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The long non-coding RNA-DANCR exerts oncogenic functions in non-small cell lung cancer via miR-758-3p

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ABSTRACT

Long non-coding RNAs (lncRNAs) have been demonstrated to be involved in the occurrence and progression of multiple cancers. In this study, we investigated the role of the lncRNA DANCR in the development of non-small cell lung cancer (NSCLC). First, we found that DANCR was markedly upregulated in NSCLC tumor tissues and cell lines compared with related normal controls. The ectopic expression of DANCR significantly increased the proliferation, migration and invasion of SPC-A1 and NCL-H1299 cells. Furthermore, we investigated whether DANCR regulates NSCLC tumor formation in vivo. Subsequently, we concluded that DANCR promotes NSCLC cell proliferation, migration and invasion by regulating the tumor suppressor miR-758-3p. These results indicated that the DANCR/miR-758-3p axis could be a potential target in the treatment of NSCLC.

1. Introduction

Lung cancer is the malignant tumor with the highest mortality rate and has become the leading cause of cancer-related deaths in China [1,2]. A study reported that 80% of lung cancers are non-small cell lung cancers (NSCLCs) [3]. In contrast to small-cell lung cancer, NSCLC has a high potential for invasion and metastasis and is usually diagnosed at advanced stages [4,5]. Consequently, the prognosis of patients with NSCLC remains poor, and the 5-year survival rate of NSCLC patients is lower than 15% [6]. Therefore, it is critical to understand the molecular mechanism of NSCLC carcinogenesis in order to search for reliable diagnostic and therapeutic targets.

Long noncoding RNAs (lncRNAs) are non-protein-coding transcripts that are longer than 200 nucleotides and serve important roles in tumorigenesis [7,8]. It has been shown that lncRNAs function in every stage of tumor progression [9] and are involved in the cell growth, invasion, and metastasis of multiple cancers including NSCLC [10,11]. Previous studies have shown that the lncRNAs GAS6-AS1, MALAT1, HOTAIR, H19 and SCAL1 are associated with the development and progression of lung cancer [5,12–14]. Our group has determined that the long non-coding RNA anti-differentiation ncRNA (lncRNA DANCR) is upregulated in NSCLC tissues compared to normal tissues (data not shown). In the present study, we explored the role of DANCR in the development of NSCLC.

DANCR was identified by Kretz et al and was found to be required for the dedifferentiation of epidermal cells [15]. Studies indicated that DANCR has a critical regulatory role in osteoblast differentiation and could promote the osteogenic differentiation of periodontal ligament stem cells [16,17]. Recently, studies have shown that high expression levels of DANCR are involved in the progression of hepatocellular carcinoma, prostate cancer and colorectal cancer [18–20]. However, the role of DANCR in NSCLC is still unknown.

In the current study, we investigated the expression and function of DANCR in NSCLC and found it could act as an oncogene in lung cancer. Moreover, we demonstrated that DANCR regulates NSCLC cell proliferation, migration and invasion by down-regulating miR-758-3p.

2. Materials and methods

2.1. Ethics statement and specimens

For this study, 128 paired NSCLC and corresponding non-tumor control tissues were collected from the Hubei Cancer Hospital. This study was approved by the ethics committee of the Hubei Cancer Hospital, and written informed consent was obtained from all patients. All tissue samples were immediately preserved at −80 °C after being washed with sterile phosphate-buffered saline (PBS).

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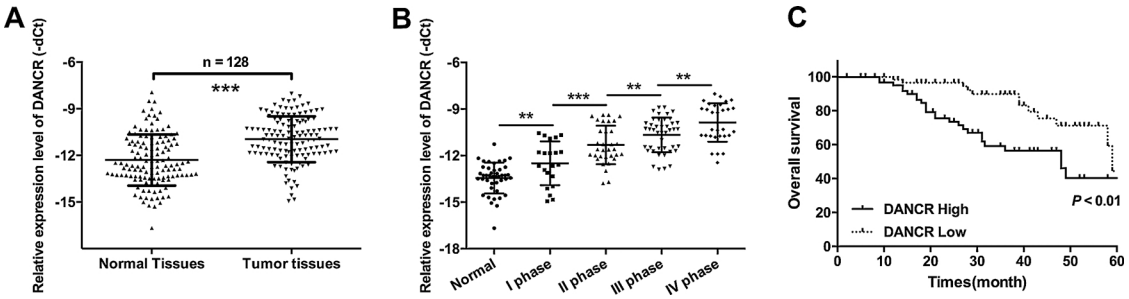

Fig. 1. Upregulation of DANCR in NSCLC. (A) Expression of DANCR in 128 matched NSCLC tissues and the corresponding adjacent normal tissues as measured by qRT-PCR (*** $P < 0.001$). (B) DANCR expression was positively correlated with advanced tumor-node-metastasis (TNM) stage (** $P < 0.01$, *** $P < 0.001$). (C) Patients with a higher expression level of DANCR had poorer prognoses than those with low expression levels. ($P < 0.01$).

2.2. Cell culture

NSCLC cell lines (SPC-A, NCL-H1650, NCL-H1975, SK-MES-1, A549, NCL-H358 and NCI-H1299) and the normal bronchial epithelial cell line (16HBE) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, CA, USA), 100 units/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 atmosphere at 37 °C.

2.3. Transfection

The pcDNA3-DANCR expression vector was constructed by Genscript (Nanjing, China). A specific shRNA against DANCR, a miR-758-3p mimic and a miR-758-3p inhibitor were designed, synthesized and validated as being effective by RiboBio (Guangzhou, China). To transfect the cells, the NSCLC cells were seeded into 6-well plates and incubated in the culture medium until they reached 70% confluence. The pcDNA3-DANCR, shDANCR, miR-758-3p mimic and negative control were individually transfected or co-transfected pairwise into the SPC-A1 or NCL-H1299 cells with Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

2.4. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA from the NSCLC tissues or cells was isolated using the TRIzol reagent (Invitrogen) after the indicated treatments were applied. Then, the reverse transcription reaction was performed with the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) and random primers to obtain the cDNA products. The thermocycling conditions for the reverse transcription were 25 °C for 5 min, 42 °C for 60 min and 70 °C for 10 min. qRT-PCR assays were performed by using the SYBR Premix Ex Taq (TaKaRa), primer and the cDNA template on the Applied Biosystems 7500 Real-time PCR System (ABI, USA). Each individual sample was run in triplicate, and the expression level was quantified by using the comparative cycle threshold (CT) method. The results were normalized to GAPDH expression, and RNA enrichments were calculated using the equation $2^{-\Delta\Delta CT}$. The specific primers for DANCR and miR-758-3p were designed and synthesized by RiboBio (Guangzhou, China).

2.5. Cell viability

Cell viability was assessed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The treated cells were seeded in a 96-well plate and cultured in complete medium. When the cells had adhered, 0.5 mg/mL MTT was added to each well and incubated at 37 °C for 4 h. Then, the supernatant was carefully aspirated, and 100 μl of DMSO was added. The absorbance was measured at 490 nm (Thermo, MA, USA).

2.6. Cell cycle was detected by flow cytometric analysis

The treated cells were washed with 1 × PBS, trypsinized and fixed with 70% ethanol for 30 min on ice. The RNA was degraded with 20 mg/ml RNase (Sigma-Aldrich) for 1 h at 37 °C. The DNA was then labeled with 20 mg/ml propidium iodide (PI, Sigma-Aldrich) and was assessed by FlowJo software.

2.7. Tumor formation assay in nude mice

Four-week-old male nude mice were purchased from the National Laboratory Animal Center (Shanghai, China) and divided into four groups for subcutaneous injection using SPC-A1 or NCL-H1299 cells. The SPC-A1 cells were infected with control plasmids or DANCR overexpression plasmids. The NCL-H1299 cells were transfected with control plasmids or DANCR knockdown plasmids (shDANCR). The animals were sacrificed 40 days after the injection, and the tumors were collected. The volume of each tumor was measured at 10, 15, 20, 25, 30, 35 and 40 days. The volume was calculated by the following formula: volume (mm^3) = length × $width^2/2$.

2.8. Colony formation assay

For the colony formation assay, SPC-A1 and NCL-H1299 cells were seeded at a density of 500 cells per well in 6-well plates after being transfected with different vectors. After being cultured for 7 days, the cells were fixed with a 4% paraformaldehyde solution and stained with crystal violet. The total number of colonies in each plate from three independent transfections was counted under an inverted microscope to evaluate the colony formation ability.

2.9. Wound-healing assay

SPC-A1 or NCL-H1299 cells were transfected with different vectors and seeded in 6-well plates. Small linear wounds were created by removing a line of cells with a disinfected Eppendorf tube tip. After removing the cell debris by washing with FBS-free medium, the wound areas were photographed under a microscope. The distance between the two edges of the wound was calculated at three different positions and analyzed by image analysis software (National Institute of Health, Bethesda, MD).

2.10. Migration and invasion assays

Cell migration and invasion assays were performed by using the Transwell assay according to the manufacturer's instructions. SPC-A-1 or NCL-H1299 Cells (5×10^4) were transfected with different vectors, seeded in the upper compartment and incubated in serum-free media; the lower compartment was filled with complete medium supplemented