinoma for their special mechanisms and functions [16–18]. Likewise, this study postulated that dysregulated lncRNA ASPA1-IT1 could improve CCA progression by regulating hedgehog signaling pathway. LncRNA ASAP1-IT1 has been reported in bladder cancer [19], epithelial ovarian cancer [20] and non-small cell lung cancer [21] for its oncogenic properties. However, the function and mechanism of it in CCA are still unclear. ASAP1-IT1 was found to be dysregulated in CCA tissues and cells. The high expression of ASAP1-IT1 predicted the unfavorable prognosis for CCA patients. loss-of function assays were carried out in two CCA cells to prove the specific function of ASAP1-IT1 in CCA cell activities. Hedgehog signaling pathway has been certified to positively modulate tumorigenesis of human cancers [22–24], including CCA progression [25]. However, the

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interaction between hedgehog signaling pathway and lncRNAs was rarely elucidated. This study aims to investigate the interaction between ASAP1-IT1 and hedgehog pathway in CCA cells. According to all experimental results, we concluded that ASAP1-IT1 acted as a tumor facilitator in CCA via modulating hedgehog signaling pathway.

2. Materials and methods

2.1. Tissues samples

68 pairs of human CCA tissues and the adjacent normal tissues were collected from patients who were diagnosed with CCA in Department of General surgery, First Affiliated Hospital of Jiamusi University. We had received ethics consents from each patient before the study. This study had acquired the approval from ethics committee of First Affiliated Hospital of Jiamusi University

2.2. Cell culture and transfection

Cell lines used in this study were all bought from the American Type Culture Collection (Manassas, VA). Cell lines including one normal cell line (KMBC) and five CCA cell lines (HuH-28, QBC939, HuCCT1, CCLP1, RBE). CCA cells were preserved in DMEM medium (Invitrogen, Shanghai, China) which had been mixed with 10% FBS, 100U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen). Cell culture was accomplished at 37 °C in a moist air with 5% CO_2.

To reduce the expression level of ASAP1-IT1 in CCA cells, specific shRNAs and NC-shRNA were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). To overexpress the expression of Smo and Gli1, pcDNA3.1 expression vectors were used to introduce the relative cDNA sequence of Smo and Gli1. All transfections were performed with Lipofectamine® 2000.

2.3. RNA extraction and qRT-PCR analysis

According to the instructions, Trizol Reagent (Invitrogen, CA, USA) was used to extract total RNA from paraffin-embedded tissues or cells. RNA quality was evaluated by using Nanodrop 2000 (Wilmington, DE, USA). RNA integrity was assessed by using Agarose gel electrophoresis. Next, total RNA (1 μg) was reversely transcribed into cDNA by a High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). All reactions were processed on the Bio-Rad CFX96 real-time PCR System with TaqMan assays (Bio-Rad, Foster City, CA, USA). GAPDH was taken as internal reference. The expression levels of genes were calculated with the $2^{-\Delta\Delta Ct}$ method and normalized to control group.

2.4. Cell proliferation assay

Cell viability was efficiently measured by MTT assay. Cells (5000/well) were seeded in a 96-well flat-bottomed plate for 24 h. After cells were transfected as needed, they were maintained in the normal medium. Then, each well was added into MTT solution (5 mg/ml, 20 μl) at different time points (0, 24, 48, 72 and 96 h) and incubated for 4 h. Next, took away the media and added into 100 μl DMSO into each well. The relative number of surviving cells was evaluated by measuring the optical density (OD) of cell lysates at 560 nm.

For colony formation assay, cells were plated in six-well plates at a density of 3000 cells per well. They were then incubated in DMEM (containing 10% FBS) at 37 °C. Two weeks later, cells were washed by PBS and fixed by methanol for 0.5 h. After fixed dots were stained by 1% crystal violet, the number of colonies was manually calculated.

2.5. Cell migration assay

Transwell assay was carried out to assess cell migration ability. 48 h after transfection, cells (5×10^4) were removed from a serum-free medium into the upper chamber of an insert (8 μm pore size; Millipore). At the same time, a medium (containing 10% FBS) was added into the lower chamber.

24 h after incubation, cells stayed on the upper membrane were removed with a cotton wool. However, cells that migrated into the lower membrane were stained with methanol and 0.1% crystal violet. The migrated cells were imaged and calculated with an IX71 inverted microscope (Olympus, Tokyo, Japan).

2.6. Western blot assay

Two days after transfection, CCA cells were digested with trypsin (Beyotime, Beijing, China) and lysed by RIPA lysis buffer (Beyotime, Beijing, China) which had been replenished with protease inhibitors. SDS-PAGE vertical electrophoresis was used to separate the equal amounts of extracted proteins. Next, proteins were transferred onto a 0.45 μm PVDF membrane (GE Healthcare, Piscataway, NJ, USA). Next, the membranes were blocked with 5% defatted milk and diluted in Tris-buffered saline (containing 0.05% Tween-20) for 1.5 h at room temperature. Next, the membrane was incubated with the primary antibodies (E-cadherin, N-cadherin, Smo and Gli1) (Abcam, USA) overnight. After washing, the secondary antibody was used for incubation. Finally, the blots were images by BeyoECL Plus Kit (Beyotime, Beijing, China).

2.7. Statistical analysis

All data were displayed as mean ± SD from more than two independent assays. Prism5 (GraphPad, USA) software was used for statistical analysis. Differences between groups were analyzed with Student's t test or one-way ANOVA. Survival curves were generated with Kaplan Meier method (the log-rank test). Cox proportional hazards model was created to identify the clinicopathological factors correlated with overall survival of CCA patients through a multivariate survival analysis. Data were thought to be statistically significant only when P value less than 0.05.

3. Results

3.1. Dysregulated ASAP1-IT1 is a prognostic factor for CCA patients

To understand the basic role of ASAP1-IT1 in CCA, qRT-PCR analysis was conducted to examine the expression levels of ASAP1-IT1 in different tissues and cell lines. At first, the expression of ASAP1-IT1 was separately tested in CCA tissues and their adjacent normal tissues (Fig. 1A). The high expression levels of ASAP1-IT1 were detected in CCA tissues. Similarly, the expression level of ASAP1-IT1 was tested in both one normal cell and five CCA cells (Fig. 1B). As a result, ASAP1-IT1 was expressed higher in CCA cells than that in normal cells. The highest level of it was examined in CCLP1 and RBE cells. Therefore, these two cell lines were chosen to do next assays. After the expression levels of ASAP1-IT1 were detected in all cancerous tissues, the mean value of ASAP1-IT1 expression was calculated. Using the mean value as the threshold, the experimental samples were divided into two groups: ASAP1-IT1 high expression group (n = 37) and ASAP1-IT1 low expression group (n = 31). The correlation between ASAP1-IT1 expression and clinicopathological features of CCA patients was analyzed. The result indicated that the levels of ASAP1-IT1 expression was correlated with TNM stage, postoperative recurrence, and lymph node metastasis, but not with age, gender and other elements (Table 1). Proportional hazards analysis verified that high level of ASAP1-IT1 expression was a prognostic factor for CCA patients (P = 0. 028*, Table 2). Kaplan Meier method analyzed that CCA patients with high expression of ASAP1-IT1 had lower overall survival rate than those with low expression of ASAP1-IT1 (Fig. 1C).