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Original article

miR-154 inhibits prostate cancer cell proliferation by targeting CCND2

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Abstract

Background: Research has shown reduced expression levels of miR-154 in prostate cancer (CaP). However, the function and molecular mechanisms of miR-154 in this cancer type remains unknown.

Objective: The aims of this study were to examine the functional significance of miR-154 in CaP cells and to identify the novel molecular targets regulated by miR-154.

Materials and methods: miR-154 expression significantly decreased in primary CaP samples compared with nonmalignant samples measured by quantitative reverse transcription polymerase chain reaction. Restoration of miR-154 lowered the potential of CaP cell lines to grow and proliferate in vitro evaluated by CCK-8 assay, colony formation assay, and flow cytometry. miR-154 down-regulated the expression of CCND2 by binding to its 3′-untranslated region by luciferase reporter assay.

Conclusions: miR-154 plays a prominent role in CaP proliferation by suppressing CCND2, and it may provide a new approach to the treatment of CaP. © 2014 Published by Elsevier Inc.

Keywords: miR-154; Prostate cancer; CCND2

1. Introduction

Prostate cancer (CaP) is the most frequently diagnosed malignancy and the second leading cause of cancer-related death in American men [1]. The incidence of CaP has been increasing in China recently [2,3]. However, the mechanisms implicated in the initiation and progression of CaP remain unclear.

MicroRNAs (miRNAs) are a class of small noncoding RNAs 20 to 22 nucleotides long. They are involved in post-transcriptional regulation of gene expression by binding to the 3′-untranslated region (UTR) of target mRNAs, resulting in mRNA degradation or repression of translation [4]. Recently, an accumulating body of evidence has suggested that miRNAs are involved in carcinogenesis, including development, differentiation, apoptosis, and proliferation [5].

miRNAs have also been demonstrated to be dysregulated in CaP. For example, hsa-let-7c, hsa-miR-21, and hsa-miR-375 were found to be significantly up-regulated in CaP, whereas hsa-miR-143, hsa-miR-145, and hsa-miR-221 were determined to be significantly down-regulated [6]. Reduced expression levels of mir-154 in CaP compared with normal tissues have been reported [7]. However, little is known about the mechanisms of miR-154 in CaP. In silico analysis of 3′-UTRs identified CCND2 as a putative target of miR-154. CCND2 is a crucial cell cycle-regulatory gene, and its aberrant expression can lead to abnormal cell proliferation. Aberrant expression of CCND2 has been observed for testicular germ cell tumor cell lines [8], gastric cancer [9], and colorectal cancer [10,11], and its overexpression has been reported in CaP tissues and cell lines [12]. In this study, we used in vitro approaches to prove that miR-154 has growth-suppressive activity in CaP cell lines and that this activity is largely triggered by the down-regulation of CCND2.

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2. Materials and method

2.1. Cell culture and tissue specimens

The human CaP cell lines DU145 and PC-3 were purchased from KeyGene Biotech (Nanjing, China). Cells were cultured in RPMI-1640, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, and at 37 °C in a humidified atmosphere with 5% CO_2. CaP tissues and nonmalignant prostate tissues were collected from patients who underwent radical prostatectomy at the First Affiliated Hospital of Nanjing Medical University, Department of Urology, Nanjing, China. The specimens were immediately frozen in liquid nitrogen after surgery and stored at −80°C before further analysis. The use of clinical specimens has been approved by the medical ethics committee of our hospital. Detailed information of each tissue donor is provided in Table 1.

2.2. Transfection

Cells were seeded in 6-well plates at 70% confluence on the day before transfection. Cell transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Six hours post-transfection, the culture medium was replaced with RPMI-1640 containing fetal bovine serum. The following sequences of miR-154 mimics were used: sense, 5′-UAGGUUAUCCGUGUUGCCUUCG-3′; anti-sense, 5′-AAGGCAACACGAUAACCUAUU-3′. The RNA with no sequence homology to any human genomic sequence was used as negative control (NC): sense, 5′-UCCUCCGAACGUGUCACGUTT-3′; anti-sense, 5′-ACGUGACACGUUCGGAGAATT-3′. The sequences of miR-154 inhibitor were: 5′-CGAAGGGAACACGGAUAACCUA-3′; the sequences of NC inhibitor were: 5′-CAGUACUUUUGUGUAGUACAA-3′; the sequences of the small interfering RNA (siRNA) targeting CCND2 (si-CCND2) were as follows: sense, 5′-UCAAUUACCUGGACCGUUUCUUGGC-3′; antisense, 5′-GCCAAGAAACGGUCCAGGUAAUUCAUG-3′. All miRNA mimics or siRNA oligonucleotide duplexes were designed and synthesized by GenePharma (Shanghai, China).

Table 1
Patients characteristics

Characteristic	CaP (n = 27)
Age, yr	
Median (range)	70 (62–78)
T stage	
T1	3
T2	12
T3	11
T4	1
N stage	
N0	24
N1	3
M stage	
M0	27
M1	0
Gleason score	
<7	9
=7	10
>7	8

2.3. RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from tissues and cell lines using Trizol (Invitrogen) according to the manufacturer's instructions. RNA concentration was measured using NanoDrop (Thermo Scientific). For mir-154, total RNA (10 ng) was transcribed into cDNAs using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Real-time RT-PCR was performed with a TaqMan MicroRNA Assay Kit (Applied Biosystems). The expression of U6 was used as a control. For CCND2, RNA was reverse-transcribed into cDNAs using a PrimeScriptOne-Step RT-PCR Kit (Takara, Dalian, China) according to the manufacturer's instructions. The expression level of CCND2 was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers for CCND2 were 5′-CAGCCGTCCACTTCAGC-3′ and 5′-TGCCTTTGGGTCTTCC-3′, whereas those for GAPDH were 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′ (synthesized by GeneRay, Shanghai, China). The following PCR conditions for detecting mRNAs were used: 95°C for 30 seconds, 40 cycles of 95°C for 5 seconds, and 60°C for 30 seconds. The PCR program for detecting miRNAs was as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. All reactions were performed in triplicate, and NC reactions that lacked cDNA were included.

2.4. Western blot analysis

Cell lines and human CaP specimens were lysed using radio immunoprecipitation assay buffer (Keygene, Nanjing, China) supplemented with protease inhibitors at 4°C for 1 hour. The protein samples were electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane (Millipore), and then blocked for 1 hour with 5% nonfat milk at room temperature. After incubation with primary antibodies at 4°C overnight, the polyvinylidene fluoride membranes were washed 3 times with TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.01% Tween-20) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at room temperature for 2 hours. Among the related antibodies used were anti-GAPDH (Bioworld) and rabbit anti-CCND2 (Cell Signaling Technology). The blots were detected using chemiluminescence (Thermo