Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Increased circular RNA hsa_circ_0012673 acts as a sponge of miR-22 to promote lung adenocarcinoma proliferation

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ARTICLE INFO

Article history:
Received 17 January 2018
Accepted 19 January 2018
Available online xxx

Keywords:
Lung adenocarcinoma
Circular RNA
hsa_circ_0012673
ErbB3
Proliferation

ABSTRACT

Recent reports have indicated that circular RNA (circRNA) may regulate Lung adenocarcinoma (LAC) development. Our previous studies showed that hsa_circ_0012673 was up-regulated in a circRNA microarray. However, its expression level in LAC has not been verified, and the underlying molecular mechanisms in LAC are unknown. In this study, we found that the expression of hsa_circ_0012673 was up-regulated in LAC tissues compared to pair-matched adjacent non-tumor tissues ($P = 0.0079$), and that the expression level was associated with tumour size ($P = 0.015$). Furthermore, hsa_circ_0012673 was primarily localized in the cytoplasm and promoted cell proliferation of LAC cells by sponging miR-22, which targeted erb-b2 receptor tyrosine kinase 3 (ErbB3) in LAC. Hsa_circ_0012673 promotes LAC proliferation by suppressing miR-22, which targets ErbB3.

1. Introduction

Although there have been advances in the management of non-small cell lung cancer (NSCLC), it remains one of the leading causes of death worldwide, with an overall 5-year survival rate of 17% [1]. In recent decades, lung adenocarcinoma (LAC) has increased yearly and has become the highest incidence type of NSCLC [2]. However, most patients are diagnosed in the advanced stage, when local invasion is serious or distant metastases have already occurred. The molecular mechanism of LAC tumorigenesis is unclear. Thus, for elucidation of the molecular mechanism of LAC and development of personalized therapeutic strategies, detailed molecular characterization of LAC tissues is extremely important.

Circular RNA (circRNA) is a special type of endogenous non-coding RNA that is formed by back-splicing events through exon or intron circularization [3]. These molecules have multi-modal features, including conservation, abundance, stability and tissue-specific expression in organisms [4]. Many studies have revealed that circRNAs have numerous biologic functions, such as acting as microRNA sponges, forming RNA-protein complexes, and regulating targeted gene transcription and splicing [5]. Several circRNAs have also been shown to play important roles in tumour processes. However, the roles of circRNAs in LAC are largely unknown.

In our previous study, we demonstrated that circRNA microarray profiles in the early stage of LAC had unique characteristics [6]. Among the 59 differentially expressed circRNAs, hsa_circ_0012673 (fold change 3.21 times compared to pair-matched adjacent non-tumor tissues) was chosen for this study based on the bioinformatics analysis results. The gene of hsa_circ_0012673 is located at chr1:55340765-55341720, and its associated gene symbol is DHCR24. However, thus far, little is known about the expression and biological function of hsa_circ_0012673 in tumors, especially LAC.

Here, we sought to confirm the expression of hsa_circ_0012673 in LAC and to investigate the relationships between hsa_circ_0012673 expression and clinicopathological parameters of LAC. Furthermore, we investigated its biological function and mechanisms.

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https://doi.org/10.1016/j.bbrc.2018.01.126
0006-291X/

2. Materials and methods

2.1. Patients and specimens

The present study design and use of samples were approved by the Research Ethics Board of Zhongda Hospital affiliated with Southeast University (Nanjing, China). The experiments were carried out with the understanding and written consent of all subjects. A total of 33 paraffin-embedded LAC samples from stage I-III patients collected between June 2013 and December 2014 were tested to validate hsa_circ_0012673. All the methodologies of this study conformed to the standards set by the Declaration of Helsinki.

2.2. Total RNA extraction and RT-qPCR

Total RNA of cells was extracted using a TRIzol kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The primer sequences are provided in Supplementary Table S1.

2.3. Cell culture and transfection

The cells used in this research were purchased from Cobioer Biosciences Co., Ltd. (Nanjing, China) and were regularly confirmed by short tandem repeat profiling. H1792, A549, PC9, H1703, H1793, H1299 and the normal human bronchus epithelium cell line HBE were cultured in RPMI1640 supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. A549 and PC9 cells were transfected with siRNA (sequence in Supplementary Table S2.) targeting hsa_circ_0012673 using siRNA-Mate (GenePharma, Shanghai, China). The overexpression vector of hsa_circ_0012673 (pLCDH-ciR-hsa_circ_0012673) was constructed by Geneseed Co., Ltd. (Guangzhou, China). miR-NC, miR-22, miR-320, miR-760, miR-602 mimic and miR-22 inhibitor were all purchased from RiboBio Co., Ltd. (Guangzhou, China).

2.4. Cell proliferation assay

Cell proliferation assays were performed using the Cell Counting Kit-8 (CCK-8, Dojindo). The transfected cells were seeded in 96-well plates at a density of 2×10^3 each well and cultured for 24, 48, 72, or 96 h. CCK-8 was added to each well and then incubated at 37 °C for 2 h. The OD_{450} was measured at a wavelength of 450 nm using a microplate reader instrument (Bio-Rad, MA, USA). The EdU assay was performed with a Cell-Light EdU DNA Cell Proliferation Kit (RiboBio, Guangzhou, China) as described previously [6].

2.5. Xenograft model

All experimental protocols were approved by the Animal Ethics Committee of Zhongda Hospital affiliated with Southeast University (Nanjing, China). All animal experiments conformed to the guidelines for the Care and Use of Laboratory Animals (Ministry of Health, China, 1998). A total of 12 BALB/c nu/nu male mice (4 weeks old) were provided by the Experimental Animal Centre of Qinglongshan (Nanjing, China) and were fed a regular standard diet and housed under controlled conditions with comfortable temperature and humidity. For the xenograft model, A549 cells were injected into the mice (4×10^6 cells per animal, 6 mice per group). Each mouse was intratumorally injected with siRNA or si-NC (10 nmol each time) once every 3 days. Tumour formation was monitored every 3 days by measuring tumour size. After 4 weeks, all mice were sacrificed, and the tumours were excised and weighed. The tumors were dehydrated and fixed for immunohistochemical examination.

2.6. Western blotting analysis

Western blotting was conducted according to standard methods as described previously [6]. The antibodies used in this experiment were anti-ErbB3 rabbit antibody and goat anti-rabbit antibody (Cell Signaling Technology, Beverly, MA, USA).

2.7. Luciferase reporter assay

HEK-293T cells transfected with hsa_circ_0012673 or hsa_circ_0012673-mutant were seeded in a 96-well plate and co-transfected with the reporter vector and 50 nM miR-NC, miR-22, miR-320, miR-760 or miR-602 mimic using Lipofectamine 2000 (Life Technologies). Firefly and Renilla luciferase activities were detected by the Dual-Luciferase Reporter system (Promega). The effect of miRNA on the luciferase reporter with hsa_circ_0012673 was normalized to hsa_circ_0012673-mutant, and the fold change was calculated.

2.8. RNA-fluorescence in situ hybridization (FISH)

The RNA-FISH assay was conducted according to methods described previously [7]. Briefly, the hsa_circ_0012673 probe was labelled with biotin for RNA labelling, and the miR-22 probe was labelled with digoxin. After pre-hybridization, A549 cells were hybridized in hybridization buffer with probes at 88 °C for 5 min, followed by incubation with anti-digoxin-FITC and anti-biotin-Rhodamine antibodies at 37 °C. The images were acquired using a confocal microscope (Olympus, Tokyo, Japan).

2.9. Statistical analysis

The expression levels of hsa_circ_0012673 between LAC tissues and their adjacent tissues were normalized to the reference gene GAPDH and compared using paired sample *t*-tests. All statistical analyses were performed with SPSS statistical software version 24.0 (IBM, SPSS, Chicago, USA). A two-sided $P < 0.05$ was considered statistically significant for all the statistical calculations.

3. Results

3.1. The circular form of hsa_circ_0012673

Hsa_circ_0012673 is located on chromosome 1q31.1 and is composed of two exons (Fig. 1A). To examine the expression of hsa_circ_0012673, we designed a set of specific divergent primers. To determine whether the PCR method is reliable, we performed Sanger sequencing of the amplified RT-qPCR products. The results confirmed the back-splice junction of hsa_circ_0012673 (Fig. 1B). To further confirm the circular characteristics of hsa_circ_0012673, we used the enzyme RNase R, which does not act on circular RNA [8]. As expected, the circular RNA was resistant to RNase R treatment compared with the linear control gene GAPDH (Fig. 1C). These results indicated that there is a naturally circular RNA form of hsa_circ_0012673.

3.2. Clinical and pathological characteristics of LAC patients

The clinical and pathological characteristics of all 33 LAC patients (10 women and 23 men) are shown in Table 1. Among them, 19 (57.58%) were older than 60 years; 23 (69.70%) had lymphatic metastasis and 10 (30.30%) had no lymphatic metastasis. Additionally, 22 (66.67%) patients had stage I/II disease, and 11 (33.33%) patients had stage III/IV disease.

Please cite this article in press as: X. Wang, et al., Increased circular RNA hsa_circ_0012673 acts as a sponge of miR-22 to promote lung adenocarcinoma proliferation, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.01.126