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Sirtuin7 has an oncogenic potential *via* promoting the growth of cholangiocarcinoma cells

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ABSTRACT

Accumulating evidence indicates that sirtuin7 (SIRT7) plays an oncogenic role in the main types of liver cancer, hepatocellular carcinoma (HCC). Nevertheless, the clinical significance of SIRT7 and its role in cholangiocarcinoma (CCA) is largely undiscovered. Here, we found that SIRT7 displayed higher expression in CCA tissues compared to intrahepatic normal bile duct and surrounding liver tissues based on The Cancer Genome Atlas (TCGA) data. Our data further confirmed that SIRT7 was overexpressed in CCA patient tissues and cell lines. Clinical analysis revealed that high SIRT7 expression was correlated with large tumor size and advanced tumor-node-metastasis (TNM) stage. Furthermore, SIRT7 overexpression independently predicted poor prognosis of CCA patients. Functionally, we demonstrated that SIRT7 knockdown suppressed proliferation and cell cycle progression of HUCCT1 cells *in vitro* and *in vivo*. SIRT7 restoration promoted the growth of QBC-939 cells. Mechanistically, SIRT7 reduced p21 expression and increased the levels of Cyclin D1 and cyclin dependent kinase 2 (CDK2) in CCA cells. Furthermore, microRNA-125b-5p (miR-125b-5p) was recognized as a direct negative regulator of SIRT7 and reduced SIRT7 abundance in CCA cells. Notably, miR-125b-5p restoration showed similar effects to SIRT7 knockdown on the growth of CCA cells. Taken together, we demonstrate for the first time that miR-125b-5p regulation of SIRT7 functions as an oncogene and a potential prognostic biomarker in CCA.

1. Introduction

According to data of Global Cancer Statistics, the incidence and mortality of cholangiocarcinoma (CCA) is increasing around the world [1,2]. Unfortunately, surgery is the only option for curative treatment of CCA [3]. CCA with invasive and metastatic characteristic is a lethal disease with a dismal survival rate [4], which highlights the urgent need for exploring new biomarkers involved in clinical diagnosis and therapy of CCA.

Sirtuins (also known as class III histone deacetylases), nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases, modulate several cellular processes including cell senescence, metabolism, cell cycle, DNA repair, stress response and apoptosis [5]. The essential role of sirtuins has been recognized in various human diseases, such as neurodegenerative disease, cardiovascular disease, diabetes and cancer [6]. Sirtuin 7 (SIRT7), a novel cancer-related gene, plays an essential role in several biological processes including proliferation, cell cycle, chromatin regulation, chemotherapy resistance, cellular transformation and stress response [7]. SIRT7 facilitates anchorage-independent growth and contact inhibition escape of human cancer cells *via* selective deacetylation of acetylated lysine 18 of histone H3 (H3K18Ac) [8]. SIRT7 acts as an oncogene by promoting proliferation and cell cycle progression *in vitro* and enhancing *in vivo* growth of hepatocellular carcinoma (HCC) [9]. Furthermore, miR-125a-5p and miR-125b are recognized as negative regulators of SIRT7 in HCC and bladder cancer [9,10]. And C/EBPα functions as a negative regulator of SIRT7 by recruiting histone deacetylase 3 (HDAC3) to its upstream promoter in HCC cells [11]. Up-regulating SIRT7 expression promotes proliferation, colony formation and mobility of colorectal cancer (CRC) cells *via* activating mitogen-activated protein kinase (MAPK) pathway [12]. Otherwise, 5-Fluorouracil (5-FU) induced downregulation of SIRT7 increases radiosensitivity of human CRC [13]. SIRT7 enhances the metastatic behaviors of non-epithelial sarcoma and epithelial prostate carcinoma cells [14]. While, the role of SIRT7 in tumorigenesis is

Abbreviations: SIRT7, sirtuin7; HCC, hepatocellular carcinoma; CCA, cholangiocarcinoma; TCGA, The Cancer Genome Atlas; TNM, tumor-node-metastasis; CDK2, cyclin dependent kinase 2; NAD^+, nicotinamide adenine dinucleotide; H3K18Ac, acetylated lysine 18 of histone H3; HDAC3, histone deacetylase 3; CRC, colorectal cancer; MAPK, mitogen-activated protein kinase; 5-FU, 5-Fluorouracil; HNSCC, head and neck squamous cell carcinoma

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controversial. SIRT7 expression is downregulated in head and neck squamous cell carcinoma (HNSCC) with advanced stages [15]. Low levels of SIRT7 expression in nuclear are correlated with an aggressive phenotype of pancreatic cancer [16]. These studies suggest that SIRT7 plays a tumor suppressive role in the context of HNSCC and pancreatic cancer. To date, the clinical significance of SIRT7 and its role in CCA are largely unknown.

In this study, on the basis of SIRT7 expression difference between CCA and non-tumor tissues from The Cancer Genome Atlas (TCGA) database, we found that SIRT7 overexpression was associated with malignant clinical features and poor survival of CCA patients. We presented evidence that SIRT7, a direct target of microRNA-125b-5p (miR-125b-5p), promoted proliferation and cell cycle progression of CCA cells probably by modulating p21, Cyclin D1, and cyclin dependent kinase 2 (CDK2) levels. To conclude, our results reveal that SIRT7 is a promising valuable prognostic biomarker and drug target for CCA patients.

2. Materials and methods

2.1. Clinical samples

This study included 80 pair-matched CCA and adjacent normal liver tissues and all samples were collected from patients who underwent operative treatment in the First Affiliated Hospital of Xi'an Jiatong University. The patients did not receive radiofrequency ablation or chemoembolization before surgery. Samples were conserved in liquid nitrogen and 10% formalin, and pathologically confirmed as CCA. The clinicopathologic parameters of patients were shown in Table 1. Ethical approval was obtained from the Research Ethics Committee of Xi'an Jiaotong University. All the patients signed informed consent forms.

2.2. Cell culture and transfection

Human CCA cell lines including HUCCT1, RBE, 9810, QBC-939 and the human intrahepatic biliary epithelial cell line (HiBEC) were obtained from American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured under standard conditions. SIRT7 shRNA, non-targeting (NT) shRNA, SIRT7 expression plasmid (pcDNA3.1-SIRT7) and empty vector were designed and synthesized by GenePharma (Shanghai, China). Precursor miR-125b-5p, precursor miR-152-3p, miR-125b-5p inhibitor and corresponding negative control vectors were purchased from GeneCopoeia™ (Guangzhou, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was employed to perform transfection according to manufactures' protocol.

Table 1
Correlation between the clinicopathological features and SIRT7 expression in cholangiocarcinoma (n = 80).

Characteristics		Total No. of patients	SIRT7 expression		P
			High level (n = 40)	Low level (n = 40)	
Age (years)	< 60	39	16	23	.117
	≥60	41	24	17	
Sex	Male	39	19	20	.823
	Female	41	21	20	
Differentiation grade	Well/ Moderate	68	31	37	.060
	Poor	12	9	3	
Tumor size (cm)	< 5	56	23	33	.015[a]
	≥5	24	17	7	
Metastasis	No	36	14	22	.072
	Yes	44	26	18	
TNM tumor stage	I/II	48	18	30	.006[a]
	III	32	22	10	

TNM, tumor-node-metastasis.
[a] Statistically significant. Data were analyzed by Chi-squared test.

2.3. Immunohistochemistry (IHC)

The paraffin sections of CCA tissues were deparaffinized in xylene and rehydrated in a graded series of ethanol. Antigen retrieval was performed in pH 6.0 citric acid buffer by heat treatment. After blocking in 10% goat serum, the sections were incubated with SIRT7 primary antibody (sc-365344, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. The sections were washed and incubated with biotinylated secondary antibody (ZSGB-BIO, Beijing, China) for 30 min. The staining was detected with the DAB detection system. The final score of each sample was decided by two pathologists depending on the basis of extent and intensity of staining. The intensity of staining was evaluated and scored as follows: no staining = 0, weak staining = 1, moderate staining = 2, and strong staining = 3. The proportion of positively staining cells was divided into three levels and scored as follows: < 25% = 1, 25%–75% = 2, and > 75% = 3. Staining scores for SIRT7 were calculated as follows: intensity of staining multiplied by the percentage of positively staining cells.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted and prepared for qRT-PCR as previously described [9]. Then the cDNA was obtained according to the protocols of PrimeScript Reverse Transcriptase kit (Takara, Dalian, China). PCR amplification of SIRT7 mRNA and miR-125b-5p was carried out by using the SYBR Premix Ex Taq™ Kit (Takara, Shiga, Japan) in ABI 7900HT sequence detection machine (Bio-Rad, CA, USA). GAPDH and U6 were employed as reference genes to normalize the expression of SIRT7 mRNA and miR-125b-5p, respectively. The primers of miR-125b-5p, U6, SIRT7 and GAPDH were synthesized and purchased from Sangon Biotech (Shanghai, China).

2.5. Proliferation assay

The measurement of cell proliferation was carried out by using Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China) assay. Cells were plated in a 96-well plate in triplicate with 5×10^3 cells/well. Then CCK8 solution was added to the well and incubated for 2 h at 37 ℃. The absorbance at 490 nm was evaluated by VICTOR3™ Multilabel Plate Readers (PerkinElmer Inc., Foster City, CA, USA). For EdU incorporation assay, CCA cells transfected with different vectors were stained with 5-Ethynyl-2′-deoxyuridine (EdU) and then were detected following the manufacturer's instruction (Thermo Fisher Scientific, Waltham, MA, USA). As for colony formation assay, 2000 SIRT7 modified CCA cells were seeded on 6-well plates. 14–21 days after cell seeding, cell colonies with crystal violet staining were counted.

2.6. Cell cycle analysis

4×10^5 CCA cells that were transfected with corresponding vectors were seeded onto 60 mm dishes. 48 h after transfection, the cells were obtained and fixed in ethanol (70%). After PBS washing, cells were subsequently resuspended in 200 μl of PBS containing 50 μg/mL propidium iodide (BD biosciences, San Jose, CA, USA), 0.05% Triton X100 and 1 mg/ml RNase, and incubated away from light for 30 min at room temperature. The results were finally detected and analyzed using the flow cytometer and CellQuest Pro software (BD Biosciences).

2.7. Western blotting

Total cell lysates were prepared with radioimmunoprecipitation assay buffer (RIPA buffer, Thermo Fisher Scientific, MA,USA) and