



The Circular RNA circFOXK2 Enhances the Tumorigenesis of Non-Small Cell Lung Cancer Through the miR-149-3p/IL-6 Axis

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Abstract

Circular RNAs (circRNAs) are the non-coding types of RNAs and are thought to be linked with human cancer progression. circFOXK2 is believed to be associated with cancers, however, the molecular mechanisms of circFOXK2 in non-small cell lung cancer (NSCLC) are still unclear. Here we firstly reported that circFOXK2 enhances the tumorigenesis of NSCLC through the miR-149-3p/IL-6 axis. The expression of circFOXK2, microRNA-149-3p (miR-149-3p) and IL-6 were assessed by qRT-PCR and western blot. Transwell, colony formation, wound healing, and CCK-8 assays were used to elucidate NSCLC cells' proliferation, migration, and invasion. MiR-149-3p interaction with circFOXK2 was confirmed by dual-luciferase reporter gene assay (DLRGA). Furthermore, the biological effect of circFOXK2 on NSCLC progression was detected by tumor xenograft assay. CircFOXK2 were upregulated in NSCLC tissues and cells, miR-149-3p were downregulated in NSCLC tissues and cells. In addition, circFOXK2 stimulated NSCLC cell proliferation, migration and invasion in vitro. Mechanical analysis indicated that circFOXK2 modulated IL-6 via miR-149-3p sponging. Furthermore, circFOXK2 overexpression promoted tumor growth in vivo. Overall, this research verified that circFOXK2 enhances the tumorigenesis of NSCLC through the miR-149-3p/IL-6 axis.

Tongwei Xiang and Liping Chen have contributed equally to this work.

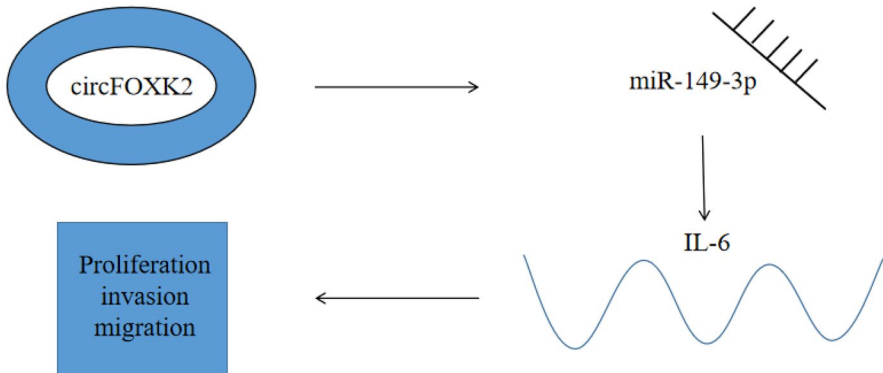
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Graphical Abstract



Keywords circFOXK2 · miR-149-3p · Non-small cell lung cancer · IL-6

Abbreviations

CircRNAs	Circular RNAs
NSCLC	Non-small cell lung cancer
microRNA-149-3p	MiR-149-3p
IL-6	Interleukin -6
ncRNAs	Non-coding RNAs
miRNAs	MicroRNAs
qRT-PCR	Quantitative real-time polymerase chain reaction
GLRGA	Dual-luciferase reporter gene assay
SD	Standard deviation

Background

Lung cancer is one of the reasons for enhanced global mortality and morbidity (Siegel and Miller 2021; Nasim et al. 2019). Non-small cell lung cancer (NSCLC) makes up to 80% of total lung cancers (Herbst et al. 2018) and has high mortality even after advancements in treatment strategies (Alexander et al. 2020). Therefore, elucidating the mechanism underlying NSCLC progression carries immense significance for novel treatment strategies.

Circular RNAs (circRNA) are novel non-coding RNAs (ncRNAs), which as the name implies have circular closed-loop structures (Jiang et al. 2022), which allows them to resist RNase degradation, indicating their advantage as a stable molecular bio-index for pan-cancer (Liu et al. 2022; Meng et al. 2022; Wang et al. 2022). Numerous research have confirmed that circRNAs play significant physiological roles. It is widely acknowledged that circRNAs are known to act as regulators in gene expression as decoys, regulating binding of miRNAs to their target mRNAs, and so affect translation of mRNAs. Rather than sponging miRNAs, circRNAs also

elicit their functions through interactions with proteins, regulation of genes transcription, mRNAs translation, and epigenetic regulatory mechanisms (Najafi 2022; Najafi 2022; Shirvani et al. 2023). CircRNAs are also crucially involved in cancer mechanisms' regulation (Zhou et al. 2021; Liu et al. 2022; Wang et al. 2021). For instance, circRNA_102231 knockdown inhibited gastric cancer proliferation and invasion (Yuan et al. 2021). Furthermore, the circ-CPA4/ let-7 miRNA/PD-L1 axis has been reported to modulate cell immune evasion, drug resistance, growth, and stemness in NSCLC (Hong et al. 2020). However, only a few studies suggest the association of circFOXK2 with NSCLC, and the underlying mechanism, therefore, it needs proper exploration.

MicroRNAs (miRNAs) are eukaryotic endogenous ncRNAs of about 20–25 nucleotides, with regulatory functions (Hill and Tran 2021; Lai et al. 2019; He et al. 2020). These recognize target mRNA by complementary base pairing and then degrade the target mRNA by guiding the silencing complex or else inhibit their translation at different complementary degrees (Ahadi 2021; Abdel Rhman and Pmo 2022; Adil et al. 2021). Multiple investigations indicate that miRNAs are also linked with cancers, and miRNAs play an significant role in suppressing tumors (Ali Syeda et al. 2020). For example, miR-149-3p inhibits the proliferation of oral squamous cell carcinoma cancer chemoresistance via the post-transcriptional suppression of AKT2 (Shen et al. 2021).

In this investigation, we found that circFOXK2 enhances the tumorigenesis of NSCLC through the miR-149-3p/IL-6 axis *in vitro* and *vivo*.

Methods and Materials

Tissue Samples

NSCLC individuals' tumors ($n=24$) and corresponding adjacent normal tissues were obtained from The Affiliated People's Hospital of Ningbo University after surgery and immediately stored in liquid nitrogen. surgical specimens of NSCLC tissues were dissociated to isolate corresponding adjacent normal tissues. Tissue 2 cm away from tumor was considered adjacent tissue (Wang et al. 2021). Before surgery, all patients did not undergo radiotherapy or chemotherapy treatment. Then, they were first informed about the study and they were asked to sign the consent letter. This investigation was authorized by the Clinical Research Ethics Committee of Ningbo University.

Cell Lines and Cell Culture

NSCLC (H661, SPC-A1, H460, and A549) and normal human lung epithelial (BEAS-2B) cell lineages were taken from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and propagated in 5% CO₂ incubator at 37 °C, propagated in DMEM media (VivaCell Biosciences, China, C3118-0500) augmented with 100 µg/mL streptomycin, 100 U/mL penicillin

(Procell, China, PB180120), and 10% heat-inactivated fetal bovine serum (Gibco, USA, 16,140,071). Every 3rd day the media was refreshed. For sub-culturing, 0.25% trypsin (Gibco, USA, 25,200,056) was used (Zhang et al. 2020).

Cell Transfection

The circular transcript expression vector comprised front and back circular frames, designed to contain inverted repeat sequences flanks. For circFOXK2 overexpression, its whole cDNA was amplified and cloned into a distinct vector between the two aforementioned frames. For circFOXK2 knockdown, based on the circFOXK2 junction sequence siRNAs were established for its specific target. The overexpression vector and siRNAs were acquired from GenePharma (Shanghai, China). Furthermore, miR-149-3p knockdown and overexpression was achieved using miR-149-3p mimics (miR-149-3p GenePharma, China), its inhibitor (anti-miR-149-3p, GenePharma, China), and their respective controls (NC and anti-NC). All transfection were used 50 nM of miRNA mimics, inhibitors and siRNAs. SPC-A1 cells were propagated for 24 h in a 6-well plate (3×10^5 cells/ml) and then transfected using Lipofectamine® 2000 (Invitrogen, USA, 11,668,019) by following the protocol. The protocol: Dilute Lipofectamine® 2000 in MEM, and dilute mimics, inhibitors, or siRNAs in MEM, then add diluted mimics, inhibitors, or siRNAs to diluted Lipofectamine® 2000. Incubate for 5 min at room temperature. Add the mixed liquid to cells. Incubate cells for 1–3 days at 37 °C. Then analyze transfected cells. The transfection efficiency was elucidated via qRT-PCR (Yuan et al. 2021). The siRNA targeting circFOXK2 sequence used in Table 1:

qRT-PCR

Whole RNA from both the tissues and the cells were obtained with the help of TRIzol® solution (Invitrogen, USA, 15596026CN). Subsequently, RNA (1 µg) was transcribed reversely into cDNA via TransScript First-strand cDNA Synthesis SuperMix (TransGen Biotech, China, AT301-02) for qRT-PCR. And reverse transcription of miRNA into cDNA via TransScript miRNA First-Strand cDNA Synthesis SuperMix (TransGen Biotech, China, AT351-01), which was carried out with Perfect-Start Green qPCR SuperMix (TransGen Biotech, China, AQ602-01) on ABI 7500 real-time PCR system (Applied Biosystems, USA). The relative circFOXK2, miR-149-3p, and IL-6 expression were assessed by the $2^{-\Delta\Delta CT}$ method (Fathi et al. 2022). The primers were designed and blasted by National Library of Medicine (<https://www.ncbi.nlm.nih.gov/>). The primers used in this investigation were in Table 2:

Table 1 siRNA sequences

siRNA	targeting circFOXK2 sequence (5'–3')
si-circFOXK2	5'-GAAGGUGCACAUUCAGGUUTT-3'

Table 2 Primers sequences

Name	Forward primers (5'–3')	Reverse primers (5'–3')
circFOXK2	TAATGGCTGACAACCTACA	GCTTCTCTCTCTCTCGCT
circFOXK2-149MUT	CACCTCATCAGCCCTCTGCGGAGG GCCACGGGAACCATCAGCGC	GCGCTGATGGTCCCGTGGCC CTCC GCAGAGGGCTGATGAGGTG
GAPDH	TCAGTGGTGGACCTGACCTG	TGCTGTAGCCAAATTCGTTG
miR-149-3p	AGGGAGGGACGGGGCTGTGC	GCGAGCACAGAATTA ATACGAC
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
IL-6	TTCTCCACAAGCGCCTTC	AGAGGTGAGTGGCTGTCTGT

CCK-8 Experiment

Cell multiplication was appraised by the TransDetect[®] Cell Counting Kit (TransGen Biotech, China, FC101-01) according to the provided protocol. Thereafter, 2×10^3 SPC-A1/well were propagated in a 96-well plate for 24 h (regarded as 0 days). Then, each well was inoculated with CCK-8 (10 μ l) for 2 h. The absorbance (OD_{450nm}) was assessed via the microplate reader (cMax plus, USA) on the 1st, 2nd, and 3rd days, respectively (Liu et al. 2019).

Wound Healing Assays

The wound healing assay was conducted by transfecting 90% confluent SPC-A1 cells, propagated overnight in 6-well plates. The propagated cells were scraped by the tip of a sterile 200 μ l pipette (record 0 h) to generate cell-free zones, then the cells were left for 48 h in a serum-free media. Cell migration was assessed under the light microscope at 24 and 48 h and the distances were elucidated via Image J software (National Institutes of Health, USA). This protocol was repeated thrice (Huang et al. 2020).

Transwell Analysis

For determining invasion activity, Transwell chambers (8 μ M, BD Biosciences, USA) were overlaid with matrigel (Corning, USA) and then used for propagating cells (5×10^4 cells/well) in serum-free medium, whereas, those resuspended in 10% FBS augmented media were propagated in the 24-well plate. After 36 h, the cells which passed across the membrane, using methanol were fixed, dyed with crystal violet (Thermo Scientific, USA, R40073), and then quantified by the light microscope (Liu et al. 2019).

Colony Formation Assay

To elucidate cell proliferation, their colony formation capability was elucidated. 500 NSCLC cells/well were propagated in 6-well plates for 2 weeks. Media was

refreshed every 3–4 d. The colonies were fixed after 2 weeks by 20 min incubation in formaldehyde, stained with 0.5% crystal violet for 20 min, rinsed thrice with PBS, and then counted with naked eyes (Zhang et al. 2021).

Western Blot

With the help of RIPA buffer (Beyotime, China, P0013B) the cell lysis was carried out, and the supernatant was then centrifuged at high speed to acquire proteins, which were quantified using BCA reagent (Beyotime, China, ST2222-25 g). The samples were heated at 100 °C for 10 min for denaturing proteins. Thereafter, 30 µg proteins/lane were used for SDS-PAGE gel (EpiZyme, China, PG111), moved to a 0.45 µm polyvinylidene difluoride membrane (Immobilon®-p, Germany), blocked in a skim milk solution (5%) for 2 h at an ambient temperature, subsequent by overnight incubation in IL-6 (Proteintech, China, 21,865-1-AP) and β-actin (Abcam, UK, ab8226) antibodies at 4 °C (Farahzadi et al. 2023). Then, using the TBST solution the membranes were rinsed, kept for 1 h with Goat Anti-Rabbit IgG H & L (Abcam, UK, ab6702) at an ambient temperature, rinsed again, and then the bands were observed by Clarity™ Western ECL Substrate (BIO-RAD, USA, 1,705,060).

DLRGA

Using the Arraystar's home-made miRNA target prediction software (<https://www.arraystar.com/>), CircInteractome database (<https://circinteractome.irp.nia.nih.gov/>) and Starbase (<https://starbase.sysu.edu.cn/index.php>), the miR-149-3p binding relationship with circFOXK2 3' un-translated region (3' UTR) was predicted. This prediction was then validated by the DLRGA. The Renilla pGL3 vector (Promega, USA, E1751) was utilized for the wild-type (WT) luciferase reporter vectors (circFOXK2-WT) structure by incorporating the circFOXK2 harboring miR-149-3p binding sequence fragments. Then, we used mutation primers to perform point mutation on circFOXK2-WT. Which was called the mutant-type (MUT) vector (circFOXK2-MUT) (Liu et al. 2020). And the SPC-A1 cells were incorporated with these vectors along with miR-149-3p or miR-NC. The luciferase function was elucidated after 48 h by the DLRGA System (Promega, USA, E1910).

Tumor Xenograft Assay

BALB/C nude mice (males of age = 5 weeks) (Vital River Laboratory, China) were acquired after authorization from the Animal Ethics Committee of the Affiliated People's Hospital Ningbo university. The animals were housed in sterile conditions and randomly categorized into 2 groups (6 mice, respectively). The flank of the mice was subcutaneously injected with SPC-A1 cells (6×10^6 in concentration) with pEX-3 (GenePharma, China) and pEX-3-circFOXK2 (GenePharma, China), respectively. Subsequently, after every 3rd day, the size of the tumor was analyzed using a caliper. The formula to measure tumor volume was: $\text{volume} = \text{length} \times \text{width}^2 / 2$.

Post 21 days of injection, the tumors were dissected for weight and volume assessment (Liu et al. 2021).

Statistical Analyses

All the analyses were conducted thrice and the data were assessed via GraphPad Prism7 (USA). The values are given as the mean ± standard deviation (SD). The association of miR-149-3p, circFOXK2, and IL-6 expressions was determined by Pearson correlation analysis. Significant variabilities of the two groups were measured by Student’s *t*-test and for multiple groups, one-way analysis of variance (ANOVA) with Tukey’s tests was applied. $P < 0.05$ was deemed statistically important.

Results

Expression of circFOXK2 was Upregulated in Cells and Tissues of NSCLC

It was discovered that circFOXK2 had one of the highest relative expressions of circRNA through chip detection results in the early stage (Fig. 1A). To elucidate circFOXK2 expression in NSCLC, qRT-PCR was conducted. As Fig. 1B represents, circFOXK2 was upregulated in NSCLC tissues than the adjacent normal tissues. Furthermore, when compared with normal BEAS-2B, the relative expression of circFOXK2 in the lung cancer cell lineages were upregulated, and it was the highest in SPC-A1 (Fig. 1C). Altogether, the relative expression of circFOXK2 were upregulated in both NSCLC tissues and cells. circFOXK2 could regulate NSCLC cells proliferation, invasion and migration in vitro

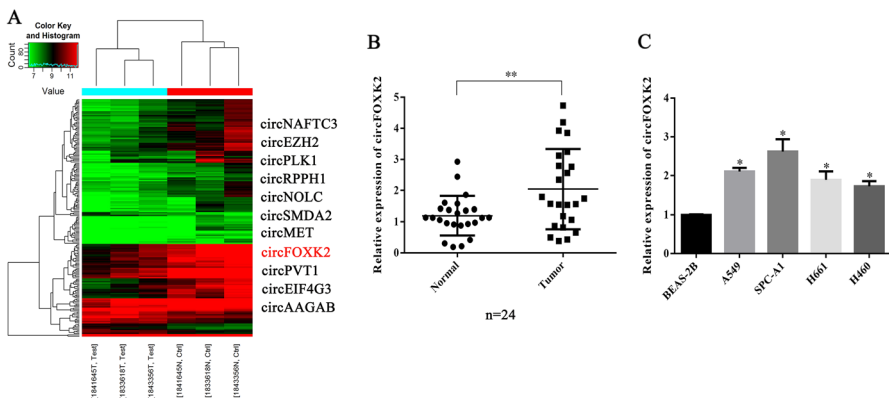


Fig. 1 CircFOXK2 expression was upregulated in NSCLC tissues and cells. **A** Detection of circRNA levels in NSCLC and adjacent normal tissues (3 pairs) by circRNA microarray experiment. **B** qRT-PCR analysis for identifying the circFOXK2 levels in NSCLC and adjacent normal tissues ($n = 24$). **C** qRT-PCR assessment of circFOXK2 levels in lung cancer cell lines and BEAS-2B. * $P < 0.05$, ** $P < 0.01$

To estimate the *in vitro* physiological role of circFOXK2 in NSCLC, SPC-A1 was selected as it presents the highest relative expression of circFOXK2. The transfection efficiency of si-circFOXK2 and pEX-3-circFOXK2 were assessed and shown in Fig. 2A. Colony formation and CCK-8 assays revealed that circFOXK2 deficiency repressed SPC-A1 growth and clone number more than the control groups. However, when circFOXK2 was overexpressed, it had the opposite effect (Fig. 2B–D). Furthermore, the transwell analysis indicated that circFOXK2 deficiency suppressed the invasion ability of SPC-A1 cells versus their counterparts (Fig. 2E) and had an opposite response when circFOXK2 was overexpressed (Fig. 2F). The wound healing experiment elucidated showed that the SPC-A1 cells migration activity was reduced due to the circFOXK2 knockdown versus their counterparts (Fig. 2G) and under overexpression, this effect was reversed (Fig. 2H).

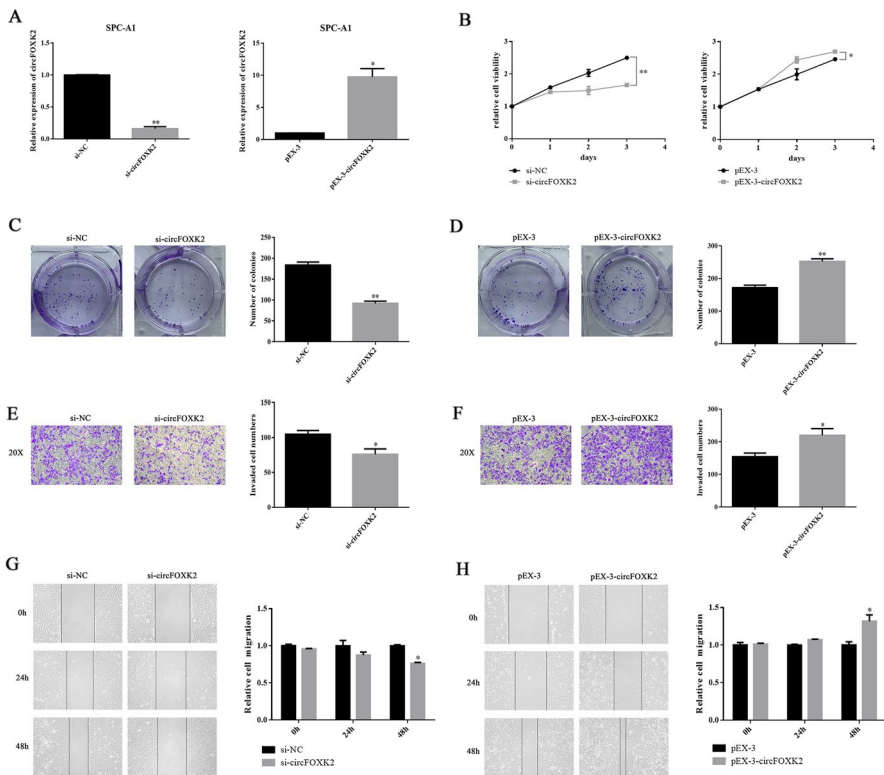


Fig. 2 The impact of circFOXK2 on NSCLC cells' colony formation, proliferation, invasion and migration activities. **A** Relative circFOXK2 levels were assessed in si-NC, si-circFOXK2, pEX-3, and pEX-3-circFOXK2 transfected SPC-A1 cells by qRT-PCR. **B** CCK-8 experiment was conducted to elucidate transfected SPC-A1 cell proliferation. **C, D** The cell colony formation experiment was carried out to elucidate the clone number in transfected SPC-A1 cells. **E, F** To determine transfected SPC-A1 cells' ability to invade, the transwell assay was carried out. **(G, H)** To determine transfected SPC-A1 cells' ability to migrate, the wound healing test was conducted. * $P < 0.05$, ** $P < 0.01$

CircFOXK2 Could Directly Interact with miR-149-3p

Further, to identify circFOXK2's mechanism of action, the putative interaction of circFOXK2 with miRNAs was observed via the Arraystar's home-made miRNA target prediction software (<https://www.arraystar.com/>), CircInteractome database (<https://circinteractome.irp.nia.nih.gov/>) and Starbase (<https://starbase.sysu.edu.cn/index.php>). We found that miR-149-3p, miR-449a, miR-449b-5p and miR-762 could simultaneously interact with circFOXK2. Subsequently, we screened them by DLRGA. As shown in Fig. 3A, miR-149-3p overexpression reduced wild-type reporter (circFOXK2-WT) luciferase activity, but there was no statistically significant difference in other miRNAs. Which revealed that miR-149-3p may bind to circFOXK2. To further confirm circFOXK2 was directly interacted with miR-149-3p, we used mutation primers to perform point mutation on circFOXK2-WT. The data suggested that miR-149-3p overexpression markedly reduced the luciferase activity of wild-type reporter (circFOXK2-WT) but not that of mutant reporter (circFOXK2-MUT) (Fig. 3B). To confirm circFOXK2 interaction with miRNA at the mRNA level, circFOXK2 was knocked out and overexpressed in cells, and miR-149-3p expression was measured by qRT-PCR. As Fig. 3C indicates, when circFOXK2 was knocked out, the miR-149-3p levels were upregulated, whereas, when circFOXK2 was overexpressed, the miR-149-3p levels were downregulated. Additionally, it was noted that in NSCLC cells, miR-149-3p was downregulated, which was negatively associated with circFOXK2 (Fig. 3D). To elucidate miR-149-3p expression in NSCLC, qRT-PCR was conducted. As Fig. 3E represents, miR-149-3p was downregulated in NSCLC tissues than the adjacent normal tissues. And Pearson's correlation analysis indicated a substantial negative association between circFOXK2 and miR-149-3p in NSCLC tissues (Fig. 3F).

CircFOXK2 Regulated the Progression of NSCLC by miR-149-3p Interaction in Vitro

To distinguish whether miR-149-3p participated in NSCLC progression stimulated by circFOXK2, rescue assays in SPC-A1 cells were performed. Moreover, the knockdown efficiency of anti-miR-149-3p and the overexpression efficiency of miR-149-3p were assessed respectively (Fig. 4A). CCK-8 assay indicated that circFOXK2 knockdown impaired SPC-A1 cell proliferation ability was abrogated after miR-149-3p knockdown (Fig. 4B). However, its improved proliferation by circFOXK2 overexpression was reduced notably after miR-149-3p overexpression (Fig. 4B). The colony formation test further validated the data of the CCK-8 assay (Fig. 4C, D). The transwell assay indicated that circFOXK2 knockdown induced a notable decline in the invasion, while miR-149-3p knockdown reversed this effect in SPC-A1 cells (Fig. 4E). Conversely, circFOXK2 overexpression could substantially increase invasion, whereas, miR-149-3p overexpression reverses this impact (Fig. 4F). According to the data of the wound healing analysis, circFOXK2 knockdown caused a marked reduction in migration, whereas miR-149-3p knockdown altered this effect in SPC-A1 cells (Fig. 4G). Conversely, circFOXK2 overexpression markedly increased

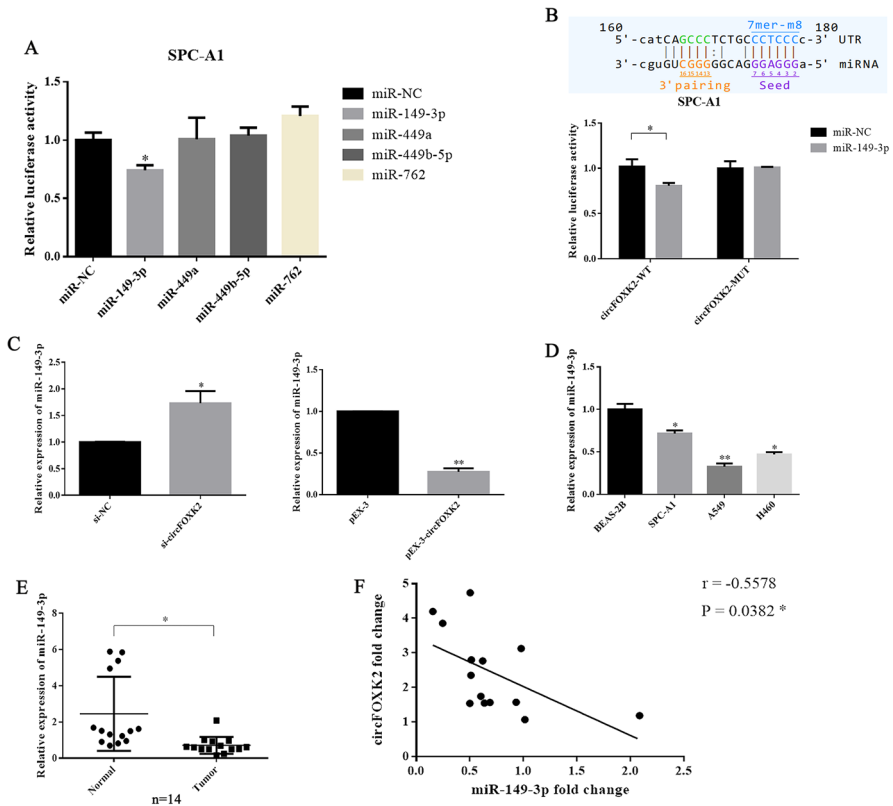


Fig. 3 CircFOXK2 directly binds miR-149-3p. **A** MiRNAs which could simultaneously interact with circFOXK2 were screened by DLRGA. **B** Schematic of a putative target miR-149-3p sequence in circFOXK2 and mutated miR-149-3p-binding sites. And with the help of DLRGA, the binding relationship in SPC-A1 cells was verified. **C** miR-149-3p expression in si-NC, si-circFOXK, pEX-3, and pEX-3-circFOXK2 transfected SPC-A1 cells was calculated by qRT-PCR. **D** miR-149-3p level was identified in BEAS-2B, A-549, and H460 cells using qRT-PCR. **E** qRT-PCR analyzed the miR-149-3p levels in NSCLC and adjacent normal tissues ($n = 14$). **F** Correlation analysis determined the expression correlation of circFOXK2 with miR-149-3p in NSCLC tissues ($n = 14$). * $P < 0.05$, ** $P < 0.01$

migration, while miR-149-3p overexpression altered this impact in SPC-A1 cells (Fig. 4H). These results reveal that circFOXK2 knockdown can suppress NSCLC proliferation, invasion and migration by targeting miR-149-3p in vitro.

CircFOXK2 Promoted IL-6 by Sponging miR-149-3p

According to previous reports, The potential target genes of miR-149-3p as follows: IL-6, CCDC6, KIF2A, E2F1 and PKP3 (Wang et al. 2021, 2020; Jin et al. 2021; He et al. 2020; Li et al. 2018). Then, circFOXK2 was knocked out and overexpressed respectively, We found that only circFOXK2 could regulate IL-6 expression by qRT-PCR assay (Fig. 5A). It was identified that IL-6

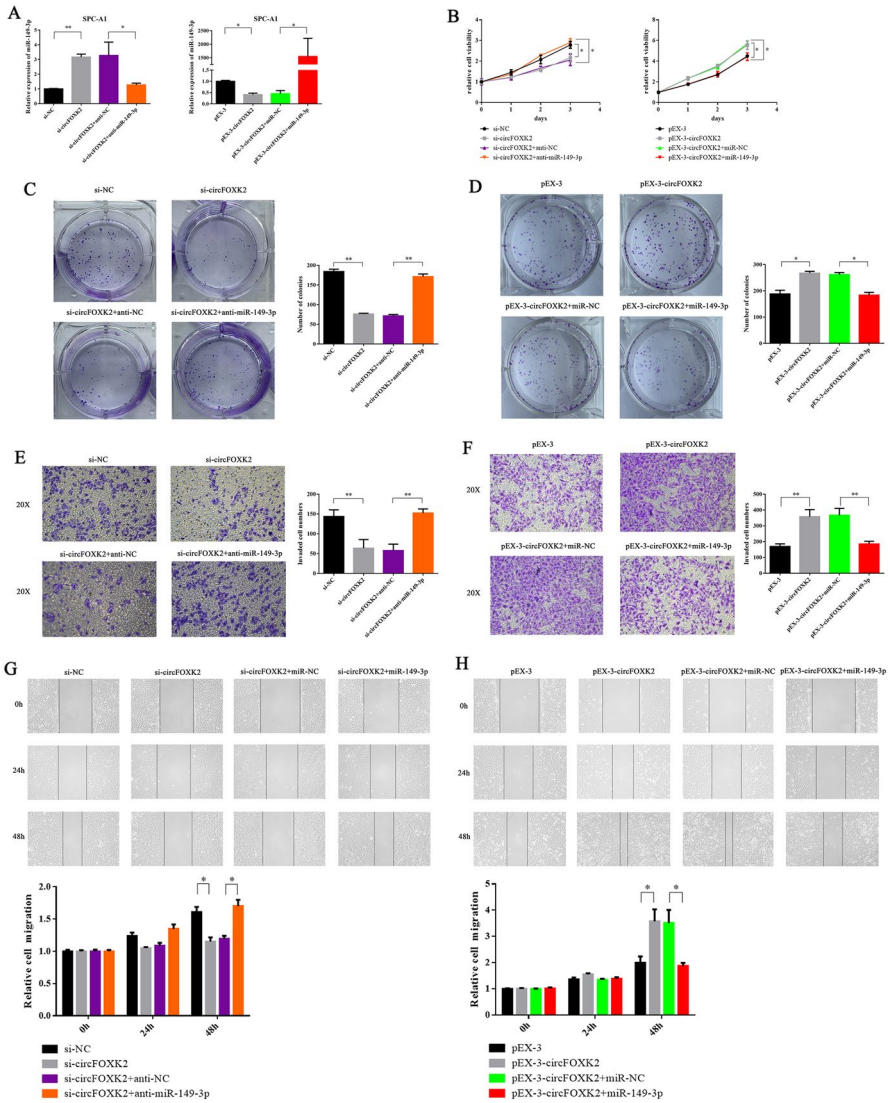


Fig. 4 miR-149-3p knockdown abolished the impact of circFOXK2 knockdown on proliferation, invasion, and migration abilities of NSCLC cells. **A** SPC-A1 cells were transfected with si-NC, si-circFOXK2, si-circFOXK2+anti-NC, si-circFOXK2+anti-miR-149-3p, pEX-3, pEX-3-circFOXK2, pEX-3-circFOXK2+miR-NC, and pEX-3-circFOXK2+miR-149-3p. **B** The proliferation of transfected SPC-A1 cells was elucidated by the CCK-8 test. **C, D** The transfected SPC-A1 cell colony numbers were quantified by colony formation analysis. **E, F** The transfected SPC-A1 cell's invading property was identified by the transwell analysis. **G, H** The transfected SPC-A1 cell migration capability was assessed by a wound healing test. * $P < 0.05$, ** $P < 0.01$

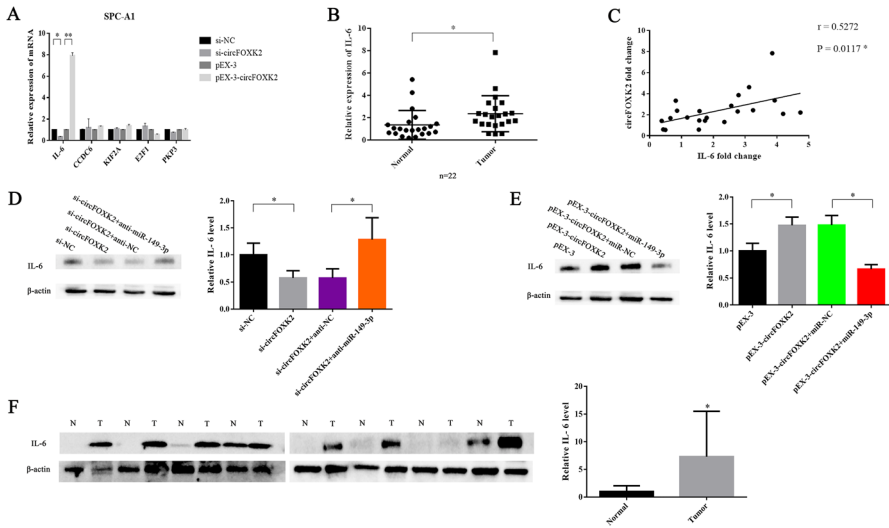


Fig. 5 IL-6 served as a direct miR-149-3p target. **A** The potential target genes of miR-149-3p expression was elucidated by qRT-PCR assay. **B** qRT-PCR analyzed the IL-6 levels in NSCLC and adjacent normal tissues ($n=22$). **C** Correlation analysis determined the expression correlation of circFOXK2 with IL-6 expression in NSCLC tissues ($n=22$). **D**, **E** IL-6 proteins were quantified by western blot in si-NC, si-circFOXK2, si-circFOXK2 + anti-NC, si-circFOXK2 + anti-miR-149-3p, pEX-3, pEX-3-circFOXK2, pEX-3-circFOXK2 + miR-NC and pEX-3-circFOXK2 + miR-149-3p transfected SPC-A1 cells. **E** Western blot quantified IL-6 protein levels as measured in 8 pairs of NSCLC and adjacent normal tissues. $*P < 0.05$, $**P < 0.01$

might be targeted by miR-149-3p. Subsequently, to elucidate IL-6 expression in NSCLC, qRT-PCR was conducted. As Fig. 5B represents, IL-6 was upregulated in NSCLC tissues than the adjacent normal tissues. And Pearson's correlation analysis indicated a substantial positive association between circFOXK2 and IL-6 in NSCLC tissues (Fig. 5C). Furthermore, western blot indicated that circFOXK2 knockdown could block IL-6 at the protein level, however, anti-miR-149-3p co-transfection counteracts this effect in SPC-A1 cells (Fig. 5D). In contrast, the circFOXK2 overexpression enhanced IL-6 at the protein level, and miR-149-3p co-transfection altered this impact in SPC-A1 cells (Fig. 5E). IL-6 levels in NSCLC and adjacent normal tissues ($n=8$, respectively) of NSCLC patients were also elucidated by western blot, which indicated an increase in the relative IL-6 levels (Fig. 5F). These statistics imply that circFOXK2 can act as a miR-149-3p sponge to modulate IL-6 expression.

CircFOXK2 Promoted Tumor Growth in Vivo

To verify the inferred above speculation that circFOXK2 overexpression might promote NSCLC growth in vivo, mice xenograft NSCLC models were

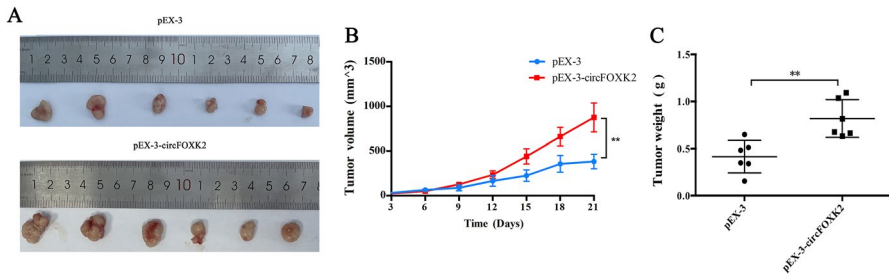


Fig. 6 CircFOXK2 promoted in vivo cell growth of NSCLC. **A** The pEX-3 or pEX-3-circFOXK2 transfected SPC-A1 cells were introduced into nude mice subcutaneous. **B, C** Xenografts’ tumor weight and volume were elucidated. * $P < 0.05$, ** $P < 0.01$

established (Fig. 6A). Results indicated that tumor volume and weight increased after circFOXK2 overexpression (Fig. 6B, C), indicating that circFOXK2 overexpression promoted NSCLC cell growth in vivo.

Discussion

Literature confirms that circRNA is closely linked with the incidence and development of cancer including lung (Yuan et al. 2022; Chen et al. 2022; Tang and Hann 2020). CircRNAs are considered as a potential biomarker because of its high stability and abundance unlike linear RNA (Yu et al. 2019). And multiple investigations indicate that circFOXK2 dysregulation widely participates in cancer occurrence and progression, but not including NSCLC (Li et al. 2022; Zhao et al. 2021).

It is also suggested that circRNAs could be used as miRNA sponges to reduce their binding with their gene targets (Singh et al. 2022). circFOXK2 has been few studies about cancers in recent years. For instance, circFOXK2 stimulates the growth and pancreatic ductal adenocarcinoma metastasis by modulating RNA binding protein via the miR-942 sponge (Wong et al. 2020). Additionally, circFOXK2 interacts with miR-370 and IGF2BP3 (an RNA-binding protein) to synergistically promote breast cancer metastasis (Zhang et al. 2021). However, its function, expression levels, and regulatory mechanism are still unclear in NSCLC. Therefore, we hypothesized that circFOXK2 can promote NSCLC progression as well. In this study, we found that circFOXK2 was upregulated in NSCLC and its deletion delayed NSCLC cell proliferation, invasion and migration in vitro. However, circFOXK2 overexpression altered this effect. To explore the circFOXK2 mechanism, miR-149-3p was focused, as it is probably sponged by circFOXK2. It was discovered that miR-149-3p has multiple functions in regulating cancer cell proliferation, metastasis, and apoptosis. For instance, miR-149-3p inhibits the proliferation of oral squamous cell carcinoma cells through the post-transcriptional suppression of AKT2 (Shen et al. 2021). And LRP11-AS1 is upregulated in triple-negative breast cancer indicated an oncogenic impact probably by sponging miR-149-3p and regulating the miR-149-3p/NRP2 axis (Li et al. 2022). Therefore, this investigation confirms that circFOXK2 sponge miR-149-3p and miR-149-3p can partially counteract

this circFOXK2 activity by promoting NSCLC development. Moreover, some studies have shown that IL-6 can promote the development of NSCLC. For example, IL-6 induces NSCLC metastasis by TIM-4 upregulating via NF- κ B (Liu et al. 2020). Furthermore, circRBM33 regulates IL-6 to stimulate gastric cancer progression by targeting miR-149 (Wang et al. 2020). So we further discovered that circFOXK2 enhances the tumorigenesis of NSCLC through the miR-149-3p/IL-6 axis in this study. Consistently, the overexpression of circFOXK2 promoted NSCLC growth in nude mice, indicating the ability of circFOXK2 to promote NSCLC occurrence and development both in *in vivo* and *in vitro*. Compared with other recent studies, we discovered that circFOXK2 enhances the tumorigenesis of NSCLC through the miR-149-3p/IL-6 axis. Unfortunately, we did not explore the mechanism of circFOXK2 *in vivo*, which is the aim of future our studies.

As compared to previous NSCLC data (Wang et al. 2020), the novelty of this investigation is that it not only enriches the miRNA network of circRNA based on competitive endogenous RNA but also explores its function in the nude mouse experiment, providing a crucial preclinical basis for NSCLC treatment.

Conclusion

It was concluded that circFOXK2 enhances the tumorigenesis of NSCLC through the miR-149-3p/IL-6 axis.

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Author Contributions WY and JL hypothesized and designed the project. TX, LC, TY, and JL performed the experiments and analyzed the data. TX and JL wrote the manuscript. LC, TL and HW provided advice and critical comments. WY is responsible for research supervision and funding acquisition. All the authors read and approved the final manuscript.

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Data Availability The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing Interests The authors declare that there are no competing interests.

Ethical Approval This investigation was authorized by the Clinical Research Ethics Committee of Ningbo University and was carried out according to the guidelines of Declaration of Helsinki.

Research Involving Human and Animal Participants Animal experiment was ratified by the Clinical Research Ethics Committee of Ningbo University and performed in accordance with the guidelines of the National Animal Care and Ethics Institution.

Consent for Publication Not applicable.

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