Original Paper

CircRNA Expression Profile in Early-Stage Lung Adenocarcinoma Patients

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Key Words

Lung cancer • Lung adenocarcinoma • CircRNA • Microarray

Abstract

Background/Aims: Lung adenocarcinoma, a form of non-small cell lung cancer with high lethality at an advanced stage, is becoming more common in women, non- or never-smokers, and even young adults. At present, there are still no effective early diagnosis methods for patients to be cured in a timely manner. Circular RNAs (circRNAs), which are stable and conserved non-coding RNA in mammalian cells, have been reported to be widely involved in the processes of cancer disease. However, it is still a puzzle as to which specific circRNAs are involved in the development of early-stage lung adenocarcinoma. **Methods:** Tumor samples and paired adjacent normal tissues from 4 patients with early-stage lung adenocarcinoma were selected to investigate the expression profile of circRNAs by using a high-throughput circRNA microarray. Bioinformatic analyses were conducted to screen those differentially expressed circRNAs. qRT-PCR and sequencing were performed to assure the microarray data. **Results:** A total of 357 circRNAs were dysregulated in the tumor samples, which suggests potential roles in lung cancer. qRT-PCR detection showed that five selected circRNAs were identical to the microarray data, and the potential circRNA-miRNA interactions were predicted. **Conclusion:** This work illustrates that clusters of circRNAs are aberrantly expressed in early-stage lung adenocarcinoma, which might offer potential targets for the early diagnosis of this disease and new genetic insights into lung cancer.

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Introduction

As the main leading cause of death among men and women, lung cancer is becoming a prevalent disease worldwide [1]. It is mainly divided into non-small cell lung cancer (NSCLC) (approximately 85% among lung cancers) and small cell lung cancer (SCLC) (approximately 15%), according to histologic types and prognosis. Current standard treatments for lung cancer include surgical resection, platinum-based dual chemotherapy and target therapy. However, these treatments rarely achieve the purpose of curing lung cancer, and the 5-year survival rate is still only 17% for NSCLC and even lower in SCLC (6%) [2, 3]. Lung adenocarcinoma is the most common subtype of NSCLC, for which the morbidity has been increasing year by year, especially in women, never-smokers and young adults [4, 5]. Therefore, it is urgent to seek effective diagnostic biomarkers for early-stage lung adenocarcinoma to ensure that the patients can be cured in a timely manner or survive after the appropriate treatments.

CircRNAs are a class of covalently closed circular RNAs that have been originally identified in plant viroid [6, 7], virus [8, 9], archaea [10], and metazoans [11]. Unlike linear RNAs, circRNAs exhibit the remarkable characteristic of being covalently closed continuous loops without 5' to 3' polarity and without a polyadenylated tail [12], which allows circRNAs to resist the degradation of major RNA enzymes. CircRNAs are characterized to be well-expressed, conserved and stable in cytoplasm [13], and they show tissue-specific and developmental stage-specific expression in mammalian cells [14]. As reported, circRNAs have mainly originated from protein-coding genes, and they can be categorized into three types according to their components, namely, exonic circRNA, intronic circRNA and exon-intron circRNA (or ElciRNA) [15]. Exonic circRNAs consist of one or more exons, and they primarily are localized in the cytoplasm and function as microRNA sponges [15]. Intronic circRNAs are produced by connecting two or more introns; when exons are circularized with introns 'retained' between exons, they are termed exon-intron circRNAs or ElciRNAs. Both intronic circRNAs and ElciRNAs are abundant in the nucleus and can promote transcription of their parent genes [16, 17]. In addition to being microRNA sponges to regulate gene expression at a transcriptional, post-transcriptional or translational level, circRNAs also have the capacity to translate proteins, according to the latest research [18, 19], whereas specific translational circRNAs usually produce proteins through the IRES-dependent mechanism on account of lacking the 5'-cap structure and poly-A tails [18]. In addition, circRNAs can insert into the genome and change the genomic DNA composition as they process pseudogenes [20]. However, the biological functions of circular RNAs remain largely unknown. However, increasing evidence has suggested that circRNAs could play a significant role in the development of cancer [21-25]. Nevertheless, little is known about their associations with lung adenocarcinoma at present.

Here, we have revealed the expression profile of circRNAs in early-stage lung adenocarcinoma by comparing it to normal adjacent tissue on microarray analysis. The identification of novel differentially expressed circRNAs can provide new insights into the early diagnosis and treatment of lung adenocarcinoma.

Materials and Methods

Patient samples

This work was conducted in accordance with the China Human Genome/Gene Research guidelines and was approved by the Shanghai 9th People's Hospital Research Ethics Committee, Shanghai Jiao Tong University School of Medicine.

Table 1. Clinical characteristics of the patients

Patient No.	Age	Gender	Histologic differentiation	TNM Stage	Smoking History
Case 1	58	Male	Poorly	T1bN0M0 IA	No
Case 2	66	Female	Well	T1bN0M0 IA	No
Case 3	70	Female	Well-moderately	T1bN0M0 IA	No
Case 4	68	Female	Moderately	T1aN1M0 IIA	No

All of the 14 samples from patients with Lung Cancer were collected from Shanghai 9th People's Hospital between May 2015 and July 2016. Among them, 4 tumors and paired adjacent normal tissue from non-smoking patients with lung adenocarcinoma were selected for the microarray analysis of circRNAs (detailed clinical data, see Table 1). Each sample was frozen in liquid nitrogen immediately after resection until it was used for detection.

RNA Extraction

Eight total RNA samples from tumors and paired adjacent normal tissues were extracted separately using TRIzol reagent (Invitrogen, USA), according to the manufacturer's protocol. gDNA contamination of each total RNA sample was further removed by using an RNase-Free DNase Set (QIAGEN, Germany), and then it was purified using an RNeasy Mini Kit (QIAGEN, Germany). The RNA integrity and quantity were assessed using standard denatured agarose gel electrophoresis and NanoDrop ND-1000 (ThermoFisher, USA), respectively.

Circular RNA microarray and computational analysis

Eight tissue samples were sent to KANGCHEN (Shanghai, China) for Arraystar circRNA Microarray analysis. The Human 8x15K circRNA Array, which contains 9114 circRNA probes, is manufactured by Arraystar Technologies (Rockville, MD, USA). Each circRNA was identified by using a specific probe that targets the specific splice junction of circRNA. Sample labeling and array hybridization were performed according to the manufacturer's protocol (Arraystar Inc.). The R software package (R version 3.1.2) was used to normalize the raw data and subsequent data processing. Two groups of profile differences (tumor samples *versus* adjacent normal tissue samples), and the absolute fold change (aFC) for each circRNA was computed.

qRT-PCR

500 ng total RNA from each sample of tumor or adjacent normal tissue was reverse-transcribed into cDNA with a random primer by using the SuperScript™ IV First-Strand Synthesis System (Invitrogen, USA) according to the manufacturer's instructions. Specific primers designed for circRNAs were listed in Table 2. Five differentially expressed circRNAs were measured by qRT-PCR using the 2xSYBR Green qPCR Master Mix (Biotool, USA) on the ROCHE LightCycler® 480 qPCR system. The reaction conditions were as follows: 95 °C for 10 min and 40 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 20 s. The human GAPDH reference gene was used to normalize the RNA levels. All of the reactions were conducted in triplicate. All of the PCR products with a single peak were purified and cloned into a pEasy-T3 vector (Transgen Biotech, China) for sequencing to assure the accuracy of the circRNA detection.

Statistical analysis

The statistical significance of the difference is conveniently estimated by a *t*-test on the microarray analysis. circRNAs that show fold changes (FC ≥ 2 and *p*-values < 0.05) are selected as being significantly differentially expressed, and the false discovery rate (FDR) was calculated to correct the *P* value on the microarray analysis. Each circRNA expression was presented as a fold change using the 2^{-ΔΔCT} method on qRT-PCR analysis. Differences between individual groups are analyzed using the *t*-test (two-tailed and unpaired). A *p*-value of < 0.05 (*), < 0.01 (**) or < 0.001 (***) is considered to be significant.

Table 2. Primers used for qRT-PCR analysis of circRNA and reference genes

circRNA ID	Primer sequence 5'-3'
has_circRNA_404833	Forward: CCCATTTTCAGCATCCCACTC Reverse: TAGTCTGGCTTGCAAGGAGGC
has_circRNA_406483	Forward: GTACCAACTTCCGTCAGTTGC Reverse: GCAGCCTTATGCACCAATTC
has_circRNA_006411	Forward: GCACCAGGTTCTTCGAAAAC Reverse: GAGGCCAATCCTTTCTGAC
has_circRNA_401977	Forward: TTCAGGGTAAACCCATCTAATCAC Reverse: ATCACTGGCTCCAAAGATGTCC
has_circRNA_001640	Forward: GATCGGAAGACTGATTCTGCTG Reverse: CTAGATGGCACCAGATACAGC
GAPDH	Forward: ATGAGGTCCACCCTGTT Reverse: CTCAAGGGCATCTGGGCTA

Results

Pathology of patient tumor samples

To promise the sample homogeneity, Hematoxylin and Eosin staining was performed, and the results showed that acinar-like tumor cells were stained and presented in the four tissue samples (Fig. 1A). According to the NCCN Guideline (Non-Small Cell Lung Cancer, Version 3.2017), TTF1 and Napsin A, which are highly specific markers for lung adenocarcinomas, were positively expressed in the tumor samples by detection using immunohistochemistry (Fig. 1B, lower panel). However, p63 and p40, which are the specific markers for lung squamous cell carcinoma, were negative or weakly expressed (Fig. 1B, upper panel). Together with the clinical characteristics of the patients in Table 1, the results indicated that all four tumor samples are homologous and belong to early-stage lung adenocarcinoma.

Overview of circRNA expression profiles

To dissect the circRNA profile in lung adenocarcinoma, the microarray assay was performed to compare the circRNA expression between tumor and paired normal tissues. Almost the same distributions for the dataset of the circRNA profiles are shown as a box plot after normalization using the R software package (Fig. 2A). The raw variations in the circRNA expression profile between the two groups were assessed by a scatter plot (Fig. 2B). The volcano plot filtered and identified the differentially expressed circRNAs with statistical significance between the two groups (Fig. 2C). Afterward, we found that 356 circRNAs were differentially expressed ($\text{aFC} > 2.0$ and $p < 0.05$) between lung adenocarcinoma samples and paired adjacent normal tissues. Among these, 204 circRNAs were up-regulated and 152 circRNAs were down-regulated in tumor samples, of which dysregulated circRNAs were summarized and classified according to their categories; most of them belong to exonic circRNAs (Fig. 2D). Hierarchical clustering is one of the simplest and widely used clustering techniques for the analysis of gene expression data, and it allows us to hypothesize about the relationships among the samples. Hierarchical clustering was performed, and the top 20 differentially expressed circRNAs were graphed (Fig. 2E). The raw and analyzed microarray data, formatted as Microsoft Excel files, were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO). The approved GEO accession number is GSE101684 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE101684>).

Validation of circRNAs expression

To confirm the microarray data, we randomly selected five differentially expressed circRNAs, including two up-regulated (hsa_circRNA_406483 and hsa_circRNA_404833) and three down-regulated (hsa_circRNA_001640, hsa_circRNA_006411 and hsa_circRNA_401977) circRNAs, to perform the qRT-PCR analysis in both tumor tissues and lung cancer cell lines (A549 & H1299), compared to normal tissues or human pulmonary bronchial

Fig. 1. Pathology of the tumor samples. A. Tumor tissue sections were stained with hematoxylin and eosin, and the acinar-like cells are indicated with arrows. Scale bar: 100 μm . B. By using the IHC method, tumor tissue sections were stained with weak p63 (#ab735, Abcam) or negative p40 (#ab166857, Abcam) expression (upper two panel), and with strong TTF-1 (#ab220211, Abcam) or Napsin A (#ab73021, Abcam) expression (lower two panel). Scale bar: 100 μm .

Fig. 2. A snapshot of the microarray signatures. A. The distributions of circRNA expression values ($\log_2$ ratios) were compared among the samples after normalization. (C: cancer tissue or tumor; AT: adjacent tissue). B. The Scatter-Plot assessed the circRNA expression variation between the tumor samples and paired adjacent normal tissue samples. The values of the X and Y axes in the Scatter-Plot represent the averaged normalized signal values of groups of samples ($\log_2$ scaled). The circRNAs above the top green line and below the bottom green line indicate that the fold change of the circRNAs is more than 2.0 between the two compared samples. C. Volcano Plots visualizing the differential expression of circRNAs. The vertical lines correspond to 2.0-fold up and down, and the horizontal line represents a p-value of 0.05. The red points in the plot represent the differentially expressed circRNAs with statistical significance (fold change > 2.0 and $p < 0.05$). D. The differentially expressed circRNAs were classified in the pie charts (right panel: up-regulated circRNAs; left panel: down-regulated circRNAs). E. Hierarchical clustering was performed based on “All Targets Value - CircRNAs” and showed a distinguishable circRNA expression profiling among 8 samples. The dendrogram hypothesizes the relationships among the expression levels of the samples.

epidermal cell lines (BEAS-2B, B2B). The results indicated that the expression patterns of selected circRNAs were consistent with the microarray data in the tumor samples (Fig. 3A), but the expression of hsa_circRNA_406483 and hsa_circRNA_001640 in the lung cancer cell lines did not match well with the microarray data (Fig. 3B). To assure the qRT-PCR results, the PCR products were cloned into a pEasy-3T vector for further sequence identification, and the results showed that those PCR products were exactly the indicated circRNAs with a back spliced junction, and the cyclic patterns of circRNAs were described according to the associated gene exons (Fig. 3C).

circRNA/miRNA interaction analysis

As reported, circRNAs could function as a miRNA sponge to regulate the gene expression [26, 27] and be involved in cancer processes [28–30]. To dissect the selected circRNAs’ potential functions, the circRNA and miRNA interactions were predicted by using Arraystar’s homemade miRNA target prediction software based on TargetScan and miRanda. Five confirmed circRNAs (hsa_circRNA_406483, hsa_circRNA_404833, hsa_circRNA_006411, hsa_circRNA_001640 and hsa_circRNA_401977) were annotated in detail using the circRNA/miRNA interaction information (Fig. 4A–E).

Fig. 3. Validation of the circRNA expression. A–B. The relative expression levels of selected circRNAs in tumor samples (A) or cell lines (B) were detected by the real time RT-PCR method. C. The PCR products with a splice junction were sequenced. Additionally, the generation models of the circRNAs were described according to the relative gene exons.

Fig. 4. Prediction of circRNA/miRNA interaction. A. hsa_circRNA_404833. B. hsa_circRNA_406483. C. hsa_circRNA_006411. D. hsa_circRNA_401977. E. hsa_circRNA_001640. 8mer: the bases from number 2 to 8 match perfectly, and the number 1 base is A; 7mer-m8: the bases from number 2 to 8 match perfectly, and the number 1 base is not A; 6mer: the bases from number 2 to 7 match perfectly, and the number 1 base is not A; offset 6mer: the bases from number 3 to 8 match perfectly; imperfect match: there is an imperfect base match from number 2 to 7; M: circRNA/miRNA interaction can be predicted by miRanda; T: circRNA/miRNA interaction can be predicted by TargetScan.

Discussion

Lung adenocarcinoma, a form of NSCLC, is the most common type of lung cancer in women and in never-smokers or non-smokers [5, 31]. Recently, it has also been frequently found in young people among Asians [4]. Considering the high morbidity and mortality of lung adenocarcinoma, many clinical trials are in progress, looking for new ways to diagnose this cancer at an early stage and to offer hope for a good chance to be cured by surgery.

Circular RNAs, a novel class of endogenous non-coding RNAs, have been shown to participate in the initiation and progression of tumors and could possibly function as biomarkers in cancers [32]. As reported in NSCLC, circ-ITCH functions as a “sponge” to block miR-7 or miR-214 and assures the stable expression of the ubiquitin E3 ligase ITCH to inhibit the Wnt/ β -catenin signaling pathway, which can indirectly inhibit the proliferation of lung cancer cells through promoting the ubiquitination and degradation of Dvl2 [33]. In addition to being a microRNA “sponge” or regulator of gene transcription [34], circRNAs not only can produce pseudogenes to alter the genomic DNA composition [18, 20, 35] but also possess a protein translation function [19]. Moreover, as prevalent molecules in eukaryotic cells, circRNAs are expressed abnormally in many types of cancer diseases and are closely related to the occurrence and development of cancer [32, 36, 37]. This evidence reveals that circRNAs can provide potential targets for the clinical diagnosis and prognosis of cancer.

By using microarray and qRT-PCR analysis in this study, we compared the expression pattern of circRNAs between tumor samples and paired adjacent normal tissue from patients diagnosed with early-stage lung adenocarcinoma. The microarray data revealed that a total of 357 circRNAs were dysregulated ($\text{aFC} > 2.0$ and $p < 0.05$) and might be involved in the occurrence of the early-stage lung adenocarcinoma. To confirm the microarray data, selected circRNAs were detected and verified in tissues or cell lines by the qRT-PCR methods. However, some circRNAs in cell lines were not totally matched with the array results, which might be due to the cell lines being isolated from patients with advanced lung carcinoma or the genetic variation. Among those dysregulated circRNAs in the tumor samples of patients, the expression levels of hsa_circRNA_404833 and hsa_circRNA_406483 were dramatically enhanced but almost undetectable in the adjacent tissues, which hinted that both might be outstanding potential candidates for early diagnosis markers of lung adenocarcinoma.

In terms of the mechanism, a majority of circRNAs are generated from back-splicing ligates of a downstream (3') splice donor site in reverse with an upstream (5') splice acceptor site, thus resulting in a covalently closed circRNA transcript, which is different from the canonical splicing that joins an upstream splice donor site with a downstream splice acceptor site [38]. Here, the aberrant expression of circRNAs could be a trigger of healthy cells to cancerous cells, and it could also be the consequence of the dysregulation of proteins or enzymes in cancer cells. However, a large amount of experimental evidence is still needed to trace those issues that could be solved from the perspective of cell regulation, including genetic mutation, DNA methylation, cell cycle disorder, and protein or enzyme activity change.

Given that circRNAs could function as miRNA sponges [33] and most of the circRNAs contain one or more miRNAs binding sites, the dysregulated circRNAs in NSCLC were likely due to an involvement in miRNA blockage, thereby contributing to the development of disease. The circRNA/miRNA interaction predicted that hsa_circRNA_404833 is potentially able to interact with miR-149-5p, which reduces cell motility and is involved in the acquired gefitinib resistance in lung adenocarcinoma [39]. However, additional research is required to clarify whether these circRNAs are involved in NSCLC through interacting with specific miRNAs.

In conclusion, we uncovered the circRNAs expression profile in early-stage lung adenocarcinoma by comparing it with adjacent normal tissue. Additionally, five dysregulated circRNAs expression levels in tissue were verified via qRT-PCR or sequencing, which is consistent with the microarray data. Taken together, we provide potential targets for the future diagnosis of early lung adenocarcinoma and novel genetic insights into lung cancer.

Disclosure Statement

None of the authors have conflicts of interest.

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