Biochemical and Biophysical Research Communications xxx (2017) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Long non-coding RNA PCAT7 regulates ELF2 signaling through inhibition of miR-134-5p in nasopharyngeal carcinoma

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ARTICLE INFO

Article history:
Received 14 July 2017
Accepted 15 July 2017
Available online xxx

Keywords:
Prostate cancer associated transcript 7 (PCAT7)
miR-134-5p
ELF2
Nasopharyngeal carcinoma (NPC)
Tumorigenesis

ABSTRACT

While some long noncoding RNAs (lncRNAs) might promote nasopharyngeal carcinoma (NPC) initiation and progression, the involved molecular mechanisms remain largely unclear. Here, we discovered the novel LncRNA, prostate cancer associated transcript 7 (PCAT7), which was overexpressed and associated with worse prognosis in NPC. Decreased PCAT7 expression was found to significantly suppress tumor cell proliferation in vitro, and inhibited tumor growth and reduced the expression of proliferation antigen Ki-67 in vivo. Rescue assay was performed to further confirm that PCAT7 contributed to the progression of NPC through regulating miR-134-5p/ELF2 signal pathway. These results indicated that PCAT7 might contribute to the tumor progression in NPC by functioning as a ceRNA to sponge miR-134-5p.

1. Introduction

Nasopharyngeal carcinoma (NPC), mainly prevalent in Southeast Asia and Southern China, is a unique type of the head and neck cancer [1–3]. Radiation therapy is the effective treatment way for NPC, especially for early-stage NPC patients. But, a large ratio of NPC patients are diagnosed at advanced stage. The general 5-year survival rate of NPC patients has not been explicitly improved over the last few decades, and it remains less than 50% [4]. It is of great urgency to explore the molecular mechanisms in NPC, which are crucial for identifying new biomarkers and helping to develop new effective treatment strategies.

Long non-coding RNAs (lncRNAs), above 200 nucleotides (nt) in length and with no protein-coding capacity, played critical effects in human tumorigenesis. The recent discoveries of lncRNAs have gained widespread attention as new players in regulation of biological processes. Until now, a number of cancer-associated lncRNAs are reported to involved in tumor growth, invasion and metastasis, and have been revealed as potential alternative biomarkers and therapeutic targets for human cancers [5–9]. Several studies have reported that lncRNAs, including LOC100129148 [10], LOC553103 [11], and EWSAT1 [12] are related to NPC tumorigenesis. However, its detail mechanism underlying NPC tumorigenesis is still remain unclear. PCAT7 (NR_121566) is a 1937-bp lncRNA that maps at chromosome 9q22.32 (https://www.ncbi.nlm.nih.gov/gene/?term=PCAT7). Recently, Zhang et al. have reported that PCAT7 is over-expressed in NPC patients [13]. While to date, whether PCAT7 is involved in NPC tumorigenesis has not been investigated yet. Thus, the functions and underlying molecular mechanisms for PCAT7 in NPC are still needed to be further explored.

Here, we demonstrated that PCAT7 was up-regulated in NPC tissues and cell lines. PCAT7 up-regulated the miR-134-5p target gene ELF2 by function as a competitive endogenous RNA (ceRNA) for miR-134-5p and then promoted the proliferation of NPC cells. Given its significance in the ELF2 signaling pathway, PCAT7/miR-134-5p represents a novel and potential therapeutic target for the treatment of NPC.

2. Materials and methods

2.1. Ethical statement and tissue collection

50 patients diagnosed as NPC were involved in this study. All histologic diagnoses were performed by the pathology department in the Renmin Hospital of Wuhan University. Normal oral mucosal tissues were obtained from non-tumor adjacent tissues or patients

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http://dx.doi.org/10.1016/j.bbrc.2017.07.093
0006-291X/

with reshaping of gingival tissues. Informed consent was obtained from all subjects. All experimental protocols were approved by the Ethics Committee at the Renmin Hospital of Wuhan University. None of these patients received any pre-operative chemotherapy or radiotherapy.

2.2. Cell lines and plasmids

The human NPC cell lines, namely, SUNE-1, CNE-1, HNE-1, CNE-2, C666-1 and HONE-1 were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10%. fetal bovine serum (FBS). The human immortalized nasopharyngeal epithelial cell line NP69 was cultured in keratinocyte/serum-free medium (Invitrogen) supplemented with bovine pituitary extract. Cells were routinely cultured as previously described [14]. All cells were cultured at 37 °C in a 5% CO_2 atmosphere and maintained in 10% Fetal bovine serum (FBS, Kibbutz BeitHaemek, Israel). Plasmid pcDNA3.1-ELF2 (NM_006874) and pcDNA3.1-PCAT7 were prepared by ourselves. RNAi sequence: **PCAT7**: sh-1 target GAACATGCAGTCTAGGAACCGGCAT; sh-2 target AGTCTAGGAACCGGCATGCGCATAA; sh-3 target AGCAACATGAAGAGAGATGCCAGGA.

2.3. Cell transfection and stable cell lines

Cells were transfected with DNA plasmids using TransFast ™ transfection reagent lipofectamine 2000 (Invitrogen) according to manufacturer's instructions [15–22]. For screening stable cell lines, 48 h after transfection, cells were plated in the selective medium containing G418 (1000–2000 μg/mL, Invitrogen, Ltd., U.K) for the next 4 weeks or so, and the selective media were replaced every 3 days.

2.4. Western blot analysis

Western blot was conducted by the protocol described previously [23–25]. Briefly, cells were harvested, rinsed with PBS and lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM MgCl2, 0.5% NP-40, 1 mM Na3VO4, 1 mM NaF, protease inhibitors cocktail). Cell lysates were separated on SDS–polyacrylamide gel, transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories) and immunoblotted using the following primary antibodies. Primary antibody of ELF2, GAPDH (Santa Cruz, CA) were used for WB analysis.

2.5. qRT-PCR

Total cellular RNA was isolated using Trizol (Invitrogen) reagent and cDNA was generated using SuperScript II first-strand synthesis system (Invitrogen). Real-time qPCR analysis was performed on the Bio-Rad iQ5 Real-Time PCR detection system (Bio-Rad) with Maxima SYBR Green qPCR Master Mix (Fermentas) or on the Rotor-Gene Q instrument (Qiagen) with QuantiFast SYBR Green PCR Kit (Qiagen). All expression levels, unless otherwise specified, were normalized against the GAPDH mRNA level. Real-time qPCR for miR-31 was performed using miScript PCR Starter Kit and hsa-miR-134-5p miScript Primer Assay according to manufacturer's instructions (Qiagen). The primer sequences used in this study are as follows: human ELF2 (NM_006874): sense: 5′-GAGTGCGTGAGTGTGTGTCG-3′, antisense: 5′-TGAACTGATGCTTCCACGGC-3′, product length is 198; human PCAT7 (NR_121566): sense: 5′-AAACAAGCCAACCGCACAAT-3′, antisense: 5′-CCTGCTTGCTGTGTTACTGC-3′ product length is 179; human GAPDH: sense: 5′-AAGACCTTGGGCTGGGACTG-3′, antisense: 5′-ACCAAATCCGTTGACTCCGA-3′, product length is 219.

2.6. Cell viability analysis and colony formation assay

Viability of cells transfected with indicated shRNA or plasmid constructs was determined by trypan blue dye exclusion and CCK-8 assay. Colony formation assay was performed with CNE-1 and SUNE-1 cells transfected with sh-NC and sh-PCAT7 for 10 days followed by crystal violet staining.

2.7. Luciferase reporter assays

Luciferase reporter assays was conducted as described previously [26–29].

2.8. Biotinylated RNA pull-down assay

The biotinylated RNA pull-down assay was performed as described previously [30,31]. Briefly, biotin-labelled RNAs was in vitro transcribed with AmpliScribe T7-Flash Biotin-RNA Transcription Kit (Epicentre), treated with RNase-free DNase I and purified with an RNeasy Mini Kit (Qiagen). The lambda transcript was generated with the control plasmid provided by the Transcription Kit. To form the proper secondary structure, biotinylated RNA supplied with RNA structure buffer (10 mM Tris pH 7, 0.1 M KCl and 10 mM MgCl2) was heated to 90 °C for 2 min, incubated on ice for 2 min and then shifted to room temperature (RT) for 20 min. The RNA was then mixed with hypoxic HeLa nuclear extract or purified proteins and incubated at RT for 1 h, followed by incubating with Streptavidin Mag Sepharose (GE Healthcare) at RT for 1 h. After subsequent washes, the pull-down complexes were analyzed by standard western blot technique.

2.9. Statistical analysis

The results were analyzed using GraphPad Prism (GraphPad Software Inc, La Jolla, CA) and the SPSS 23.0 software (SPSS, Chicago, IL, USA). The significance of differences was assessed using Student's *t*-test or a one-way ANOVA. All data are expressed as the means ± SEM. $P < 0.05$ indicates a significant difference.

3. Results

3.1The expression of PCAT7 is up-expressed and implies a worse prognosis in NPC patients.

PCAT7 levels were determined by qRT-PCR and found to be higher expressed in NPC tissues compared with corresponding noncancerous tissues (N = 50) (Fig. 1A). PCAT7 was also found to be up-regulated in six NPC cell lines, including CNE-2, HNE-1, C666-1, HONE-1, CNE-1 and SUNE-1 cells, compared with a normal human oral keratinocytes cell line NP69 ($P < 0.05$), and PCAT7 expression was much higher in CNE-1 and SUNE-1 cells than other NPC cells (CNE-2, HNE-1, C666-1 and HONE-1) (Fig. 1B), thus, we performed the following experiments in CNE-1 and SUNE-1 cells. Additionally, we also discovered that higher PCAT7 expression implied a worse survival rate in NPC patients ($P < 0.05$) (Fig. 1C). These data demonstrated high PCAT7 expression was closed related to poor prognosis, and over-expression of PCAT7 might be crucial for NPC progression.

3.2PCAT7 knockdown suppresses NPC cell growth in vitro and in vivo

Next, we explored the role of PCAT7 on NPC cell growth. CNE-1 and SUNE-1 cells were transfected with sh-NC or sh-PCAT7, i.e., sh-PCAT7#1, sh-PCAT7#2, and sh-PCAT7#3. The qRT-PCR results

Please cite this article in press as: Y. Liu, et al., Long non-coding RNA PCAT7 regulates ELF2 signaling through inhibition of miR-134-5p in nasopharyngeal carcinoma, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.07.093