Effect of lncRNA THOR on proliferation and migration of colon cancer cells

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Received January 3, 2019; Accepted June 14, 2019

DOI: 10.3892/ol.2019.10585

Abstract. The effect of long non-coding RNA (lncRNA) THOR on proliferation and migration of colon cancer cells was investigated. Lentiviral vector expressing lncRNA THOR shRNA was used to establish colon cancer SW620 lncRNA THOR knockdown cell line (experimental group), and at the same time, a control vector cell line (control group) was established by empty vector virus. Proliferation ability of the two groups was analyzed by CCK8 and EdU methods. Migration ability of the cells was analyzed by Transwell method. Xenograft tumor method was used to analyze the *in vivo* proliferation ability of the two groups of cells. mRNA levels of lncRNA THOR target genes were analyzed by reverse transcription-quantitative PCR (RT-qPCR). Compared with control cells, the cell proliferation ability of the experimental group was significantly decreased (P<0.05). Compared with the control group, the cell migration ability of the experimental group was significantly decreased (P<0.05). The tumor growth rate of the experimental group in the mice was significantly lower than that of the control group (P<0.05). Compared with the control group, mRNA levels of lncRNA THOR target genes IGF2BP1, SOX9 and c-myc in the experimental group were significantly downregulated (P<0.05). The results indicated that lncRNA THOR knockdown can significantly downregulate the expression of genes involved in tumor proliferation and migration, promote tumor cell proliferation and migration, indicating that lncRNA THOR plays an important role in colon cancer.

Introduction

Colon cancer is one of the common types of nausea and digestive tract tumors, which has become a disease that seriously affects human health (1). The molecular mechanism of colon cancer development and progression is still unclear. Long non-coding RNAs (lncRNAs) are a subgroup of non-coding RNAs composed of more than 200 nucleotides, playing an important role in many biological processes such as dose-compensation effect, epigenetic regulation, cell cycle and cell differentiation regulation (2). Previously, it was believed that lncRNA genes had no biological function and were therefore called junk DNA. Recent studies have found that lncRNAs are abnormally expressed in many tumors such as lung cancer, glioma, liver cancer and breast cancer (3-6). lncRNA THOR is a conserved lncRNA identified in testicular tissue in 2017 (7). It was reported that lncRNA THOR can significantly promote the proliferation of osteosarcoma cells and renal cancer cells (8,9). In addition, it was found that lncRNA THOR could enhance the stem cell characteristics of nasopharyngeal carcinoma cells, thereby increasing the resistance of nasopharyngeal carcinoma cells to chemotherapy (10). Early high-throughput sequencing revealed that the expression of lncRNA THOR in colon cancer tissues was significantly higher than that in adjacent normal tissues. However, effect of lncRNA THOR on the biological behavior of colon cancer cells has not been reported. This study analyzed the effect of lncRNA THOR on proliferation and migration of colon cancer cells.

Materials and methods

General information. Thirty female Balb/c nude mice weighing 20 g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. The mice (6 per cage) were kept in SPF animal room at 25˚C with a 12 h light/12 h dark cycle and free access to food and water and humidity was 50%. Colon cancer cell line SW620 was purchased from ATCC. CCK8 cell proliferation assay kit and BeyoClick™ EdU-488 cell proliferation assay kit were purchased from Biyuntian Biotechnology Co., Ltd. Transwell kit was purchased from Corning. MTT assay kit and RNA extraction kit were purchased from Sigma-Aldrich; Merck KGaA. RNA extraction kit (RC101), reverse transcription kit (R323-01) and universal high-sensitivity dye quantitative PCR detection kit (Q431-02) were purchased from Nanjing Nuoweizan Biological Co., Ltd. Control shRNA lentivirus, and Lenti-THOR-shRNA lentivirus were obtained from Shandong Weizhen Biological Co., Ltd. The study was approved by the Ethics Committee of Jinan Central Hospital Affiliated to Shandong University (Jinan, China).

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Key words: lncRNA THOR, colon cancer, proliferation, migration

Table I. Sequences of primers used in PCR reactions.

Genes	Forward primers (5'-3')	Reverse primers (5'-3')
lncRNA THOR	ACAATCGAGCAAGGCAGTGA	TGGCCAAGACCTGCTGTTAG
β-actin	GGCCCAGAATGCAGTTCGCCTT	AATGGCACCCTGCTCACGCA
c-myc	CCCTCCACTCGGAAGGACTA	GCTGGTGCATTTTCGGTTGT
SOX9	GCTGCGAAGTGGAAACCATC	CCTCCTTCTGCACACATTTGAA
IGF2BP1	CAGGAGATGGTGCAGGTGTTTATC C	GTTTGCCATAGATTCTTCCCTGAGC

lncRNA THOR knockdown cell line. Colon cancer cell line SW620 [SW-620] (ATCC® CCL-227™) was cultured in DMEM medium (10% FBS) twice to restore cell status. After digestion with 0.25% trypsin, the cell concentration was adjusted to $1x10^5$ cells/ml and inoculated in 12-well plate with 1 ml per well. After 12 h of cell adherence, the control shRNA lentivirus and Lenti-THOR-shRNA lentivirus were added respectively, and the cell-to-virus titer ratio was 1:100. Culture medium was replaced by fresh medium at 24 h after transfection, followed by cell culture for additional 4 h. Then cells were treated with 1 μg/ml puromycin for 24 h, and viable cells were observed under the microscope (ZEISS) to observe GFP fluorescence. When GFP in all cells is expressed, stable cell line is successfully constructed. Lenti-THOR-shRNA stable expression cell line was used as the experimental group, and control shRNA stable expression cell line was used as the control group.

Preparation of a reverse transcription-quantitative PCR (RT-qPCR) system. The same number of living cells were collected both from the control and experimental groups. Total RNA was extracted using an RNA extraction kit, and then the RNA was reverse-transcribed into cDNA using a reverse transcription kit to serve as the template for RT-qPCR. The temperature protocol for reverse transcription was 37˚C for 30 min, 85˚C for 15 sec and 4˚C for storage. cDNA template was diluted 1:100 before use. PCR reaction systems included: 2X SYBR-Green premix 10 μl, template 8.8 μl, upstream primers 0.6 μl and downstream primers 0.6 μl. PCR reaction conditions were: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. Sequences of primers used in PCR reactions are shown in Table I. β-actin was the reference gene. The method of quantification was $2^{-\Delta\Delta Cq}$ (11).

Cell proliferation analysis (CCK8 and EDU methods). Control and experimental cells were trypsinized at a cell density of $5x10^5$ cells/ml, and seeded in 96-well plates with 100 μl per well. Cells were cultured for 6 h at 37˚C in a 5% CO_2 incubator. After that, 10 μl of CCK solution was added 12, 24, 36, 48 and 72 h later. After that, cells were cultivated for additional 3 h. Finally, OD values at 450 nm were measured using a microplate reader (BioTek) to reflect cell proliferation.

Cells of the control and experimental groups were digested and inoculated into a 12-well plate. After the cells were completely adhered, the EdU was diluted to 50 μM with a cell culture medium, and 100 μl was added to each well. Cells were incubated for 2 h in a 37˚C and 5% CO_2 incubator. After that, cells were washed three times with PBS, and then fixed with 4% paraformaldehyde at room temperature for 20 min. After washing three times with PBS, nuclei were stained with DAPI. After washing three times with PBS, cells were observed under a microscope.

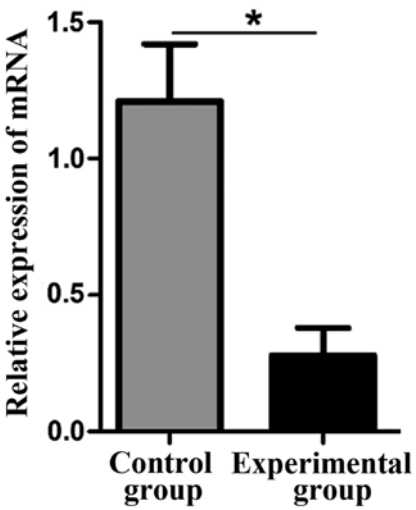

Figure 1. Comparison of lncRNA THOR levels between the control and experimental groups. *P<0.05 vs. the control group. lncRNA, long non-coding RNA.

Cell migration analysis (Transwell method). The control and experimental cells were trypsinized to prepare a single cell suspension, and the cell density was adjusted to $5x10^5$ cells/ml, and inoculated into the Transwell upper chamber, 100 μl per well. The lower chamber was filled with fresh cell culture medium. Cells were incubated in a 37˚C and 5% CO_2 incubator for 24 h, and were fixed with paraformaldehyde. Then the upper chamber membrane was fixed with 1% crystal violet (MXB Biotechnologies) at room temperature for 5 min. Migrating cells were observed under an optical microscope, and 20 visual fields were selected for both the experimental and control groups to calculate the average number of migrating cells.

Xenograft tumor. After cells of the control and experimental groups were trypsinized, cell density was adjusted to $1x10^8$/ml. Thirty Balb/c nude mice were randomly divided into the control and experimental groups. Tumor cells (100 μl) were injected into the fat pad of each mouse, and mice were raised in SPF-level animal house. Tumor formation and growth in the mice were observed. Tumor length (L) and width (W) were

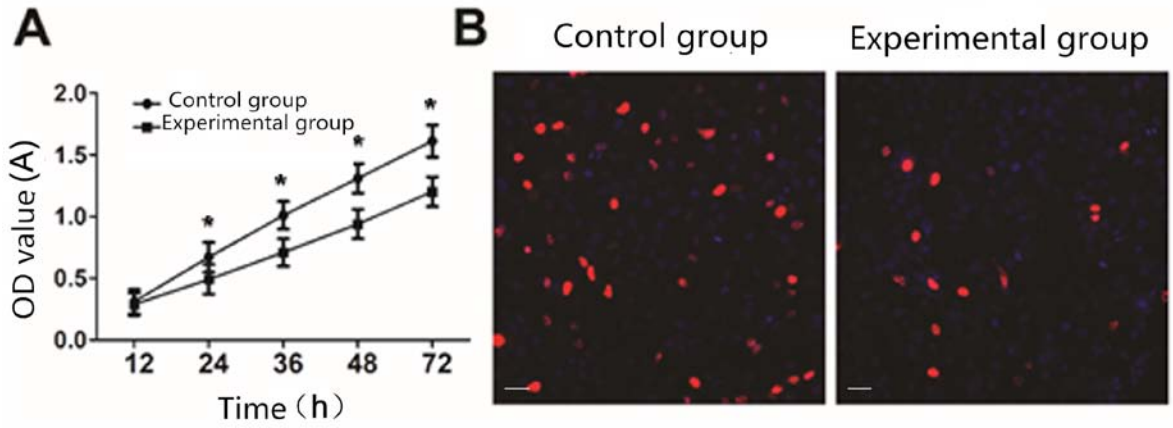

Figure 2. Effect of lncRNA THOR on the proliferation of colon cancer cells. (A) Cell proliferation between the control shRNA group and Lenti-THOR-shRNA experimental group measured using CCK-8 assay. *P<0.05 vs. the control group. (B) Fluorescent micrographs of EdU staining (red) between the control shRNA group and Lenti-THOR-shRNA experimental group. Cell enlargement, x100. Scale bar, 20 μm. lncRNA, long non-coding RNA.

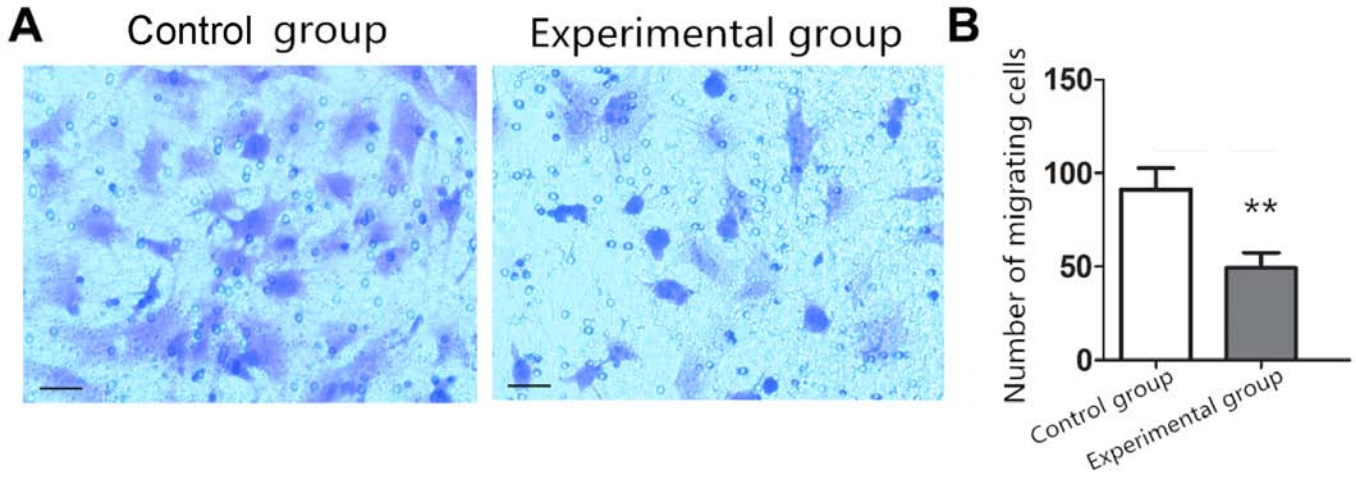

Figure 3. Effect of lncRNA THOR on cell migration. (A) Cell migration between shRNA control group and Lenti-THOR-shRNA experimental group analyzed by Transwell. Cell enlargement, x200. Scale bar, 20 μm. (B) Quantitative analysis of the average number of migrating cells in Fig. 2A. **P<0.01 vs. the control group. lncRNA, long non-coding RNA.

measured. Tumor volume was calculated by the following formula: $V = 1/2 \times L \times W^2$.

Statistical analysis. All data were analyzed by SPSS 17.0 statistical software (SPSS, Inc.). Measurement data were expressed as mean ± standard deviation and compared by t-test. P<0.05 indicated a difference with statistical significance.

Results

Comparison of lncRNA THOR levels in the control and experimental groups. The lncRNA THOR knockdown stable cell line was established by lentiviral-mediated THOR-shRNA. The expression level of lncRNA THOR in the control group (1.21±0.21) was significantly higher than that in the experimental group (0.28±0.10), and the difference was statistically significant (P<0.05) (Fig. 1).

Effect of lncRNA THOR on cell proliferation. CCK8 analysis showed that compared with the control cells, cell proliferation ability of the experimental group was significantly reduced (P<0.05) (Fig. 2A). EdU staining analysis showed that the cell proliferation ability of the experimental group was significantly decreased compared with that of the control group (P<0.05) (Fig. 2B).

Effect of lncRNA THOR on cell migration. Compared with the number of migrating cells in the control group (91.23±11.44), the number of migrating cells in the experimental group (49.32±8.14) decreased significantly (P<0.05) (Fig. 3).

Effect of lncRNA THOR on tumor growth. Control and experimental group cells were inoculated into nude mice, and cell proliferation rate *in vivo* was analyzed. Compared with the control cells, growth rate of tumors in the experimental group was significantly reduced (P<0.05) (Fig. 4).

Effect of lncRNA THOR knockdown on its target genes. Effect of lncRNA THOR knockdown on its target genes at mRNA level was further analyzed by RT-qPCR. Compared with

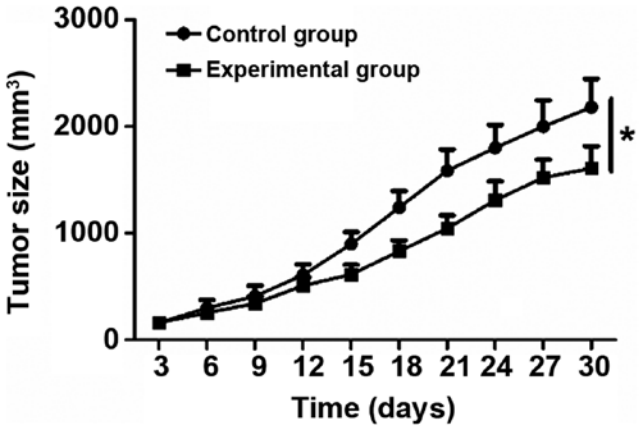

Figure 4. Effect of lncRNA THOR on tumor growth by *in vivo* tumor formation experiment. *P<0.05 vs. the control group. lncRNA, long non-coding RNA.

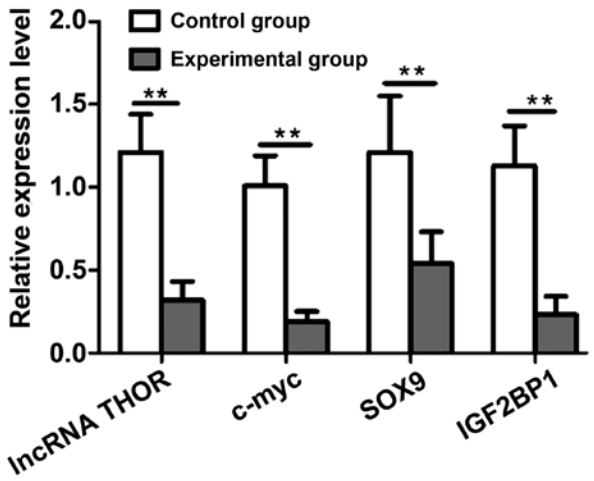

Figure 5. Effect of lncRNA THOR knockdown on the relative expression level of target gene mRNA by RT-qPCR. **P<0.01 vs. the control group. lncRNA, long non-coding RNA.

the control group, the mRNA levels of IGF2BP1, SOX9 and c-myc in the experimental group were significantly reduced (P<0.05) (Fig. 5).

Discussion

Colon cancer, as the third most common type of malignant tumor in the world, seriously affects human health (12). Despite the efforts made in cancer diagnosis, many cancer patients are still diagnosed at advanced stages. Therefore, novel early diagnosis biomarkers are needed (13). Studies have shown that lncRNAs participate in many biological processes by regulating gene expression (14). It has been reported that lncRNA CCTA1, ATB and HOTAIR participate in the regulation of cell behavior of colon cancer by mediating the downstream signaling pathways (15,16). Because of the important role of lncRNA in tumorigenesis, lncRNAs have been used as a novel tumor marker and tumor therapeutic target. This study analyzed the role of lncRNA THOR in the pathogenesis of colon cancer.

At cellular level, lncRNA THOR knockdown led to significantly inhibited proliferation and migration of colon cancer SW620 cells. At animal level, lncRNA THOR knockdown mediated the significantly inhibited growth of tumors in mice. It indicated that lncRNA THOR plays an important role in the development of colon cancer.

Sox9 plays an important role in early embryonic development, cell fate determination and differentiation of tissues and organs, sex determination, occurrence and development of nervous system and cartilage (17). The high expression of Sox9 is related to the size of the tumors, TNM stage, lymph node metastasis and differentiation of colorectal cancer patients (18). lncRNA THOR maintains the stability and activity of IGF2BP1 through conservative interaction with IGF2BP1 mRNA (7). The results showed that knockdown of lncRNA THOR resulted in significant downregulation of the target genes SOX9 and IGF2BP1 mRNA, suggesting that lncRNA THOR has important significance in stabilizing SOX9 and IGF2BP1 mRNA. C-myc, as an oncogene, plays an important role in tumorigenesis (19). This study also found that knockdown of lncRNA THOR in colon cancer cells resulted in a significant decrease in c-myc mRNA level, which further demonstrated that lncRNA THOR, was involved in the occurrence and progress of colon cancer genes.

Collectively, lncRNA THOR, as an oncogene, promotes the proliferation and migration of colon cancer cells, which can be used as a therapeutic target for colon cancer treatment.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YL and XY drafted the paper and performed PCR. XY and LW were responsible for CCK8 assay and Transwell method. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Jinan Central Hospital Affiliated to Shandong University (Jinan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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