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Original article

Long non-coding RNA CCAT1 promotes human retinoblastoma SO-RB50 and Y79 cells through negative regulation of miR-218-5p

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ABSTRACT

Objective: To investigate the regulatory role and potential mechanism of long non-coding RNAs (lncRNA) in human retinoblastoma (RB).
Methods: The lncRNA profile in RB tissues were analyzed by microarray and quantitative reverse transcription PCR (qRT-PCR). One of the identified lncRNAs (LncRNA CCAT1) was selected for further experiments. SO-RB50 and Y79 cells were transfected with negative control, siRNA targeting lncRNA CCAT1 (si-CCAT1) and si-CCAT1 + miR218-5p inhibitor, respectively. lncRNA CCAT1 expression was measured by qRT-PCR. Cell proliferation, migration and invasion were detected by CCK8, wound scratching, and transwell assay, respectively. Apoptosis and cell cycle distribution were assessed by flow cytometry. Apoptosis- (cle-caspase-3, cle-caspase-9, Bax and Bcl-2) and cell cycle-related protein expression (cyclin B1, CDC2 and p-CDC2 (Thr161)) were analyzed by Western blot.
Results: lncRNA CCAT1 expression in SO-RB50 and Y79 cells was significantly inhibited after si-CCAT1 transfection ($P < 0.01$). Both RB cells exhibited significantly reduced proliferation, migration and invasion abilities, but markedly increased apoptosis at 48 h after si-CCAT1 transfection ($P < 0.05$ or 0.01). RB cells in si-CCAT1+miR218-5p inhibitor group had significantly higher proliferation, migration and invasion, but notably lower apoptosis compared with si-CCAT1 group at 24 and 48 h after transfection (all $P < 0.05$ or 0.01). si-CCAT1 significantly increased the expression of cle-caspase-3, cle-caspase-9, Bax, but decreased Bcl-2 expression ($P < 0.01$). The proportion of G2/M SO-RB50 and Y79 cells in siCCAT1 group was significantly increased compared with negative control group ($P < 0.01$). LncRNA CCAT1 interference significantly reduced the expression of cyclin B1, CDC2 and p-CDC2 (Thr161) ($P < 0.01$).
Conclusion: LncRNA CCAT1 promotes the proliferation migration and invasion, and reduces cell apoptosis of SO-RB50 and Y79 cells, probably through negative modulation of miR-218-5p. Our study suggested lncRNA CCAT1 as a potential biomarker and therapeutic target for RB.

1. Introduction

Retinoblastoma (RB) is the most common intraocular malignant tumor in children with a mortality rate of 50–70% in developing countries [1,2]. Children with RB are under a variety of life-threatening risks including RB cell invasion, malignant transformation of intracranial neuroblastoma, heterochronogenous tumor, etc. [3]. Despite the rapid development of the treatment of RB in recent years, the survival rate remains quite low primarily due to early diagnosis limitations of the disease. Reliable biomarkers can effectively improve the diagnosis of tumors. Moreover biomarkers may be potential therapeutic targets, which may improve the prognosis of tumors and markedly increase the survival rate of patients [4]. Therefore, studies on the biomarkers for RB and relevant regulatory mechanisms are important for the improvement of the treatment of the disease.

lncRNAs are a class of non-coding RNA molecules with the length of more than 200 nucleotides that are widely present in the nucleus and cytoplasm of eukaryotic cells [5]. lncRNAs regulate the expression of protein-coding genes on epigenetic, transcriptional

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and post-transcriptional levels, and are involved in a variety of regulatory processes such as X chromosome silencing, genomic imprinting, activation of proto-oncogenes to oncogenes [6–8]. Abnormal expression of lncRNAs has been detected in several diseases including cancer [9,10], and may be associated with the occurrence and development of cancer, indicating that lncRNAs may be potential tumor-specific epigenetic marker.

Studies have also suggested an important role of lncRNAs in RB [11]. Nevertheless, the regulatory role of lncRNAs in RB has not been fully clarified. In this study, the expression of lncRNAs was analyzed with microarray chips to screen lncRNAs with abnormal expression. The effect of lncRNA CCAT1, one of the selected lncRNAs, on the proliferation, migration and invasion of RB cells was investigated. Its regulatory role on the apoptosis and cell cycle of RB cells was also assessed. Previous studies have shown that lncRNA CCAT1 promotes the pathogenesis of lung cancer via negative regulation of miRNA-218-5p (miR-218-5p) [12]. We herein investigated the association between lncRNA CCAT1 and miR-218-5p in order to elucidate the underline regulatory mechanism of the long code RNA in RB cells. The current study shall shed lights on the targeted gene therapy for RB, which may be an effective method for the treatment and prognosis of malignant cancers.

2. Material and methods

2.1. Main reagents

Human lncRNA chip V3.0 including 30,586 lncRNAs and 26,109 mRNAs was purchased from Arraystar (Rockville, MD, USA). RNA extraction kit, reverse transcription kit, DreamTaq Green PCR Master Mix (2x) were purchased from Takara (Japan). The primers used for quantitative reverse transcription-PCR (qRT-PCR) were synthesized by Beyotime Institute of Biotechnology (Shanghai, China). RPMI-1640 culture media and fetal bovine serum (FBS) were purchased from HyClone Company (Waltham, MA, USA). The lncRNA-targeted siRNA, negative control, and miR-218-5p inhibitor were synthesized by GenePharma Biotech (Shanghai, China). Lipofectamine 2000 transfection reagent was purchased from Shanghai Bioleaf Technology Co (Shanghai, China). Protein extraction kit and BCA kit were purchased from Beyotime Institute of Biotechnology. CCK-8 kit was purchased from Dojindo Laboratories (Kumamoto, Japan). The AnnexinV-FITC/PI double staining kit for the detection of apoptosis was purchased from BD Biosciences (San Jose, CA, USA). Rabbit anti-human cleaved caspase-3 (cle-caspase-3), cle-caspase-9, Bax, Bcl-2, cyclin B1, CDC2, p-CDC2 (thr-161) and β-actin antibodies, and HRP-labeled goat anti-rabbit IgG were purchased from Abcam (Cambridge, MA, USA). Transwell chambers were purchased from Corning Inc. (Corning, NY, USA). Matrigel and crystal violet were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture and tissue samples

Human RB cell line SO-RB50, Y79 and WERI-RB1 were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CCCAS, Shanghai). Cells were cultured in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in an incubator with 5% CO_2. Cells in exponential phase were used for subsequent experiments. RB cancerous tissues and normal adjacent tissues were collected 15 patients who were hospitalized at Hangzhou First People's Hospital, Nanjing Medical University between July 2012 and December 2013. The collection of human tissues was approved by the Research Ethics Committee at the hospital and performed in strict accordance with international standards. All patients were required to sign the informed consent.

2.3. Microarray analysis of lncRNA profile

Three RB cancerous tissues and their normal adjacent tissues were randomly selected. Total RNA was extracted with RNA extraction kit following the manufacture's instruction and subjected to microarray analysis of lncRNA profile with human lncRNA chip V3.0 according to the manufacture's instruction. The expression profile of cancerous tissues and controls was compared by *t*-tests. LncRNAs with an expression fold change above 2 and P smaller than 0.05 were defined as those with differential expression. The screened lncRNAs with the most pronounced up- and down-regulation was further validated by qRT-PCR.

2.4. QRT-PCR analysis

Three randomly selected RB tissues and their adjacent normal tissues were subjected to qRT-PCR analysis. Total RNA was extracted using RNA extraction kit. RNA concentration was determined using a spectrometer under a wavelength of 260 nm. Total RNA was reverse transcribed into cDNA using reverse transcription kit according to the manufacture's instructions. The top 3 up-regulated (MEG3, CCAT1, and HIF1A-AS1) and down-regulated lncRNAs (MIR31HG, BCAR4 and H19), and β-actin mRNA were measured by qRT-PCR using cDNA as template and DreamTaq Green PCR Master Mix following the manual. The reaction condition: 95 °C 15 s; followed by 30 cycles of 95 °C 5 s, 60 °C 60 s. After amplification, melting curve analysis was performed: 95 °C 15 s, 60 °C 30 s, 72 °C 30 s. The sequences of primers: MEG3-forward, 5′- CTGCCCATCTACACCTCACG-3′, MEG3-reverse, 5′- CTCTCCGCCGTCTGCGCTAGGGGCT-3′; CCAT1-forward, 5′- CATTGGGAAAGGTGCCGAGA-3′, CCAT1-reverse, 5′-ACGCTTAGCCATACAGAGCC-3′; HIF1A-AS1-forward, AATGTGTTCCTTGCTCTT, HIF1A-AS1-reverse, GTATGTCTCAGTTATCTTCCT; MIR31HG-forward, TCTCTGGTGCTTCCCTCCTT, MIR31HG-reverse, GATCTAAGCTTGAGCCCCCA; BCAR4-forward, ATCTTGAGTTGTTGCCATTA BCAR4-reverse, GACCAGGTGATAGGAGTT; H19-forward, CCCACAACATGAAAGAAATGGTGC, H19-reverse, CACCTTCGAGAGCCGATTCC; β-actin-forward, GACATGGAGAAGATCTGG CA and β-actin-reverse, GGTCTTTACGGATGTCAACG. The experiment was repeated three times. Data was analyzed using the 2-ΔCt method. One of the lncRNAs, lncRNA CCAT1, was selected for subsequent experiments.

2.5. Confirmation of differential expression in RB tissues and cell lines

The level of lncRNA CCAT1 in 15 RB tissues and their adjacent normal tissues was compared by qRT-PCR as described in section 1.4. The level of lncRNA CCAT1 in RB cell line SO-RB50, Y79, WERI-RB1 and normal podiatric retina was also compared.

2.6. Transfection of siRNA

The siRNA targeting lncRNA CCAT1 (siCCAT1, 5′-CGGCAGGCATTAGAGATGAACAGCA-3′) and negative control was transfected into SO-RB50 and Y79 cells with Lipofectamine 2000 transfection according to the manufacture's instruction. Briefly, aliquots of 2 ml of SO-RB50 and Y79 cells were inoculated into each well on 6-well plates and cultured at 37 °C in a 5% CO_2 incubator. Cells at 70% confluent were transfected respectively with 100 nmol siCCAT1 and 50 nmol negative control using Lipofectamine 2000. After 6 h of incubation, culture medium was replaced with fresh medium.