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Circ-UBR5: An exonic circular RNA and novel small nuclear RNA involved in RNA splicing

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ABSTRACT

Circular RNAs (circRNAs) are class of non-coding RNAs formed by back-splicing events as loops, and could be found in all types of organisms. They play important and diverse roles in cell development, growth, and tumorigenesis, but functions of the majority of circRNAs remain enigmatic. Particularly functional phenotypes of great majority of circRNAs are not obvious. Here we randomly selected a circRNA circ-UBR5, which has no obvious functional phenotype in non-small cell lung cancer (NSCLC) cells from our previous research findings, to explore its potential function in cells. Differential expression of circ-UBR5 was detected in paired samples of tumorous tissues and adjacent nontumorous tissues from 59 patients with NSCLC by real-time quantitative reverse transcription-polymerase chain reactions (qRT-PCRs). Results showed circ-UBR5 expression was significantly downregulated in NSCLC tissues ($p < 0.001$) and was correlated with tumor differentiation ($p = 0.00126$), suggesting circ-UBR5 might serve as an index of NSCLC differentiation. Our findings indicated circ-UBR5 could bind splicing regulatory factor QKI, KH domain containing RNA binding (QKI) and NOVA alternative splicing regulator 1 (NOVA1) and U1 small nuclear RNA (snRNA) in the nucleus, revealing circ-UBR5 might be a novel snRNA involved in RNA splicing regulatory process. Moreover, we first presented a highly efficient strategy for finding specific circRNA binding proteins using Human Protein Microarray (Huprot™ Protoarray).

1. Introduction

To date more and more non-coding RNAs play important regulatory roles in tumors, such as miRNAs [1,2] and lncRNAs [3]. Circular RNAs (CircRNAs) are class of non-coding RNAs formed by back-splicing events as loops, and could be found in all types of organisms. Compared with linear RNA, its stability and conservation are higher than the former. It has great prospects as a target for drug therapy. In addition, circRNA sequence length is usually longer than miRNA and siRNA. which could be beneficial to the clinical application by reducing off-target effects. Recent evidences suggested some circRNAs could play roles in the proliferation and metastasis of various human cancers, such as colorectal cancer [4–6], hepatocellular carcinoma [7,8], bladder cancer [9], esophageal squamous cell carcinoma [10], gastric cancer [11], glioma [12], renal clear cell carcinoma [13], cholangiocarcinoma [14], oral cancer [15,16], cervical cancer [17,18], melanoma [19], lung Cancer [20–22], osteosarcoma [23,24], pancreatic carcinoma [25,26], breast Cancer [27,28], prostate cancer [29] and the growth of ovarian cancer [30].

However, we have also found many bottlenecks hindering our further study in the process of circRNA research at the same time. The following research difficulties exist: multifarious circRNA cyclization patterns are not conducive to the over-expression of partially circRNAs in vitro; circRNA expression in clinical tissue sample is usually very low, so sometime it could not be accurately

Abbreviations: circRNA, Circular RNA; NSCLC, non-small cell lung cancer; UBR5, ubiquitin protein ligase E3 component n-recognin 5; RBP, RNA binding protein; PUF60, poly(U) binding splicing factor 60; QKI, QKI, KH domain containing RNA binding; NOVA1, NOVA alternative splicing regulator 1; ANOVA, Analysis of variance; snRNA, small nuclear RNA.

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detected in clinical tissue samples by qRT-PCR test; design of the primers, siRNA and probes of circRNA were limited for removing the interference from its linear transcript, which caused some difficulties for circRNA research; functions of the majority of circRNAs remain enigmatic and most circRNAs have no obvious functional phenotype. The last one is the major problem and thus aroused our interest. Therefore, we randomly selected a circRNA circ-UBR5, which has no obvious functional phenotype in non-small cell lung cancer (NSCLC) cells from our previous research findings, to explore its potential role in cells.

In this study, we first identified circ-UBR5 and detected its expression in NSCLC, our datas showed circ-UBR5 was significantly downregulated in NSCLC and indicated low expression of circ-UBR5 in NSCLC was significantly related with tumor poor differentiation, suggesting that circ-UBR5 might serve as an index of NSCLC differentiation. Our findings indicated circ-UBR5 could bind splicing regulatory factor QKI, KH domain containing RNA binding (QKI) and NOVA alternative splicing regulator 1 (NOVA1) and U1 small nuclear RNA (snRNA) in the nucleus, revealing circ-UBR5 might be a novel snRNA involved in RNA splicing regulatory process. Moreover, we first presented a highly efficient strategy for finding specific circRNA binding proteins using Human Protein Microarray (HuprotTM Protoarray). Our research first revealed the potential role of non-functional phenotype circRNA in cells and could promote circRNA function research.

2. Materials and methods

2.1. Samples

The 59 samples of NSCLC and paired adjacent lung tissues were obtained from Shanghai Chest Hospital (Shanghai, China). All tissue specimens were stored at −80 °C until use. Clinical information was obtained under an Institutional Review Board approved study protocol and written informed consent was obtained from each subject.

2.2. Cell culture and transfection

Immortalized NSCLC cell lines (NCI-H1299, A549, NCI-H157, NCI-H1975, HCC827, NCI-H292, NCI-H1650, NCI-H460, NCI-H2170) and human lung epidermal cell line BEAS-2B, were from American Tissue Culture Collection (ATCC) (Manassas, USA). NCI-520, NCI-H358 and NCI-H226 were purchased from CAS (Chinese Academy of Sciences, China). All cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS) and a humidified atmosphere consisting of 5% CO2 and 95% air at 37 °C except for A549 cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD, USA). NCI-H1299 cells were transfected with circ-UBR5 overexpression vector PLCDH-circ-UBR5-GFP using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PLCDH-circ-UBR5-GFP was constructed by GENESEED (Guangzhou, China).

2.3. Quantitative real-time polymerase chain reaction assay

Total RNA was extracted from each sample of NSCLC tissues and paired adjacent nontumorous tissues using TRIzol Reagent (Invitrogen; Carlsbad, CA, USA) following the manufacturer's instructions. The cDNA was synthesized by reverse transcription (RT) using a Primescipt RT reagent kit with gRNA Eraser according to manufacture-provided protocols (TaKaRa) (with random primers). Real-time quantitative reverse transcription-polymerase chain reactions (qRT-PCR) were performed using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa), following manufacturer's instructions. Divergent primers for circ-UBR5 and primers for β-actin, U1, GAPDH and UBR5 were synthesized by Sangon Biotech (Shanghai, China). All qRT-PCR primer sequences see Table 1.

2.4. Sanger sequencing

Target fragment was inserted into a T vector for sanger sequencing to verify its full-length. The following Circ-UBR5-QC divergent primers were designed for use in experiments to confirm the back-splice junction of circ-UBR5: 5′-GCTGGAGAAGAGGATCATCATGATGAACAG-3′ (sense) and 5′-CACACCCTCCACCACCTCAAC-3' (antisense). Primers were synthesized by Sangon Biotech (Shanghai, China), and sanger sequencing was performed by Biosune (Shanghai, China).

2.5. RNase R treatment

1 μg total RNA was added to 10 μl Rnase R enzyme (Epicentre)

Table 1
Sequences.

Name	Sequences (5′− 3′)
Primer Circ-UBR5 F	TAGTGGGCGCAGAAGCGTTGT
Primer Circ-UBR5 R	CTTCTCCAGCCACACCCTCCAC
Primer β-actin F	AGTTGCGTTACACCCTTTCTTG
Primer β-actin R	GCTGTCACCTTCACCGTTCC
Primer U1 F	GGGAGATACCATGATCACGAAGGT
Primer U1 R	CCACAAATTATGCAGTCGAGTTTCCC
Primer GAPDH F	TGCACCACCAACTGCTTAGC
Primer GAPDH R	GGCATGGACTGTGGTCATGAG
Primer Circ-UBR5-QC-F	GCTGGAGAAGAGGATCATCATGATGAACAG
Primer Circ-UBR5-QC-R	CACACCCTCCACCACCTCAAC
Primer ubr5 F	AAGGGCAATACACTGACATGGTT
Primer ubr5 R	GGCCAGGCCAGTGCCATTTA
oligo-1	CUGCUUCUGAACCAGCAGUUGCUGCAGUGACAACGCUUCUGCGCCCACUAGCAUUAUCUUGGUUGCUGUGGUUACUUU CACUAUCACUUUCUGUUUCAGCUGCUGCU AACAAGUCCAGCUCCAUAUCACUCCCGUCUUCAUCAUGCUCAUCAUGUUGUCCCUCUGCCUCAGCAUUUUCUUCCCCGUGUUCUUCCUGU UCAUCAUGAUGAUCCUCUUCU CCAGCCACACCCUCCACCACCUCAAC
oligo-2	GCCACACCCUCCACCACCUCAACCUGCUUCUGAACCAGCAGUUGCU
oligo-NC	UGUGAACCACUGAAGGGAGACAAGUUGGAGAGGAAGCUGGUACAUUACUCACGAAUGAAUCAGACAUCCCCUCCCUCCGUGUUUGA AAAUGUCCUCGGCAUAAAGCAUGGUACAAUUCCAAACGAGGAACUAUGCUCAG
FISH-probe	CCTCCACCACCTCAACCTGCTTCTGAACCAGCAG

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