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TINCR suppresses proliferation and invasion through regulating miR-544a/FBXW7 axis in lung cancer

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ABSTRACT

Background: Long noncoding RNAs (LncRNAs) play critical roles in multiple biological processes implicated in the development and progression of cancers. Terminal differentiation-induced lncRNA (TINCR) has been demonstrated to be associated with the carcinogenesis of several cancers. However, little is known about the function and mechanism of TINCR in lung cancer.

Methods: qRT-PCR was performed to measure the expression of TINCR, miR-544a or FBXW7 mRNA in lung cancer tissues or cells. FBXW7 protein level was detected via western blot analysis. Cell Counting Kit-8 (CCK-8) and transwell invasion analysis were used to assess the proliferative and invasive ability of lung cancer cells. Bioinformatic softwares, luciferase reporter assay, and RNA immunoprecipitation (RIP) were employed to explore the relationship between TINCR, miR-544a and FBXW7.

Results: TINCR expression was downregulated while miR-544a expression was upregulated in lung cancer tissues and cells. TINCR overexpression suppressed proliferation and invasion in lung cancer cells. Moreover, TINCR was confirmed as a molecular sponge of miR-544a. We further validated that miR-544a facilitated proliferation and invasion, and miR-544a could reverse TINCR-mediated anti-proliferation and anti-invasion effect in lung cancer cells. TINCR acted as a competing endogenous RNA (ceRNA) to sequester miR-544a from its target gene FBXW7. Finally, FBXW7 suppressed proliferation and invasion, and FBXW7 knockdown abolished the inhibition of TINCR on proliferation and invasion in lung cancer cells.

Conclusion: TINCR suppressed proliferation and invasion through regulating miR-544a/FBXW7 axis in lung cancer, indicating that it might be a potential target for the therapy of lung cancer.

1. Introduction

Lung cancer is a main cause in cancer-related deaths and more than 50% patients are confirmed at a distant stage with a 5-year survival rate of 4% [1]. To date, the common treatment for lung cancer in clinical is chemotherapy, which only can prolong lung cancer patients survival time without ultimate cure [2]. So it is extremely momentous to understand the underlying pathological mechanisms of lung cancer in order to lay foundation for developing more effective diagnostic biomarkers and therapeutic strategies.

Long non-coding RNAs (LncRNAs) are a class of transcripts over 200 nt in length without protein-coding potential [3]. LncRNAs has been found to be vital regulators in various biological processes involved in the development and progression of cancers [4]. Also, previous studies showed that a variety of lncRNAs was closely associated with the development of lung cancer [5,6]. Terminal differentiation-induced lncRNA (TINCR), an lncRNA about 3.7 kb, is closely related to gene differentiation in epidermal tissue [7]. Moreover, multiple evidence reveals that TINCR can actively participate in the progression of cancer. For example, TINCR functioned as an inducer of ESCC via modulating cell proliferation, migration, and invasion by potentially targeting CLND7 and ANAX1 [8]. TINCR regulated PDK1 expression by sponging miR-375, thereby facilitating the development of gastric cancer via inhibiting apoptosis and promoting proliferation [9]. It is worth noting that TINCR was found to be down-regulated in lung cancer tissues compared to normal tissues [10]. However, little is known about the function and mechanism of TINCR in occurrence and development of lung cancer.

Abbreviations: LncRNAs, long noncoding RNAs; TINCR, terminal differentiation-induced lncRNA; ceRNA, competing endogenous RNA; miRNAs, microRNAs; RIP, RNA immunoprecipitation; SD, standard deviation

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microRNAs (miRNAs) are a type of endogenous and evolutionarily conserved non-coding RNAs with the length of 21–23 nt [11]. miRNAs have been highlighted as oncogenes or tumor suppressors by regulating many biological events such as cell differentiation, proliferation, and apoptosis [12]. MiR-544a was previously reported to accelerate cancer progression in several malignances, including gastric cancer [13] and colorectal cancer [14]. Moreover, miR-544a was observed to be up-regulated in lung cancer, and miR-544a overexpression led to the increase of invasive capability of lung cancer [15]. It also has been shown that lncRNAs could act as competing endogenous RNAs (ceRNAs) of miRNAs to regulate corresponding target mRNAs expression [16]. LncRNAs abnormal expression results in the disorder of lncRNA-miRNA-mRNA network and the disruption of steady states of gene expression, which may trigger carcinogenesis [17]. Therefore, we attempted to explore whether TINCR was implicated in lung cancer process by regulating miR-544a.

In this study, we observed a significant down-regulation of TINCR level in lung cancer tissues and cell lines. Besides, TINCR up-regulation suppressed lung cancer cells proliferation and invasion. Furthermore, we demonstrated that TINCR could regulate the expression of FBXW7 by competitively binding to miR-544a, and then exerted its tumor-suppressive function. Our study provided novel insight for better understanding of the ceRNA network in lung cancer, and elucidating a potential biomarker and therapy target for lung cancer.

2. Materials and nethods

2.1. Patients

With the informed consents of all patients, a total of 45 cases of lung cancer tumor tissues and adjacent normal tissues were collected from lung cancer patients undergoing surgical resection in our hospital. This research was ratified by the Research Ethic Committee of Huaihe Hospital of Henan University.

2.2. Cell culture and transfection

The human bronchial epithelial cells (HBE) and lung cancer-derived cell lines A549, H322, H460, GLC-82, SPC-A1 were obtained from American type culture collection (ATCC, Rockefeller, MD, USA). All cells were growing in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) in a humidified air atmosphere with 5% CO_2 at 37 °C.

To overexpress TINCR, the full length sequences of TINCR was amplified and cloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). siRNA targeting TINCR (si-TINCR) was synthesized by GenePharma Co. Ltd. (Shanghai, China) for knockdown of TINCR, with si-NC as a scramble control. miR-544a mimics, miR-544 inhibitor, scrambled mimic control (miR-con) and scrambled inhibitor control (anti-miR-con) were also purchased from Gene Pharma Co. Ltd. (Shanghai, China). A549 and H460 cells were plated in 24-well plates for 24 h, followed by transfected with indicated oligonucleotides or plasmids using Lipofectamine 2000 (Invitrogen). Approximately 48 h after transfection, cells were collected and used for further studies.

2.3. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from tissues and cells by Trizol reagent (Invitrogen). Then FBXW7 mRNA and TINCR were reversely transcribed to a single-stranded cDNA using Reverse Transcription System Kit (Takara, Dalian, China). Reverse transcription of miRNA-544a was performed using miRNA First-Stand cDNA Synthesis Kit (GeneCopoeia, Guangzhou, China). qRT-PCR reactions were carried out with Universal SYBR Green Master (Roche, Basel, Switzerland). The relative quantification of TINCR, FBXW7 mRNA and miR-544a were calculated by the $2^{-\Delta\Delta Ct}$ method, with GAPDH as an internal control for TINCR and FBXW7 mRNA, as well as with U6 as an internal reference for miR-544a.

2.4. RNA immunoprecipitation (RIP)

RIP assay was performed using Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. Ago2 antibody (Cell Signaling Technology, Danvers, MA, USA) was used to precipitate the potential substances in the RISC complex. At last, RNA in the RISC complex was purified with RNase-free DNase I and proteinase K (Thermo Fisher Scientific, Waltham, MA, USA), followed by the detection of TINCR and miR-544a using qRT-PCR assay.

2.5. Luciferase reporter assay

Partial sequences of TINCR and FBXW7 3′ untranslated region (3′UTR) containing wide-type or mutant-type miR-544a binding sites were cloned into the pGL3-Basic luciferase vector (Promega, Madison, WI, USA) to generate TINCR-WT, TINCR-MUT and FBXW7-3′UTR-WT (WT), FBXW7-3′UTR-MUT (MUT). Then the constructed luciferase vectors were respectively transfected into lung cancer cells along with pRL-TK vector (Promega) and miR-con, miR-544a, anti-miR-con or anti-miR-544a. Then dual-luciferase Reporter assay system (Promega) was used to detect luciferase activity in the cells lysates 48 h post-transfection.

2.6. Western-blot analysis

Total protein was extracted from cultured cells by cell lysis buffer (Huashun, Shanghai, China). Equal amounts of proteins were added to each hole and divided by SDS-PAGE at 80 V. Then proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA) for two hours. After blocked with 5% non-fat milk, PVDF membranes were incubated overnight at 4 °C with ki-67 or FBXW7 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Next, the membranes were further probed with HRP-conjugated anti-rabbit secondary antibody (Cell Signaling Technology) for 1 h after washed with TBST buffer for three times. Finally, the immunoreactive bands were visualized by chemiluminescence (Millipore).

2.7. Cell proliferation assay

The cell proliferation assay was performed using the Cell Counting Kit-8 (CCK-8 kit) (Dojindo, Tokyo, Japan) according to manufacturer's instructions. Lung cancer cells were inoculated into 96-well plates at a density of 5×10^3/well, and then transfected with indicated oligonucleotides or plasmids. Next, 10 μl CCK-8 reagent was added for another 2 h incubation at 0, 24, 48 and 72 h. The absorbance at 450 nm was measured to assess the relative cell viability by a microplate reader.

2.8. Cell invasion assay

Cell invasion assay was evaluated using Matrigel invasion chambers (BD Biosciences, Franklin Lakes, New Jersey, USA) based on the manufacturer's specifications. Transfected cells (5×10^4 cells/well) suspended in serum-free RPMI-1640 medium was seeded into the upper chamber, while the complete RPMI-1640 medium with 5% FBS was placed into the lower chamber. After 24 h of incubation, cells remaining on the upper membrane were removed carefully, and adherent to underside of the membrane were fixed and stained with crystal violet (Sigma, Santa Clara, CA, USA), followed by photographed and counted under a microscopy.