Circular RNA TADA2A promotes proliferation and migration via modulating of miR-638/KIAA0101 signal in non-small cell lung cancer

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Abstract. Accumulating evidence indicates that circular (circ)RNAs exhibit complex functions in diverse malignant tumors, including non-small cell lung cancer (NSCLC). The role of the circRNA transcription adaptor 2A (circTADA2A) in NSCLC remains unclear. The expression, function and mechanism of circTADA2A in NSCLC development were investigated in the present study. The results revealed that circTADA2A was upregulated in NSCLC, and that knockdown of circTADA2A inhibited cell proliferation and migration in the NSCLC cell lines A549 and H1299. Functional assays demonstrated that circTADA2A promoted proliferation and migration via interacting with microRNA (miR)-638. Bioinformatics and reverse transcription-quantitative PCR assay confirmed that miR-638 was expressed at low levels in NSCLC. In addition, it was found that miR-638 served a tumor-suppressive role and suppressed proliferation and migration via PCNA clamp associated factor (KIAA0101) inhibition in A549 and H1299 cells. Lastly, it was verified that circTADA2A promoted cell proliferation and migration via modulating miR-638/KIAA0101 signaling.

Introduction

As the most common cause of cancer-associated mortality worldwide, lung cancer accounted for ~18.4% of all cancer-associated mortality in 185 countries in 2018 (1). Small cell lung cancer (SCLC) and non-SCLC (NSCLC) are the two primary types of lung cancer. NSCLC accounts for ~85% of all histological subtypes of lung cancer (2). Despite improvements in surgical resection, chemotherapy, targeted therapy and radiotherapy, the prognosis of NSCLC remains unfavorable, since it presents with locally advanced or metastatic disease in ~70% of patients at time of diagnosis (2). Therefore, it is urgent to identify new molecules and clarify their functions in NSCLC.

PCNA clamp associated factor (KIAA0101) is located on human chromosome 15q22.31 and contains five exons. KIAA0101 has been found to serve an oncogenic role in cancer (3-6). Fan et al (7) reported that KIAA0101 is a key promoter of renal cell carcinoma malignancy induced by erythropoietin. Lv et al (4) found that KIAA0101 knockdown suppresses cell growth, colony formation and G1/S phase transition in breast cancer cells. To the best of our knowledge, there is limited research on KIAA0101 and NSCLC. By analyzing 357 NSCLC tissue microarrays via immunohistochemical staining, Kato et al (8) found that KIAA0101 is highly expressed in 33.9% (121 of 357) of cases, and that over-expression of KIAA0101 predicted poor prognosis in patients with NSCLC. However, the detailed mechanism of KIAA0101 in NSCLC remains largely unknown.

Previously, circular (circ)RNAs have attracted increasing attention in non-coding RNA research (9,10). circRNAs are a group of stable, covalently closed RNAs transcripts with gene-regulatory potential (11). circRNAs serve pivotal regulatory roles, and can be valuable diagnostic and prognostic biomarkers in NSCLC (12). The function of circRNA transcriptional adaptor 2A (TADA2A; circTADA2A; also named hsa_circ_0043278 in circBase) in cancer remains controversial.
circTADA2A serves as a tumor suppressor in several types of malignancy, including breast and colorectal cancer (13,14). Wu et al (15) reported that circTADA2A competes with cyclic AMP-responsive element-binding protein 3 (CREB3) via similar microRNA (miRNA/miR)-203a-3p response elements and promotes cancer progression and metastasis in osteosarcoma. However, the role of circTADA2A in NSCLC remains largely unknown.

The present study investigated the expression and function of circTADA2A and its potential oncogenic role in NSCLC.

Materials and methods

Patients and tissue samples. A total of 60 NSCLC (38 males and 22 females; age, 49-83 years; mean age, 61.7 years) and paired paratumor tissue specimens (2 cm from NSCLC tissue) were collected from patients following surgical resection between January 2015 and April 2018 at Liaoning Cancer Hospital and Institute (Shenyang, China). All patients provided written informed consent, and the study was performed in accordance with the Declaration of Helsinki. Approval of the study was granted by the Institute Research Medical Ethics Committee of Liaoning Cancer Hospital & Institute. All patients were histologically diagnosed by two pathologists, and classified as stage I, II, III or IV, using the Tumor-Node-Metastasis (TNM) staging system (version 8) (16).

Cell culture. The human NSCLC (A549, H460, H1299 and H1975) and normal bronchial epithelial cell line 16HBE were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences. The 16HBE cells were cultured in Airway Epithelial Cell Basal Medium (American Type Culture Collection); NSCLC cell lines were cultured in Dulbecco's modified Eagle medium (Gibco; Thermo Fisher Scientific, Inc.). All cells were incubated at a constant temperature of 37°C in a humidified atmosphere (Thermo Fisher Scientific, Inc.). All cells were incubated at 37°C for 4, 8, 12 and 24 h. Next, total RNA from A549 or H1299 cells was extracted and incubated at 37°C for 4, 8, 12 and 24 h. Next, total RNA from A549 or H1299 cells was extracted, and the levels of circTADA2A and linear TADA2A mRNA were measured by RT-qPCR.

Bioinformatics and GEO database analysis. CircPrimer (17) was used to compare the nucleotide sequences of circTADA2A and TADA2A mRNA. The clinical value of miR-632-targeted genes in The Cancer Genome Atlas was analyzed using online software UALCAN (18). Data on differentially expressed circRNAs (ncbi.nlm.nih.gov/geoo/query/acc.cgi?acc=GSE101586), miRNAs (ncbi.nlm.nih.gov/geoo/query/acc.cgi?acc=GSE19945 and ncbi.nlm.nih.gov/geoo/query/acc.cgi?acc=GSE74190) and miRNAs in NSCLC were downloaded from the GEO database (ncbi.nlm.nih.gov/geoo/query/acc.cgi?acc=GSE19188 and ncbi.nlm.nih.gov/geoo/query/acc.cgi?acc=GSE30219). Differentially expressed circRNAs, miRNAs and mRNAs were analyzed using the online software GEO2R (19).

RNome R treatment. In order to detect the stability of circTADA2A, RNome R assay was performed as previously described (15). A total of 5 U/µg RNome R (Epicentre; Illumina, Inc.) was added to 2 mg total RNA (isolated from A549 or H1299 cells) and incubated for 30 min at 37°C. Controls were untreated. Subsequently, RNome MinElute Cleaning kit (Qiagen, Inc.) was applied to purify the RNAs according to the manufacturer's instructions. Next, reverse transcription-quantitative (RT-q)PCR was performed to detect circRNAs or mRNAs.

Actinomycin D assay. Actinomycin D assay was performed as previously reported (20). Briefly, 2 µg/ml actinomycin D (Sigma-Aldrich; Merck KGaA) was added to A549 and H1299 cells and incubated at 37°C for 4, 8, 12 and 24 h. Next, total RNA from A549 or H1299 cells was extracted, and the levels of circTADA2A and linear TADA2A mRNA were measured by RT-qPCR.

RNA extraction and RT-qPCR. RNA extraction and RT-qPCR were performed as previously described (21). Total RNA from CRC tissue and cells was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. To qualify the level of circTADA2A, RNase R (3 U/1,000 ng; Epicentre; Illumina, Inc.) was added to digest linear RNAs during RNA extraction and incubated for 10 min at 37°C. A total of 500 ng RNA was reverse transcribed into cDNA using a Gosecript™ Reverse Transcription system (Promega Corporation) according to the manufacturer's instructions. Stem-loop RT was performed for miR-638 using the TaqMan MicroRNA detection kit (Thermo Fisher Scientific, Inc.). Then, qPCR was performed using SYBR Premix Ex Taq II (Takara Bio, Inc.). β-actin and U6 were employed as endogenous controls for mRNA and miR-637, respectively. PCR amplification conditions were as follows: 2 min at 95°C for one cycle, followed by denaturation for 15 sec at 95°C and extension for 60 sec at 60°C for 38 cycles. The relative expression levels of the genes were determined using the 2^ΔΔCq method (16). The primer sequences are listed in Table I.

Western blot analysis. Western blotting was performed as previously reported (21). Total protein from A549 and H1299 cells was extracted using radio immunoprecipitation assay lysis buffer (Sigma-Aldrich; Merck KGaA) and quantified into cDNA using a Gosecript™ Reverse Transcription system. Western blot analysis was performed using specific antibodies: anti-β-actin (1:2,000; cat. no. ab226255; Abcam), anti-GAPDH (1:500; cat. no. ab8245; Abcam), anti-TADA2A (1:600; cat. no. ab65623; Abcam) and bands were analyzed with Image J software version 2 (National Institutes of Health).

Plasmids and oligonucleotide transfection. Specific small interfering (si)RNAs targeting circTADA2A and KIAA0101 and scramble control siRNAs (siSCRs) were purchased from Shanghai GenePharma Co., Ltd. miR-638 mimics and miR-negative control (NC), as well as miR-638 inhibitor (inh) and inh-NC were purchased from Shanghai GenePharma Co., Ltd. circTADA2A overexpression plasmids containing miR-638.
binding sequences (oeircTADA2A) were also synthesized by Shanghai GenePharma Co., Ltd. When cells reached 80-90% confluence, 5 nM miR-638 mimics/inhibitors and 50 nM siRNAs were transfected into A549 and H1299 cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions at room temperature. Subsequent experiments were performed 24 h after transfection. Sequences of siRNAs, miR-638 mimics, miR-NC, miR-638 inhibitor and inh-NC are listed in Table I.

**Cell Counting Kit-8 (CCK-8) assay.** CCK-8 assay was performed as previously described (20). In brief, 2x10^5 A549 and H1299 cells were seeded into 96-well plates, and 200 µl culture medium was added to each well. The cells were then incubated at 37°C with 5% CO₂. Following incubation for 1-5 days, each well was supplemented with 20 µl CCK-8 solution (Dojindo Molecular Technologies, Inc.). Following 2 h incubation, a microplate reader (Bio-Rad Laboratories, Inc.) was used to measure the absorbance at 450 nm. The assays were repeated three times.

**Transwell chamber assay.** Transwell assay was conducted as previously reported (22). For invasion assays, upper chambers (Corning, Inc.) were pre-coated with Matrigel (1:8; BD Biosciences) for 1 h at room temperature. A549 and H1299 cells (5x10⁴) were incubated in the upper chambers. Serum-free DMEM was added to the upper chamber; medium in the lower chamber was filled with DMEM supplemented with 10% FBS and the cells were incubated at 37°C for 12 h. After 12 h, non-migratory cells were removed and while migratory or invasive A549 and H1299 cells were fixed with 4% paraformaldehyde at room temperature for 30 min, stained with crystal violet at 25°C for 1 min and counted using an inverted microscope (Olympus Corporation).

**RNA fluorescence in situ hybridization (FISH).** The assay was performed as previously described (20). Specific probes targeting circTADA2A or miR-638 were synthesized by Shanghai GenePharma Co., Ltd. The assay was conducted using a FISH kit (Shanghai GenePharma Co., Ltd.) according to the manufacturer’s instructions. Cy3-labeled circTADA2A and Dig-labeled locked nucleic acid miR-638 probes (Shanghai GenePharma Co., Ltd.) were measured using the aforementioned FISH kit, followed by visualization with a confocal microscope (Carl Zeiss AG).

**Immunohistochemistry (IHC).** IHC assay was performed using a specific HRP/DAB (ABC) Detection IHC kit (Abcam) as previously described (23). NSCLC tissues were sliced following 4% paraformaldehyde fixation for 4 h at room temperature, 10% EDTA (pH, 7.3) decalcification, dehydration and paraffin embedding. Then, the tissue sections (4-µm thick) were incubated with anti-KIAA0101 antibody (1:500; cat.no. ab226255; Abcam) at 4°C overnight. The tissue sections were incubated with secondary antibodies (1:1,000; cat. no. K060911-8; Dako; Agilent Technologies, Inc.) at 37°C for 30 min. Subsequently, the tissue sections were incubated with streptavidin HRP for another 30 min at room temperature (Labeled Streptavidin Biotin kit; cat. no. K060911-8; Dako; Agilent Technologies, Inc) at room temperature and stained with 0.05% 3,3-diaminobenzidine for 60 sec at room temperature. Finally, the slides were counterstained with 10% hematoxylin for 3 min at room temperature, dehydrated in a graded ethanol series (absolute ethyl alcohol for 3 min, 95% ethanol for 3 min and 85% ethanol for 3 min), then observed under a light microscope.

**Dual luciferase reporter assay.** Reporter plasmids containing wild-type (wt) or mutant (mut) circTADA2A sequences (luc-circTADA2A-wt and luc-circTADA2A-mut), as well as reporter plasmids containing wt or mut KIAA0101 sequences (luc-KIAA0101-wt and luc-KIAA0101-mut) were synthesized by Shanghai GenePharma Co., Ltd. The procedures were performed as previously described (15). When A549 and H1299 cells reached 70% confluence, reporter plasmids and miR-638 mimics or miR-NC were co-transfected into A549 and H1299 cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h, luciferase activity

### Table I. Primer and oligonucleotide sequences.

#### A. Primers

| Name        | Sequence, 5’→3’                        |
|-------------|---------------------------------------|
| circTADA2A  | F: TTGTCACCAAGACCAAGGAAG            |
|             | R: AGGAAACATCTGAAGAGTGA              |
| circNOL10   | F: TGCTTCCTGACCGCCAAATGAA           |
|             | R: AACAGGCCCAACTCTGTTTCA            |
| TADA2A      | F: CCAAAATAATATGTAAGGCA             |
|             | R: AAGCAGTTTTTGCJAAGAACA            |
| KIAA0101    | F: TACGTTCCTTCTCTCCTCC             |
|             | R: TAGTGGCAGAGGTGGAAGAAC             |
| β-actin     | F: CTTCTCAAAATGAGCTGGT              |
|             | R: TACATGAGTAATGCTAGTCAGG            |
| miR-638     | F: ATCCAGTGGCTGTCGTG                |
|             | R: TGGTAAGGATCAGGGCGGAT              |
| U6          | F: CTCGCTTCCGCGAGCAAGACA           |
|             | R: AACAGCTTCAGAATTTGCGT             |

#### B. Oligonucleotides

| Name                        | Sequence, 5’→3’                        |
|-----------------------------|---------------------------------------|
| sicircTADA2A-1              | CCAUUUCACUGAGAGGAUGUC dTdT            |
| sicircTADA2A-2              | CACUGCAGAUUGUAGCAAC dTdT              |
| siSCR                      | UUCUCGGAACUGAGCUACCAGGTUT             |
| miR-638                    | AGGGAUCGCGGGCGGGCGGGCGGG             |
| mimics                     | AGGCGCGCCCACGCCCGGCAUCC              |
| miR-638 inhibitor           | CAGUACUUUUGUGUAGUACAA                 |
| miR-NC                     |                                      |

stained with 0.05% 3,3-diaminobenzidine for 60 sec at room temperature. Finally, the slides were counterstained with 10% hematoxylin for 3 min at room temperature, dehydrated in a graded ethanol series (absolute ethyl alcohol for 3 min, 95% ethanol for 3 min and 85% ethanol for 3 min), then observed under a light microscope.
was measured and compared with Renilla luciferase activity using the Dual-Luciferase Reporter Assay system (Promega Corporation) according to the manufacturer's protocol.

**RNA immunoprecipitation (RIP) assay.** RIP assay was performed as previously reported (24) using a Magna RIP RNA-Binding Protein Immunoprecipitation kit (EMD Millipore). Argonaute (Ago)2 plasmid or vector were transfected into A549 and H1299 cells. Subsequently, RIP Lysis Buffer combined with a protease inhibitor cocktail and RNase inhibitors was used to lyse A549 and H1299 cells. Cell lysates (200 µl) were incubated with 5 µg antibody against Ago2 (EMD Millipore) or rabbit IgG-coated beads at 4˚C overnight with rotation. RNeasy MinElute Cleanup kit (Qiagen, Inc.) was used to extract the immunoprecipitated RNA and extracted RNAs were reverse transcribed and subjected to qPCR to detect the abundance of circTADA2A.

**RNA pull-down assay.** The targeted binding effect between circTADA2A and miR-638 was verified by RNA pull-down assay as previously reported (25). Biotinylated miR-638 probe was purchased from Shanghai GenePharma Co., Ltd. A549 and H1299 cells (1x10⁶) were harvested, lysed by 200 µl Pierce™ IP lysis buffer (Thermo Fisher Scientific, Inc.) or rabbit IgG-coated beads at 4˚C overnight with rotation. RNeasy MinElute Cleanup kit (Qiagen, Inc.) was used to extract the immunoprecipitated RNA and extracted RNAs were reverse transcribed and subjected to qPCR to detect the abundance of circTADA2A.

**Statistical analysis.** All data are presented as the mean ± SD (n=3). Statistical analysis was performed with GraphPad Prism v5.0 (GraphPad Software, Inc.) and SPSS 19.0 statistical software (IBM Corp.). Survival analysis was performed by Kaplan-Meier analysis using log-rank test. The clinical value of circTADA2A was measured by receiver operating characteristic (ROC) curve analysis. The association between circTADA2A and miR-638 was analyzed by Spearman correlation analysis. For comparison of two groups, χ² or paired Student’s t test were performed. For the comparison of multiple groups, one-way ANOVA followed by post hoc Dunnett’s or Tukey’s multiple comparisons test were performed, as appropriate. P<0.05 was considered to indicate a statistically significant difference.

### Table II. Association between circTADA2A and clinicopathological characteristics of patients with non-small cell lung cancer.

| Characteristic               | Cases (n=60) | circTADA2A expression | P-value |
|-----------------------------|--------------|-----------------------|---------|
|                             |              | High | Low |                     |
| Age, years                  |              |      |    |                     |
| ≤60                         | 23           | 12   | 11  | 0.791               |
| >60                         | 37           | 18   | 19  |                     |
| Sex                         |              |      |    |                     |
| Male                        | 38           | 21   | 17  | 0.284               |
| Female                      | 22           | 9    | 13  |                     |
| Smoking history             |              |      |    |                     |
| Yes                         | 39           | 19   | 20  | 0.787               |
| No                          | 21           | 11   | 10  |                     |
| Pathology                   |              |      |    |                     |
| Adenocarcinoma              | 28           | 16   | 12  | 0.301               |
| Squamous carcinoma          | 32           | 14   | 18  |                     |
| TNM stage                   |              |      |    |                     |
| I + II                      | 28           | 10   | 18  | 0.038               |
| III + IV                    | 32           | 20   | 12  |                     |
| Lymph node metastasis       |              |      |    |                     |
| Yes                         | 38           | 25   | 13  | 0.001               |
| No                          | 22           | 5    | 17  |                     |
| Tumor size, cm              |              |      |    |                     |
| ≤3                          | 29           | 10   | 19  | 0.020               |
| >3                          | 31           | 20   | 11  |                     |

Data were analyzed by Pearson Chi-square or Fisher's exact test. circTADA2A, circular RNA transcription adaptor 2A.
Results

circTADA2A is upregulated in patients with NSCLC. By analyzing the profile of circRNA expression in five lung adenocarcinoma and paired normal lung tissue samples from a genome-wide study (GSE101586), it was found that 22 upregulated circRNAs and 17 downregulated circRNAs were significantly differentially expressed in lung cancer compared with normal tissue (cut-off criteria: \(P<0.05\) and \(|\log_{2}\text{FC}|>1.2\); Fig. 1A; Table S1). circTADA2A (probe ID: ASCRP002384; circBase ID: hsa_circ_0043278) was selected for further study since it was the most significantly upregulated circRNA (Fig. 1B; Table S1). The expression of circTADA2A was determined in 60 NSCLC tissue specimens and cell lines, circTADA2A was upregulated in NSCLC tissue specimens and cell lines (Fig. 1C and D). Additionally, it was uncovered that the changes of circTADA2A in A549 and H1299 were more significantly than in H460 and H1975 cells. A549 and H1299 were selected for use in subsequent experiments. In comparison with its parental gene, circTADA2A was found to form a covalently closed continuous loop, and comprised exons 5 and 6 of the TADA2A gene, using the online software CircPrimer (17) (Fig. 1E). RNase R and actinomycin D assays were performed to detect the stability of circTADA2A. circTADA2A was more stable than its parental TADA2A mRNA following treatment with RNase R and actinomycin D (Fig. 1F-I).

Upregulated circTADA2A predicts aggressive clinicopathological characteristics and poor prognosis in patients with NSCLC. In order to determine the clinical role of circTADA2A, the association between circTADA2A expression levels and clinicopathological characteristics of patients with NSCLC was analyzed. Higher levels of circTADA2A were more frequently observed in patients with lymph node metastasis and advanced stage disease (Fig. 2A and B). Furthermore, high expression of circTADA2A was associated with clinicopathological characteristics of patients with NSCLC, particularly TNM stage and lymph node metastasis. (Table II). Kaplan-Meier analysis indicated that patients with NSCLC who had a higher circTADA2A level had poorer overall survival (Fig. 2C). circTADA2A may be a valuable biomarker in NSCLC according to the results of ROC curve analysis (area under the curve=0.8606; Fig. 2D).

circTADA2A suppression inhibits proliferation and migration of A549 and H1299 cells. In order to evaluate the role of circTADA2A on proliferation and migration of NSCLC cells, siRNAs targeting the back-splice sites were used to knock down expression of circTADA2A in A549 and H1299 cells. circTADA2A was significantly knocked down, while TADA2A mRNA was not changed following transfection with circTADA2A siRNAs (Fig. 3A and B). sicircTADA2A-1 was selected as the silencing tool for subsequent experiments as it exhibited the greatest efficacy. Next, the proliferation, migration and invasion of A549 and H1299 cells were detected following knockdown of circTADA2A. The results indicated a significant delay in the proliferation, migration and invasion of A549 and H1299 following knockdown of circTADA2A compared with cells transfected with siSCR (Fig. 3C-F).

circTADA2A serves as a sponge for miR-638. circRNAs have been reported to exert their functions by acting as miRNAs sponges in various cancer types (26). It was hypothesized that circTADA2A functions via a similar mechanism in NSCLC. circTADA2A was primarily located in the cytoplasm of A549 and H1299 cells (Fig. 2E and F). RIP assay showed that circTADA2A was significantly enriched in Ago2 protein (Fig. 4A). Online prediction software Circular RNA Interactome (27), was used to predict potential miRNAs that may interact with circTADA2A. A total of 8 potential miRNAs that could bind to circTADA2A were found (Table SII). Among them, only miR-638 was expressed at low levels in NSCLC tissue according to genome-wide studies using GSE74190 and GSE19945 datasets (Fig. 4B-D, and Tables SIII and SIV). Furthermore, miR-638 was expressed at low levels in NSCLC tissue and cells (Fig. 4E and F). Additionally, an inverse correlation between circTADA2A and miR-638 was observed (Fig. 4G). RNA FISH assay indicated that circTADA2A and miR-638 co-localized in the cytoplasm (Fig. 4H). A luciferase reporter assay was conducted to confirm the targeted binding effect between circTADA2A and miR-638. Compared with that of miR-NC, miR-638 mimic significantly decreased luciferase reporter activity (Fig. 4I and J). When the predictive target sites for miR-638 were mutated, the change in luciferase activity was insignificant. Ago RISC catalytic component 2 (AGO2) is a key RNA binding protein for the ceRNA theory of RNA-RNA interactions among miRNAs, long non-coding RNAs and circRNAs (28-30). In order to determine the mechanism of circTADA2A, RIP assay was performed, using IgG as a control. The enriched circTADA2A and circNOL10 [a circRNA reported not to bind to Ago2 (31)] were analyzed. The results indicated that, compared with the NC probe, circTADA2A but not circNOL10 was pulled down by the miR-638 probe (Fig. 4K and L). Lastly, it was observed that circTADA2A was not affected by up- or down-regulation of miR-638. Similarly, the level of miR-638 was not affected either by up- or downregulation of circTADA2A (Fig. 4M and N).

miR-638 suppresses cell proliferation and migration by targeting KIAA0101 in A549 and H1299 cells. In order to identify the downstream genes of miR-638, the online prediction results of TargetScan (32) and upregulated genes according to two genome-wide studies, GSE19188 and GSE30219 (cut-off criteria: \(P<0.05\) and \(\log_{2}\text{FC} \geq 2\)), were combined. It was found that four mRNAs, matrix metalloproteinase 1, BUB1 mitotic checkpoint serine/threonine kinase, KIAA0101 and steroid 5 alpha-reductase 1, overlapped (Fig. 5A; Tables SV-SVII). KIAA0101 has been reported to be implicated in NSCLC proliferation and metastasis (8). Among the aforementioned four genes, KIAA0101 was more closely correlated with shorter OS rate in lung cancer according to Kaplan-Meier analysis using the online software UALCAN (18) (Fig. S1). Therefore, KIAA0101 was selected for subsequent experiments. KIAA0101 was upregulated in NSCLC according to the results of IHC staining and genome-wide studies using GSE19188 and GSE30219 datasets (Fig. 5B-D). Furthermore, up- and downregulation of miR-638 inversely regulated KIAA0101 protein expression (Fig. 5E and F). Functionally, upregulation of miR-638 suppressed A549 and H1299 cell
proliferation and metastasis. When the level of KIAA0101 was elevated (following co-transfection of miR-638 mimic and specific KIAA0101 overexpression plasmids), the suppressive effect of miR-638 was abolished (Fig. 5G-J). Lastly, it was verified that KIAA0101 was a direct target of miR-638 via six base pair binding sites (positions 1,473-1,479; Fig. 5K-M).

**circTADA2A promotes cell proliferation and migration partially via the miR-638/KIAA0101 pathway in A549 and H1299 cells.** The present study investigated whether the oncogenic role of circTADA2A in A549 and H1299 cell proliferation and migration was achieved, at least partially, via the miR-638/KIAA0101 signaling pathway. The protein expression of KIAA0101 following overexpression or knockdown of circTADA2A was analyzed by western blotting. Overexpression of circTADA2A positively regulated KIAA0101 protein expression and vice versa (Fig. 6A and B). circTADA2A sponged miR-638, and KIAA0101 was a downstream target of miR-638. The present study investigated whether circTADA2A regulated KIAA0101 via miR-638 sponging. circTADA2A, circular RNA transcription adaptor 2A; circRNA, circular RNA; NSCLC, non-small cell lung cancer; RT-q, reverse transcription-quantitative; ANT, adjacent non-tumor tissue; FC, fold change; LAD, lung adenocarcinoma.
Discussion

Increasing evidence indicates that non-coding RNAs are involved in various human diseases, including cancer (33). As a novel type of non-coding RNAs, circRNAs, which are abundant in the human transcriptome, exhibit diverse biological functions associated with their specific structural features (34-37). circTADA2A (circBase ID hsa_circ_0043278)
is derived from exons 5 and 6 of the TADA2A gene (15). The role of circTADA2A in cancer is not well known, and its function remains controversial. Wu et al (15) reported that circTADA2A is upregulated in osteosarcoma tissue specimens and cell lines and serves as a tumor promoter and increases malignant tumor behavior via miR-203a-3p/CREB3 signaling in osteosarcoma. In another study, Xu et al (14) found that circTADA2A is consistently and significantly decreased in a large cohort of patients with breast cancer, and its downregulation is associated with poor patient survival for triple-negative breast cancer. In the present study, the differentially expressed circRNAs in NSCLC were analyzed according to the GEO dataset GSE101586. It was found that circTADA2A was upregulated in five NSCLC tissue samples compared with paired paratumor tissue. RT-qPCR verification revealed that circTADA2A was upregulated in 38 NSCLC tissue samples.
Figure 5. miR-638 suppresses proliferation and migration by targeting KIAA0101 in A549 and H1299 cells. (A) Schematic illustration exhibiting overlapping target mRNAs of miR-638 predicted by TargetScan and the mRNAs that were highly expressed in genome-wide studies GSE19188 and GSE30219. KIAA0101 expression in NSCLC tissues was higher than that in healthy and NTL tissue according to the results of genome-wide studies (B) GSE19188 and (C) GSE30219. ****P<0.01. (D) KIAA0101 expression in three NSCLC and paired paratumor tissue samples was evaluated by immunohistochemistry. KIAA0101 protein expression in (E) A549 and (F) H1299 cells following different miR-638 treatments was evaluated by western blot assay. **P<0.01. Proliferation of (G) A549 and (I) H1299 cells were determined by Cell Counting Kit-8 assay. Migration and invasion of (H) A549 and (J) H1299 cells were measured by Transwell assay. Magnification, x20; scale bar, 200 µm. *P<0.05. (K) Diagram of the constructed luciferase reporter plasmids. A luciferase assay was conducted to verify the targeted binding effect between miR-638 and KIAA0101 in (L) A549 and (M) H1299 cells. All data are presented as mean ± SD (n=3). **P<0.01 vs. miR-NC. miR, microRNA; NSCLC, non-small cell lung cancer; KIAA0101, PCNA clamp associated factor; NTL, non-tumor lung; NC, negative control; BUB1, BUB1 mitotic checkpoint serine/threonine kinase; SRD5A1, steroid 5α-reductase 1; inh, inhibitor; OD, optical density; wt, wild-type; mut, mutant; n.s., not significant.
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and four NSCLC cell lines. CCK-8 and Transwell assays verified that circTADA2A promoted NSCLC cell proliferation and migration.

circRNAs function via multiple mechanisms, including serving as sponges of miRNAs and proteins, protein translation and derivation of pseudogenes (38). Since the majority of circRNAs are located in the cytoplasm and function as competitive endogenous (ce)RNAs for miRNAs, circRNA research has attracted attention (39,40). Here, RNA-FISH assay demonstrated that circTADA2A and miR-638 were primarily located in the cytoplasm. miR-638, which is located at human chromosome 19p13.2, is a well-known tumor-suppressor miRNA that is implicated in multiple types of malignant tumor including hepatocellular carcinoma, breast, gastric, cervical and colon cancer, as well as glioma (41-46). Xia et al (47) reported that miR-638 is expressed at low levels in NSCLC, and downregulation of miR-638 promotes proliferation, invasion and epithelial-mesenchymal transition via SRY-box transcription factor 2 regulation in NSCLC. The present study indicated that circTADA2A communicated with KIAA0101 and regulated KIAA0101-mediated proliferation and migration of A549 and H1299 cells via miR-638.

In summary, the present study provides evidence that circTADA2A promoted proliferation and migration via regulation of the miR-638/KIAA0101 axis in NSCLC cells. circTADA2A and its downstream miR-638/KIAA0101 signaling pathway may be novel therapeutic targets for the molecular treatment of 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

XL and YW conceived the study. YZ, HY, YL and LY performed the experiments. XL and YZ confirmed the authenticity of all the raw data. LZ and JC analyzed the data. YW wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institute Research Medical Ethics Committee of Liaoning Cancer Hospital & Institute. All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018.

2. Molina JR, Yang P, Cassivi SD, Child SE and Adjei AA: Non-small cell lung cancer: Epidemiology, risk factors, treatment, and survivorship. Mayo Clin Proc 83: 584-594, 2008.

3. Lei H, Wang K, Jiang T, Lu J, Dong X, Wang F, Li Q and Dong H: KIAA0101 and UbcH10 interact to regulate non-small cell lung cancer cell proliferation by disrupting the function of the spindle assembly checkpoint. BMC Cancer 20: 957, 2020.

4. Lv W, Su B, Li Y, Geng C and Chen N: KIAA0101 inhibition suppresses cell proliferation and cell cycle progression by promoting the interaction between p53 and Spl in breast cancer. Biochem Biophys Res Commun 503: 600-606, 2018.

5. Jiao M, Zhang L, Patterson EE and Kebebew E: KIAA0101 is overexpressed, and promotes growth and invasion in adenral cancer. PLoS One 6: e26866, 2011.

6. Tanttwireunandet A, Panvichian R, Sornmayura P, Leelaudomlipi S and Macoska JA: PCNA-associated factor (KIAA0101/PCLAF) overexpression and gene copy number alterations in hepatocellular carcinoma tissues. BMC Cancer 21: 295, 2021.
28. Yu J, Xu QG, Wang ZG, Yang Y, Zhang L, Ma JZ, Sun SH, Yang F and Zhou WP: Circular RNA cSMARCA5 inhibits growth and metastasis in hepatocellular carcinoma. J Hepatol 68: 1214-1227, 2018.
29. Cai H, Liu X, Zheng J, Xue Y, Ma J, Li Z, Xi Z, Li Z, Bao M and Liu Y: Long non-coding RNA taurine upregulated 1 enhances tumor-induced angiogenesis through inhibiting microRNA-299 in human glioblastoma. Oncogene 36: 318-331, 2017.
30. Song YX, Sun JX, Zhao JH, Yang YC, Shi JX, Wu ZH, Chen XW, Gao P, Miao ZF and Wang ZN: Non-coding RNAs participate in the regulatory network of CLDN4 via ceRNA mediated miRNA evasion. Nat Commun 8: 289, 2017.
31. Liu X, Zheng J, Xue Y, Ma J, Li Z, Xi Z, Li Z, Bao M and Liu Y: Long non-coding RNA taurine upregulated 1 enhances tumor-induced angiogenesis through inhibiting microRNA-299 in human glioblastoma. Oncogene 36: 318-331, 2017.
32. Agarwal V, Bell GW, Nam JW and Bartel DP: Predicting effective microRNA target sites in mammalian mRNAs. Elife 4: e05005, 2015.
33. Anastasiadou E, Jacob LS and Slack FJ: Non-coding RNA networks in cancer. Nat Rev Cancer 18: 5-18, 2018.
34. Kristensen LS, Andersen MS, Stagsted LVW, Ebbesen KK and Hansen TB: The biogenesis, biology and characterization of circular RNAs. Nat Rev Genet 20: 675-691, 2019.
35. Salzman J: Circular RNA Expression: Its potential regulation and function. Trends Genet 32: 309-316, 2016.
36. Su M, Xiao Y, Ma J, Tang Y, Tian B, Zhang Y, Li X, Wu Z, Yang D, Zhou Y, et al: Circular RNAs in Cancer: Emerging functions in hallmark cancers, stemness, resistance and roles as potential biomarkers. Mol Cancer 18: 90, 2019.
37. Cui C, Yang J, Li X, Liu D, Fu L and Wang X: Functions and mechanisms of circular RNAs in cancer radiotherapy and chemotherapy resistance. Mol Cancer 19: 58, 2020.
38. Li X, Yang L and Chen LL: The biogenesis, functions, and challenges of circular RNAs. Mol Cell 71: 428-442, 2018.
39. Yang Q, Li F, He AT and Yang BB: Circular RNAs: Expression, localization, and therapeutic potentials. Mol Ther 29: 1683-1702, 2021.
40. Zhong Y, Du Y, Yang X, Mo Y, Fan C, Xiong F, Ren D, Ye X, Li C, Wang Y, et al: Circular RNAs function as ceRNAs to regulate and control human cancer progression. Mol Cancer 17: 79, 2018.
41. Cheng J, Chen Y, Zhao P, Li N, Lu J, Li J, Liu Z, Lv Y and Huang C: Dysregulation of miR-638 in hepatocellular carcinoma and its clinical significance. Oncol Lett 13: 3859-3865, 2017.
42. Li M, Wang J and Liu H: Downregulation of miR-638 promotes progression of breast cancer and is associated with prognosis of breast cancer patients. Onco Targets Ther 11: 6871-6877, 2018.
43. Shen Y, Chen H, Gao L, Zhang W, He J, Yang X, Qin L, Xue X and Guo Z: MiR-638 acts as a tumor suppressor gene in gastric cancer. Oncotarget 8: 108170-108180, 2017.
44. Wei H, Zhang JH and Tang QL: MiR-638 inhibits cervical cancer metastasis through Wnt/b-catenin signaling pathway and correlates with prognosis of cervical cancer patients. Eur Rev Med Pharmacol Sci 21: 5587-5593, 2017.
45. Yan S, Dang G, Zhang X, Jin C, Qin L, Wang Y, Shi M, Huang H and Duan Q: Downregulation of circulating exosomal miR-638 predicts poor prognosis in colon cancer patients. Oncotarget 8: 72220-72226, 2017.
46. Zheng DH, Wang X, Lu LN, Chen DL, Chen JM, Lin FM and Xu XB: MiR-638 serves as a tumor suppressor by targeting HOXA9 in glioma. Eur Rev Med Pharmacol Sci 22: 7798-7806, 2018.
47. Xia Y, Wu Y, Liu B, Wang P and Chen Y: Downregulation of miR-638 promotes invasion and proliferation by regulating SOX2 and induces EMT in NSCLC. FEBS Lett 588: 2238-2245, 2014.
48. Yu CY, Li TC, Wu YY, Yeh CH, Chiang W, Chuang CY and Kuo HC: The circular RNA circBIRC6 participates in the molecular circuitry controlling human pluripotency. Nat Commun 8: 1149, 2017.
49. Chen H, Xia B, Liu T, Lin M and Lou G: KIAA0101, a target gene of miR-429, enhances migration and chemoresistance of epithelial ovarian cancer cells. Cancer Cell Int 16: 74, 2016.
50. Jain N, Roy J, Das B and Mallick B: miR-197-5p inhibits sarkomagenesises and induces cellular senescence via repression of KIAA0101. Mol Carcinog 58: 1376-1388, 2019.
51. Samantarrai D and Mallick B: miR-429 inhibits metastasis by targeting KIAA0101 in soft tissue sarcoma. Exp Cell Res 357: 33-39, 2017.
52. Salmena L, Poliseno L, Tay Y, Kats L and Pandolfi PP: A ceRNA hypothesis: The rosetta stone of a hidden RNA language? Cell 146: 353-358, 2011.