Abstract. To the best of our knowledge, the oncogenic role of circular RNA solute carrier family 26 member 4 (circSLC26A4) has only been reported in cervical cancer, while its role in non-small cell lung cancer (NSCLC) is unknown. The present study explored the involvement of circSLC26A4 in NSCLC. NSCLC tissues and paired adjacent non-tumor tissues were collected from 64 patients with NSCLC. The expression levels of circSLC26A4, mature microRNA-15a (miR-15a) and miR-15a precursor in these tissues were determined by reverse transcription-quantitative PCR (RT-qPCR). NSCLC cells were transfected with pcDNA3.1-circSLC26A4 vector to overexpress circSLC26A4, followed by the measurement of the expression levels of mature miR-15a and miR-15a precursor using RT-qPCR. Cell proliferation was analyzed using a Cell Counting Kit-8 assay. circSLC26A4 expression was upregulated in NSCLC tissues, and its high expression was significantly associated with poor survival of patients with NSCLC. The expression levels of circSLC26A4 were correlated with the expression levels of mature miR-15a, but not the expression levels of miR-15a precursor in NSCLC tissues. In NSCLC cells, overexpression of circSLC26A4 was associated with downregulation of mature miR-15a expression, but not miR-15a precursor expression. A cell proliferation assay revealed that overexpression of circSLC26A4 reduced the inhibitory effects of overexpression of miR-15a on cell proliferation. Therefore, circSLC26A4 may suppress the maturation of miR-15a in NSCLC to inhibit cancer cell proliferation.

Introduction

Non-small cell lung cancer (NSCLC) accounts for >80% of lung cancer cases worldwide according to the statistics of global cancer in 2018 (1). Patients with NSCLC are usually treated with surgical resection, chemotherapy and radiotherapy, and surgical resection is only appropriate for patients diagnosed at early stages, and resistance usually develops during the long-term application of chemotherapy and radiotherapy (2,3). With the emergence of targeted therapies, survival of certain NSCLC cases, especially patients diagnosed at advanced stages, has been markedly improved (4,5). However, as the molecular mechanisms of NSCLC are unclear, there remains a lack of effective targets for targeted therapy (6,7). Therefore, an improved understanding of the pathogenesis of NSCLC is required.

Most cases of NSCLC are associated with tobacco smoking (8), while ~10% of NSCLC occurs in never-smokers (9), suggesting the involvement of other factors, such as molecular factors, in the pathogenesis of NSCLC. In effect, certain molecular signaling pathways, such as the TGF-β and KRAS signaling pathways, have been demonstrated to be potential targets for targeted therapies of NSCLC, which can be performed to treat NSCLC by regulating the expression levels of NSCLC-related genes (10,11). Circular RNAs (circRNAs/circs) are single strand RNA transcripts closed by covalent bonds (12). CircRNAs have limited protein-coding capacity; however, they are involved in cancer biology by regulating gene expression, suggesting that circRNAs are potential therapeutic targets for NSCLC (13). In a recent study, circRNA solute carrier family 26 member 4 (circSLC26A4) has been reported to be an oncogene in cervical cancer (14); however, to the best of our knowledge, its role in NSCLC is unknown. Our preliminary microarray analysis revealed altered expression levels of circSLC26A4 in NSCLC and its inverse correlation with mature microRNA (miRNA/miR)-15a, which is a cancer-related miRNA (15). The present study aimed to explore the potential interaction between circSLC26A4 and miR-15a in NSCLC.

Materials and methods

Tissue collection. NSCLC tissues and adjacent non-tumor tissues (within 5 cm around tumors) were collected from 64 patients with NSCLC who underwent surgical resection [40 male and 24 female patients; 28 cases of lung adenocarcinoma (LUAD) and 36 cases of lung squamous cell carcinoma (LUSC)] at Shanghai Pulmonary Hospital (Shanghai, China) between June 2016 and May 2019. The patients had an age range of 45–69 years, with a median age of 56 years. The present study was approved by the Ethics Committee of
Therapy and follow-up. The 64 patients with NSCLC were classified into American Joint Committee on Cancer stage I or II (n=26) and III or IV (n=38) (16). The 64 patients were treated with surgical resection, radiotherapy, chemotherapy or immunotherapy according to their cancer stage and health conditions. The patients were followed up in a monthly manner until July 2020. Patient survival conditions were recorded and survival analysis was performed. All patients completed the follow-up or died of NSCLC during the follow-up.

NSCLC cells and transfection. The two human NSCLC cell lines, H1793 (LUAD) and DMS 79 (LUSC), obtained from American Type Culture Collection were used in the present study. Cells were cultured in a mixture composed of 90% RPMI-1640 medium (HyClone; Cytiva) and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) with 100 U/ml penicillin and 100 µg/ml streptomycin. The cell culture conditions were: 37°C, 5% CO₂, and 95% humidity. Cells were collected at a confluence rate of ~85% for the subsequent experiments. Vector expressing circSLC26A4 was constructed with pcDNA3.1(+)(Invitrogen; Thermo Fisher Scientific, Inc.) as the backbone. To overexpress miR-15a, miR-15a mimic (5'-UAG CAG CAC AUA AUGGUUUGUG-3') and non-specific miRNA [5'-UUC UCC GAACGUUCAGCUTT-3']; used as the negative control (NC) were purchased from Sigma-Aldrich (Merck KGaA). H1793 and DMS 79 cells were counted and 5x10^4 cells were transfected with 45 nM miRNA or 1 µg expression vector using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 24 h. Empty vector- or miRNA NC-transfections were performed to serve as NC experiments. Untransfected cells were used as the control cells. Prior to the subsequent assays, cells were cultured in fresh medium for another 48 h.

RNA preparation. Ribozol (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from tissue samples and H1793 and DMS 79 cells. RNA samples were digested with DNase I at 37°C for 2 h to remove genomicDNAs. RNA integrity was checked using 6% urea-PAGE gel electrophoresis. The optical density (OD)260/280 ratio of RNA sample was measured to reflect RNA integrity using NanoDrop 1000 instrument spectrophotometer (Thermo Fisher Scientific, Inc.). Reverse transcription-quantitative PCR (RT-qPCR). Total RNA (500 ng) was reverse transcribed into cDNA using PrimeScript RT Master Mix (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. SYBR Green Master Mix (Bio-Rad Laboratories, Inc.) was used to perform qPCR. The expression levels of circSLC26A4 were determined using GAPDH as the internal control. Expression levels of mature miR-15a and miR-15a precursor were analyzed using the All-in-One™ miRNA qRT-PCR Reagent kit (GeneCopoeia, Inc.) according to the manufacturer's protocol. Sequence-specific forward and reverse primers were used to perform RT and qPCR to determine the expression levels of miR-15a precursor.

| Characteristics          | No. |
|--------------------------|-----|
| Age, years               |     |
| ≤55                      | 34  |
| >55                      | 30  |
| Sex                      |     |
| Male                     | 40  |
| Female                   | 24  |
| Smoking history          |     |
| Smoker                   | 39  |
| Nonsmoker                | 25  |
| Pathological pattern     |     |
| LUAD                     | 28  |
| LUSC                     | 36  |
| Lymphatic metastasis     |     |
| No                       | 24  |
| Yes                      | 40  |
| Stage                    |     |
| I+II                     | 26  |
| III+IV                   | 38  |

LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

To measure the expression levels of mature miR-15a, poly (A) addition was first performed, followed by using poly (T) as the reverse primer to perform both RT and qPCR. The conditions of PCR reaction were initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 30 sec. The expression levels of miRNAs were normalized to the internal reference U6. All experiments were performed in three technical replicates. The 2^-ΔΔCq method was used to normalize Ct values of target genes to the corresponding internal control (17). The primers used in the present study were: CircSLC26A4 forward, 5'-TCCAAGTGTGCTGTCAGCAG-3' and reverse, 5'-CCATATCGGAAGAATCGC-3'; miR-15a precursor forward, 5'-GCC GAGTAGCAGCACACATAA-3' and reverse, 5'-CAGTGGCTGTCCGGAGG-3'; mature miR-15a forward, 5'-TGAGACCACATAATGG-3' and reverse, 5'-GTCAGGGCCGAGGAG-3'; and GAPDH forward, 5'-ATCACTGCCACCCAGAAGAC-3' and reverse, 5'-TTCTAAGCGGCAGCTAC-3'.

Cell Counting Kit-8 (CCK-8) assay. Transfected H1793 and DMS 79 cells were subjected to cell proliferation analysis using a CCK-8 assay (Sigma-Aldrich; Merck KGaA). Briefly, 0.1 ml medium containing 4,500 cells was added into each well of a 96-well plate. H1793 and DMS 79 cells were cultured at 37°C for 24, 48, 72 and 96 h, and then 10 µl CCK-8 regent was added for 2 h. The measurement of OD values was performed, and the absorbance was detected at 450 nm.

Statistical analysis. SPSS 22.0 (IBM Corp.) was used to conduct the statistical analysis of data. The expression levels of miR-15a were compared using the Student's t-test. A P-value of <0.05 was considered to indicate a statistically significant difference.
of circSLC26A4, mature miR-15a and miR-15a precursor in NSCLC and non-tumor tissues were presented as average values of three technical replicates. Data comparisons were performed using a paired t-test. Other data on cell transfection, RT-qPCR and CCK-8 assay were presented as the mean ± SD of three biological replicates. The comparisons of the study groups was conducted using one-way ANOVA followed by Tukey's post hoc test. Pearson's correlation coefficient was used for correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Altered expression levels of circSLC26A4, mature miR-15a and miR-15a precursor in NSCLC tissues. The expression levels of circSLC26A4, mature miR-15a and miR-15a precursor in NSCLC tissues and adjacent non-tumor tissues were measured by RT-qPCR. The results revealed that, compared with that in non-tumor tissues, circSLC26A4 expression was significantly upregulated in NSCLC tissues (Fig. 1A; P<0.01). Furthermore, the expression levels of mature miR-15a (Fig. 1B) and miR-15a precursor (Fig. 1B) were significantly downregulated in NSCLC tissues compared with in non-tumor tissues (P<0.01). Therefore, circSLC26A4 and miR-15a may be involved in NSCLC.

High expression levels of circSLC26A4 in NSCLC tissues are associated with poor survival of patients with NSCLC. Survival curve analysis revealed that, compared with patients in the low circSLC26A4 expression group, patients in the high circSLC26A4 expression group had a significantly lower overall survival rate. Therefore, high expression levels of circSLC26A4 may predict poor survival of patients with NSCLC (Fig. 2).

circSLC26A4 suppresses the maturation of miR-15a in NSCLC. Pearson's correlation coefficient analysis was used to determine
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The correlation between circSLC26A4 expression and mature miR-15a or miR-15a precursor expression in NSCLC tissues. It was observed that circSLC26A4 expression was inversely and significantly correlated with the expression levels of mature miR-15a in NSCLC tissues (Fig. 3A). However, there was no significant correlation between the expression levels of circSLC26A4 and miR-15a precursor expression in NSCLC tissues (Fig. 3B). To further explore the interaction between circSLC26A4 and miR-15a, circSLC26A4 expression vector or miR-15a mimic was transfected into H1793 and DMS 79 cells, and transfection efficiency was confirmed at 48 h after transfection. The effects of overexpression of circSLC26A4 on mature miR-15a and miR-15a precursor expression were also analyzed by reverse transcription-quantitative PCR (Fig. 3C). Overexpression of circSLC26A4 resulted in downregulation of mature miR-15a expression (P<0.05; Fig. 3D), but not in downregulation of miR-15a precursor expression (Fig. 3E).

These findings suggested that the maturation of miR-15a in NSCLC cells could be suppressed by circSLC26A4. By contrast, overexpression of miR-15a exhibited no significant effect on the expression levels of circSLC26A4 (Fig. 3F).

Overexpression of circSLC26A4 promotes NSCLC cell proliferation via miR-15a. A CCK-8 assay was performed to explore the role of circSLC26A4 and miR-15a in regulating the proliferation of H1793 and DMS 79 cells. Overexpression of circSLC26A4 significantly increased NSCLC cell proliferation, while overexpression of miR-15a decreased cell proliferation compared with their respective NCs. In addition, overexpression
of circSLC26A4 reduced the inhibitory effects of overexpression of miR-15a on cell proliferation (P<0.05; Fig. 4).

Discussion

The differential expression of circSLC26A4 in NSCLC and its interaction with miR-15a were investigated in the present study. The present data demonstrated that circSLC26A4 expression was significantly upregulated in NSCLC tissues and it could promote the proliferation of NSCLC cells by suppressing the maturation of miR-15a.

In a recent study, circSLC26A4 expression was reported to be upregulated in cervical cancer, and its high expression was significantly associated with poor survival of patients with cervical cancer (14). In addition, circSLC26A4 promotes the migration and proliferation of cervical cancer cells by sponging miR-1287-5p to upregulate HOXA7 (14). However, the regulatory effects of circSLC26A4 in NSCLC is still unclear. In the present study, to investigate the role of circSLC26A4 in the development of NSCLC, the expression levels of circSLC26A4 in NSCLC and adjacent non-tumor tissues were measured. circSLC26A4 expression was upregulated in NSCLC tissues. In addition, overexpression of circSLC26A4 increased the proliferation of both LUAD and LUSC cells, which are the two major subgroups of NSCLC. Therefore, circSLC26A4 may promote the proliferation of cancer cells, resulting in the development and progression of NSCLC.

Even with advances in the diagnosis and treatment of NSCLC, the prognosis of NSCLC is still poor (18,19). Goyal et al (19) conducted a retrospective study of patients with NSCLC diagnosed between 2004 and 2014 using the National Cancer Database, revealing that the overall 5-year survival rate of patients with stage IV NSCLC is only ~24%. This is mainly due to the low early diagnostic rate (19). Furthermore, due to the lack of sensitive markers, the early diagnosis of NSCLC is unlikely to be markedly improved in the near future (20). The present study demonstrated that high expression levels of circSLC26A4 were closely associated with poor survival of patients, suggesting a potential role of circSLC26A4 as a prognostic biomarker for NSCLC. Therefore, evaluation of the expression levels of circSLC26A4 prior to therapy may assist the determination of treatment approaches, which would in turn improve patient survival. miR-15a has been reported to be a tumor suppressor in different types of cancer, including NSCLC (21,22).

One study reported that miR-15a expression was downregulated in NSCLC tissues and cells, and overexpression of miR-15a inhibited NSCLC cell proliferation, migration and invasion (23). Another study revealed that miR-15a expression is markedly downregulated in NSCLC tissues, and overexpression of miR-15a markedly suppresses cell viability, invasion and migration, and accelerates the apoptosis of NSCLC cells (24). miR-15a not only suppresses tumor metastasis, but also increases the sensitivity of cancer cells to chemotherapy (19,20). Consistently, the present study revealed the downregulation of miR-15a expression in NSCLC and its
inhibitory effects on cell proliferation. Notably, the present study revealed that overexpression of circSLC26A4 suppressed the maturation of miR-15a in NSCLC cells. However, to the best of our knowledge, the underlying mechanism is unknown. To be cleaved into mature miR-15a, miR-15a precursor should be transported out of nucleus to enter the cytoplasm (25). The involvement of circSLC26A4 in the transportation of miR-15a will be explored in our future studies.

In conclusion, circSLC26A4 expression is upregulated in NSCLC and may promote cancer cell proliferation by suppressing the maturation of miR-15a. The current findings may help to increase the understanding of NSCLC pathogenesis and to identify potential targets for the treatment of patients with NSCLC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QC conducted the experiments and contributed to data analysis and manuscript writing. JL provided critical guidance for data analysis and manuscript writing. All authors confirmed the authenticity of the data and read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shanghai Pulmonary Hospital, Tongji University (Shanghai, China), and all patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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