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LncRNA *THOR* promotes human renal cell carcinoma cell growth

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ABSTRACT

Background: Recent studies have characterized a novel but extremely conserved long non-coding RNA (LncRNA) *THOR*. *THOR* directly associates with insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) to promote mRNA stabilization of key pro-cancerous genes.
Results: Here, we show that *THOR* is expressed in human renal cell carcinoma (RCC) tissues and established/primary human RCC cells. It was not detected in normal renal tissues nor in HK-2 and primary human renal epithelial cells. *THOR* silencing (by targeted siRNAs) or CRISPR/Cas9 knockout inhibited RCC cell growth, viability and proliferation *in vitro*. Reversely, forced over-expression of *THOR* promoted RCC cell survival and proliferation. IGF2BP1-regulated genes, including *IGF2*, *GLI1* and *Myc*, were down-regulated by *THOR* silencing or knockout, but they were upregulated after *THOR* over-expression. *In vivo*, *THOR*-knockout 786-O tumors grew significantly slower than the control tumors in nude mice.
Conclusion: *THOR* expression promotes RCC cell growth *in vitro* and *in vivo*. *THOR* could be a novel and important therapeutic target for human RCC.

1. Introduction

Renal cell carcinoma (RCC) causes significant cancer-related human mortalities each year [1–4]. Due to the lack of characteristic clinical symptoms, RCC patients are commonly diagnosed at late and/or advanced stages [1–4]. It is urgent to develop more effective anti-RCC strategies [1–4].

Long non-coding RNAs (LncRNAs) are endogenous cellular RNAs with a length over 200 nucleotides [5–7]. LncRNAs have an absent or reduced coding potential with its open reading frames (ORF) > 30 amino acids [5–7]. These polyadenylated and non-polyadenylated LncRNAs are important regulators in a number of key biological processes and a broad range of diseases [5–7]. LncRNAs are dysregulated in human RCC [8,9], which are critically associated with RCC progression [8,9].

Hosono et al., have recently characterized a novel but extremely conserved LncRNA, named *THOR* (ENSG00000226856) [10]. *THOR* is expressed in testis, but it is also re-expressed in a number of human cancers [10]. *THOR* depletion can inhibit human cancer cell growth [10]. *THOR* directly associates with insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1), which is essential for mRNA stabilization of several key pro-cancerous genes, including *IGF2 (insulin-like growth factor 2)*, *GLI1 (glioma-associated oncogene homolog 1)* and *Myc* [10]. However, the expression and potential functions of *THOR* in human RCC have not been studied. Our results demonstrate that *THOR* expression promotes RCC cell growth *in vitro* and *in vivo*.

2. Materials and methods

2.1. Chemicals and reagents

Puromycin was purchased Sigma-Aldrich (St. Louis, Mo). Cell culture reagents were obtained from Gibco Bio (Grand Island, NY). All antibodies were obtained from Cell Signaling Tech (Beverly, MA).

2.2. Human RCC tissues

Total six (6) written-informed consent RCC patients (all male, 42–68 years old), administrated at the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China), were enrolled. The patients all performed nephroureterectomy surgery, receiving no

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prior irradiation or chemotherapy. Tumor tissues and the surrounding normal renal tissues were separated carefully under the operating microscope. The tissues were then incubated with tissue lysis buffer (Biyuntian, Wuxi, China). The protocols were approved by the Ethics Review Board (ERB) of Wenzhou Medical University, and according to Declaration of Helsinki.

2.3. Cell lines

The established human RCC cell lines, 786-O, A498 and ACHN, as well as the HK-2 renal epithelial cells were provided by Dr. Zheng [11–13]. The cultures of the described RCC cells and HK-2 cells were described previously [11–13].

2.4. Primary human cells

Two different primary human RCC cells, namely "RCC1" and "RCC2", were provided from Dr. Zheng [14,15]. The primary cancer cells were derived from two primary RCC patients: Patient 1, male, 54-years old; Patient 2, male, 43-years old [14]. The primary human renal epithelial cells were provided by Dr. Zheng as well [15]. The primary human cells were cultured in the previously-described medium for primary cells [13]. The primary human cells at passage 3–9 were utilized for *in vitro* experiments. The protocols of using human cells were according to the principles of Declaration of Helsinki, and approved by the ERB of Wenzhou Medical University.

2.5. THOR siRNA

The two siRNAs against non-overlapping sequence of *THOR*, "S1": 5-CUACAUGGGUAAUCAAUAU-3 and "S2": 5-CUAUGGUGUGUGAACAUUA-3 [10] were synthesized by Min-de Biotech (Suzhou, China). *THOR* siRNA (1 μM) or the scramble control siRNA ("siR-C", Santa Cruz biotech, 1 μM) was transfected via Lipofectamine 2000 (Invitrogen, Shanghai, China) for 48 h. *THOR* silencing was confirmed by PCR assay.

2.6. THOR knockout by CRISPR/Cas9 gene editing

THOR small guide RNA (sgRNA): *sgRNA-1*, F: 5′-CACCgAGGGTGTAGCGCGGGCTAGA-3′, R: 5′-AAACTCTAGCCCGCGCTACACCCTc-3'. *sgRNA-2*, F: 5′-CACCgGTAGGTGCTGCCATGCCAG-3′, R: 5′-AAACCTGGCATGGCAGCACCTACc-3' [10]) was annealed into the *Bbs*I-linearized pSpCas9(BB)-2A-GFP (PX458) plasmid (Addgene, Cambridge, MA). RCC cells were transfected with the construct via Lipofectamine 2000. GFP-positive cells were then subjected to FACS sorting to achieve a single cell onto 96-well plate. After culturing for 3 weeks, cells were distributed onto 24-well plates, followed by genotyping of *THOR*. Two stable cell lines with complete deletion in the targeted regions of *THOR* were achieved: "KO-*THOR*-L1/L2".

2.7. Stable expression of THOR

The full-length *THOR* transcript (amplified from 786-O cells via the described primer [10]) was sub-cloned to the pLenti6-GFP vector (Invitrogen). The lentiviral pLenti6-puro-GFP-*THOR* expression vector ("LV-*THOR*") was transfected to RCC cells. Afterwards, puromycin (5.0 μg/mL) was added to select the stable cells. Two stable cell lines ("L1/L2") with the construct were established. *THOR* over-expression in the stable cells was confirmed by the qRT-PCR assay. Control cells were infected with the pLenti6-puro-GFP vector control ("LV-C").

2.8. Methylthiazol tetrazolium (MTT) assay

Cell viability was examined by the MTT (Sigma-Aldrich, St. Louis, Mo) assay according to the manufactory's recommendation [11–13]. MTT optical density (OD) at 590 nm was recorded.

2.9. Clonogenicity assay

RCC 786-O cells were initially seeded at 1×10^4 cells per 10-cm culture dish. Cells were plated in 1% agarose-containing complete medium. After incubation for 10 days, the remaining 786-O cell colonies were counted manually.

2.10. In vitro proliferation assay

Bromodeoxyuridine (BrdU) incorporation in RCC cells was tested using a commercial available BrdU ELISA assay (Cell Signaling Tech, Shanghai, China), the protocol was described in detail in our previous studies [16,17]. BrdU ELISA OD at 450 nm was recorded.

2.11. Real-time PCR

Total RNA was extracted by the SV RNA isolation kit (Promega, Madison WI). TOYOBO ReverTra Ace-a RT-PCR kit (TOYOBO, Japan) was utilized for the reverse transcription to achieve cDNA [13], which was then mixed with SYBR Green PCR Master Mix. The ABI-7600 system (Applied Biosystems) was applied for the quantitative real-time PCR ("qRT-PCR") reaction, using the $^{-2\Delta\Delta}$Ct method for the quantification. The mRNA primers for *IGF2*, *Myc* and *GLI1* were described previously [10]. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* mRNA was tested as the internal control for above mRNAs. The primers for detecting *THOR* (F: 5′-CAAGGTGCTTCTCTCTGGATTT-3′ and R: 5′-GCCAAAGTCATTTGTTGGGTAT-3′) [10] were synthesized by Min-de (Suzhou, China). The primers for *U6 mRNA* were reported early [18]. *THOR* was normalized to *U6 mRNA*.

2.12. Western blotting assay

Protein lysates (40 μg per sample of each lane) were separated by 10% SDS-PAGE gel, which were then transferred to the PVDF (polyvinylidene difluoride) membrane (Merck Millipore, Darmstadt, Germany). After blocking, the blots were incubated with applied primary and secondary antibodies. Enhanced chemiluminescence (ECL) reagents (Pierce, Shanghai, China) were utilized to detect the targeted protein bands using x-ray films. Intensity of the band was quantified via ImageJ software.

2.13. Tumor xenograft assay

The nude mice (female, 5–6 week age, 18–19g weight) were provided by the Animal Center of Wenzhou Medical University (Wenzhou, China). Mice were maintained at standard conditions. Experimental animals were kept under standard conditions, 12-h dark/12-h light cycle, 24 ± 0.5 °C temperatures, and 50 ± 2.5% humidity, with free access of water and food. Mice were injected with 786-O cells (six millions cells each mouse in 200 μl of Matrigel gel/medium) in the right flanks. Within 2–3 weeks, xenograft tumors were around 100 mm^3 in volume. Thereafter, the tumor volume was measured once every 5 days for a total of 35 days using the described method [17]. The animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Wenzhou Medical University.