Circ_0005962 functions as an oncogene to aggravate non-small cell lung cancer progression via circ_0005962/miR-382-5p/PDK4 regulatory network

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Running title: Circ_0005962 promotes NSCLC progression

Abstract

Non-small cell lung cancer (NSCLC) is a leading threat to human lives with high incidence and mortality. Circular RNAs (circRNAs) were reported to play important roles in human cancers. The purpose of this study was to investigate the role of circ_0005962 and explore the underlying functional mechanisms. The expression of

31 circ_0005962, miR-382-5p and pyruvate dehydrogenase kinase 4 (PDK4) was
 32 detected by quantitative real-time polymerase chain reaction (qRT-PCR). Cell
 33 proliferation and cell apoptosis were assessed by cell counting kit-8 (CCK-8) assay
 34 and flow cytometry assay, respectively. The protein levels of Beclin 1, light chain3
 35 (LC3-II/LC3-I), PDK4, Cleaved Caspase 3 (C-caspase 3) and proliferating cell
 36 nuclear antigen (PCNA) were examined using western blot analysis. Glycolysis was
 37 determined according to the levels of glucose consumption and lactate production.
 38 The interaction between miR-382-5p and circ_0005962 or PDK4 was predicted by the
 39 online tool CircInteractome or starbase and verified by dual-luciferase reporter assay
 40 and RNA immunoprecipitation (RIP) assay. Xenograft model was constructed to
 41 investigate the role of circ_0005962 *in vivo*. circ_0005962 expressed with a high level
 42 in NSCLC tissues and cells. Circ_0005962 knockdown inhibited proliferation,
 43 autophagy, and glycolysis but promoted apoptosis in NSCLC cells. MiR-382-5p was
 44 targeted by circ_0005962, and its inhibition reversed the role of circ_0005962
 45 knockdown. Besides, PDK4, a target of miR-382-5p, was regulated by circ_0005962
 46 through miR-382-5p, and its overexpression abolished the effects of miR-382-5p
 47 reintroduction. Circ_0005962 knockdown suppressed tumor growth *in vivo*.
 48 Circ_0005962 knockdown restrained cell proliferation, autophagy, and glycolysis but
 49 stimulated apoptosis through modulating the circ_0005962/miR-382-5p/PDK4 axis.
 50 Our study broadened the insights into understanding the mechanism of NSCLC
 51 progression.

52 **Key words:** circ_0005962, miR-382-5p, PDK4, NSCLC

54 Introduction

55 Lung cancer is a leading cause of cancer-related death worldwide (Magnuson et al.,
 56 2016). Lung cancer is the second most common cancer among men and women:
 57 second only to prostate cancer in men, and second only to breast cancer in women
 58 (Testa et al., 2018). Non-small cell lung cancer (NSCLC) and small cell lung cancer
 59 are two types of lung cancer, and NSCLC accounts for about 85% of all lung cancer
 60 cases (Zhukovsky et al., 2014). NSCLC, including large cell carcinoma, squamous

61 cell carcinoma, and adenocarcinoma, is associated with high incidence and mortality
62 (Barnett et al., 2016; Brody, 2014). Clinically, treatment modalities, including
63 chemotherapy and surgery, are used to treat NSCLC, but the 5-year overall survival
64 rate for all stages of NSCLC patients is only 16% (Laskin et al., 2005; Testa et al.,
65 2018). The severe situation of NSCLC treatment makes it urgent to further explore the
66 mechanism of occurrence and development of NSCLC to establish novel therapeutic
67 strategies.

68 Circular RNAs (circRNAs) belong to non-coding RNAs and derive from
69 alternative and back-splicing of precursor mRNAs. CircRNAs are mostly detected in
70 the cytoplasm and function as competing endogenous RNAs (ceRNAs) to work as
71 microRNA (miRNA) sponges (Hansen et al., 2013; Kulcheski et al., 2016; Salzman et
72 al., 2012). Advances of high-throughput RNA sequencing in the identification of
73 circRNAs hinted that circRNAs participated in the pathogenesis of cancers (Liang et
74 al., 2014). Recent studies stated that circRNAs played vital roles in the development
75 of NSCLC. For example, circ_100146 functioned as an oncogene, and its suppression
76 hindered cell proliferation and invasion (L. Chen et al., 2019). CircP4HB showed a
77 high level in NSCLC, promoted epithelial-mesenchymal transition (EMT), and was
78 associated with metastatic diseases (T. Wang et al., 2019). CircRNA F-circEA-2a
79 contributed to cell migration and invasion but had little role in cell proliferation (Tan
80 et al., 2018). A former study obtained dozens of circRNAs through the CircBase
81 database and CSCD database for comparison between lung adenocarcinoma tissues
82 and paired non-tumor tissues (Liu et al., 2019), and circ_0005962, back-spliced from
83 tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
84 (YWHAZ), was one of the significantly upregulated circRNAs and had valuable
85 prognostic significance. However, the understanding of the specific roles of
86 circ_0005962 in NSCLC remains lacking and requires further exploration.

87 It is well documented that miRNAs are involved in the biological processes of
88 cancers. MiRNAs are known as small non-coding RNAs with 18~23 nucleotides
89 (Jiang et al., 2014). MiRNAs play vital roles in tumor formation, growth and
90 metastasis by acting as oncogenes or tumor suppressor genes. Among these miRNAs,

miR-382-5p was frequently mentioned in different cancers, including glioma, oral squamous cell carcinoma and breast cancer (Ho et al., 2017; Sun et al., 2019; J. Wang, C. Chen, et al., 2019). Unfortunately, the role of miR-382-5p in NSCLC is unknown, and we attempted to investigate the function of miR-382-5p in NSCLC cells.

One of the important functions of pyruvate dehydrogenase kinase (PDK) is to regulate the metabolic conversion from mitochondrial respiration to cytoplasmic glycolysis (Jeoung, 2015). Glycolysis is a preferential way for tumor cells to obtain energy (Vander Heiden et al., 2009). Pyruvate dehydrogenase kinase 4 (PDK4) is one of the PDK family protein kinases and located on chromosome 7q21.3 (J. Wang, Y. Qian, et al., 2019). PDK4 was reported to be implicated in numerous cellular activities, such as cell proliferation, metastasis, drug resistance, glycolysis and autophagy in different types of cancers (Feng et al., 2019; J. Wang, Y. Qian, et al., 2019; Yang et al., 2019). The specific role of PDK4 and associated action mechanisms in NSCLC still need to be explored to expand the function of PDK4 in cancers.

In the present study, we measured the expression of circ_0005962 in NSCLC tissues and cells. Functional analyses revealed the role of circ_0005962 in cell proliferation, autophagy, glycolysis and apoptosis. Besides, we constructed circRNA-miRNA-mRNA regulatory axis to expound the potential mechanism of circ_0005962 in NSCLC. Our study aimed to provide new sights for the understanding of NSCLC development and supply promising biomarkers.

Materials and methods

Tissues and cell lines

A total of 45 tumor tissues and adjacent normal tissues from NSCLC patients were collected from Gong'an County People's Hospital. Informed consent was signed by each subject. All tissues were immediately placed into liquid nitrogen and stored at -80°C ultra low-temperature refrigerator. This research obtained the approval of the Ethics Committee of Gong'an County People's Hospital.

NSCLC cell lines (A549 and HCC827) and human bronchial epithelial cells (BEAS-2B) were purchased from Zishi Biotechnology (Shanghai, China). A549 and

121 HCC827 cells were maintained in 90% Roswell Park Memorial Institute 1640 (RPMI
122 1640; Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Sigma).
123 BEAS-2B cells were cultured in 90% Dulbecco's Modified Eagle Medium (DMEM;
124 Sigma) containing 10% FBS (Sigma). All mediums were placed at 37°C containing 5%
125 CO₂.

126

127 **Quantitative real-time polymerase chain reaction (qRT-PCR)**

128 Trizol reagent (Beyotime, Shanghai, China) was utilized to isolate total RNA from
129 tissues and cells. The complementary DNA (cDNA) was assembled using the
130 riboSCRIPT Reverse Transcription Kit (Ribobio, Guangzhou, China). Then the
131 amplification reaction was carried out using SYBR Green Master PCR mix (Beyotime)
132 through the ABI 7900 system (Applied Biosystems, Foster City, CA, USA). The
133 relative expression was normalized by Glyceraldehyde-3-phosphate dehydrogenase
134 (GAPDH) or small nuclear RNA U6 and calculated using 2^{-ΔΔCt} method. The primers
135 used were listed as below: circ_0005962, forward (F):
136 5'-AACTCCCCAGAGAAAGCCTGC-3' and reverse (R): 5'-
137 TGCTTGTGAAGCATTGGGGAT-3'; YWHAZ, forward (F): 5'- ACTTTTGGTACA
138 TTGTGGCTTCAA-3' and reverse (R): 5'-CCGCCAGGACAAACCAGTAT-3';
139 PDK4, F: 5'-GGAGCATTTCTCGCGCTACA-3' and R:
140 5'-ACAGGCAATTCTTGTCGCAAA-3'; GAPDH, F: 5'-
141 CTGGGCTACACTGAGCACC-3' and R: 5'-AAGTGGTCGTTGAGGGCAATG-3';
142 miR-382-5p, F: 5'-ATCCGTGAAGTTGTTCGTGG-3' and R: 5'-
143 TATGGTTGTAGAGGACTCCTTGAC-3'; U6, F: 5'-
144 GCUUCGGCAGCACAUAUACUAAAAU-3' and R: 5'-
145 CGCUUCACGAAUUUGCGUGUCAU-3'.

146

147 **RNase R treatment**

148 To confirm the stability and tolerance of circ_0005962, the RNA extraction was
149 probed with or without RNase R (Applied Biological Materials Inc., Vancouver,
150 Canada) at 37°C for 10 min. Then, the qRT-PCR analysis was conducted as described

151 above.

152

153 **Cell transfection**

154 Small interference RNA against circ_0005962 and negative control were synthesized
155 by Sangon Biotech (Shanghai, China). The mimics of miR-382-5p (miR-382-5p), the
156 inhibitor of miR-382-5p (anti-miR-382-5p) and respective negative control (NC or
157 anti-NC) were purchased from Ribobio (Shanghai, China). The overexpression of
158 circ_0005962 (circ_0005962) was performed as the previous study described (Y.
159 Wang, J. Zhang, et al., 2019) and constructed by Genechem (Shanghai, China), while
160 the specific plasmid without the circ_0005962 cDNA was served as control (circ-NC).
161 The vector pcDNA3.1-PDK4 (PDK4) for the overexpression of PDK4 and the control
162 pcDNA empty vector (vector) were constructed by Sangon Biotech. Lentiviral vector
163 (Lenti-short hairpin) for stable NEAT1 downregulation (Lv-sh-circ_0005962) and
164 corresponding negative control (Lv-sh-NC) were obtained from Genechem. Cell
165 transfection was conducted by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA,
166 USA). Transfection efficiency and following experiments were implemented after 48h
167 of transfection.

168

169 **Cell counting kit-8 (CCK-8) assay**

170 Cell proliferation was investigated by CCK-8 assay. A549 or HCC827 cells with
171 different transfection were seeded into 96-well plates (5×10^3 cells/well). Then, cells
172 were interacted with CCK-8 solution (Beyotime) for continuing 2 h at 24, 48, and 72
173 h. The absorbance at 450 nm was measured using a microplate reader (Bio-Rad,
174 Hercules, CA, USA).

175

176 **Flow cytometry assay**

177 Flow cytometry was applied to monitor cell apoptosis. After 48 h, A549 or HCC827
178 cells with different transfection were washed with phosphate buffer saline (PBS),
179 probed with 0.25% trypsin and resuspended in binding buffer. Afterward, the Annexin
180 V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit

(Sigma) was used to doubly stain cells for 15 min in the dark. Eventually, the apoptotic cells were sorted using CellQuest software under a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

184

185 **Western blot**

The western blot analysis was performed in line with a previous study described (W. Ren et al., 2019). In brief, total proteins were separated and transferred into polyvinylidene fluoride (PVDF) membranes (Bio-Rad). After the block, the membranes were incubated with the primary antibodies and the secondary antibodies. Finally, the seeking proteins were visualized using the enhanced chemiluminescent reagent (Beyotime) through an imaging system (Bio-Rad). The antibodies used were listed as follows: anti-Becclin 1 (1:1000; cat. no. ab210498; Abcam, Cambridge, MA, USA), anti-light chain3 (LC3) (1:2000; cat. no. ab192890), anti-PDK4 (1:1000; cat. no. ab89295), anti-Cleaved Caspase 3 (C-caspase 3) (1:1000; cat. no. ab2302), anti-proliferating cell nuclear antigen (PCNA) (1:1000; cat. no. ab92552), anti-GAPDH (1:1000; cat. no. ab8245) and the horseradish peroxidase-conjugated secondary antibodies (1:5000; cat. no. ab205718).

198

199 **The detection of glucose consumption and lactate production**

A549 or HCC827 cells with different transfection were planted into 96-well plates. After 48 h, cells were collected, washed three times with PBS, and then used for the detection of glucose consumption and lactate production using the Glucose Uptake Colorimetric Assay Kit and Lactate Assay Kit (Sigma) in agreement with the manufacturer's instructions.

205

206 **Bioinformatics analysis**

The targets of circRNA and miRNA were forecasted, and their binding sites were analyzed by the online bioinformatics tool CircInteractome (<https://circinteractome.nia.nih.gov/>) and starbase (<http://starbase.sysu.edu.cn/>).

210

211 **Dual-luciferase reporter assay**

212 Dual-luciferase reporter assay was carried out for the verification of the relationship
213 between miR-382-5p and circ_0005962 or PDK4. The partial frame of circ_0005962
214 containing the binding site or mutant binding site (wild-type or mutant-type) with
215 miR-382-5p was amplified and cloned into the downstream of pGL4 vector (Promega,
216 Madison, WI, USA) to generate circ_0005962-wt and circ_0005962-mut fusion
217 plasmids. Likewise, the 3' UTR sequences of PDK4 harboring the binding site with
218 miR-382-5p or mutant binding site (wild-type or mutant-type) were also amplified
219 and inserted into the downstream of pGL4 vector to generate PDK4-wt and PDK-mut
220 fusion plasmids. Subsequently, these fusion plasmids and miR-382-5p or NC were
221 co-transfected into A549 and HCC827 cells, respectively. After 48 h, the cells were
222 collected and detected using the Dual-luciferase assay system (Promega). The firefly
223 luciferase activity was normalized by Renilla luciferase activity.

224

225 **RNA immunoprecipitation (RIP) assay**

226 RIP assay was executed to further confirm the relationship between miR-382-5p and
227 circ_0005962 or PDK4. In one case, A549 and HCC827 cells were harvested and
228 incubated with RNA lysis buffer. Then, cell lysate was incubated with RIP binding
229 buffer containing magnetic beads coated with human Ago2 antibody or mouse IgG
230 antibody (control) (Millipore, Billerica, MA, USA). Subsequently, the levels of
231 circ_0005962 and miR-382-5p enriched in the beads were detected by qRT-PCR. In
232 another case, A549 and HCC827 cells transfected with miR-382-5p were lysed in RIP
233 buffer with anti-Ago2- or IgG-bound magnetic beads. Next, the mRNA level of PDK4
234 enriched in the beads was examined by qRT-PCR.

235

236 ***In vivo* experiment**

237 The mice experiment obtained the approval of the Animal Care and Use Committee of
238 Gong'an County People's Hospital. A total of 10 BALB/c nude mice (five-week-old,
239 male) were purchased from HFK Bioscience (Beijing, China). A549 cells with
240 Lv-sh-circ_0005962 or Lv-sh-NC transfection were subcutaneously injected into the

back right flank of nude mice, dividing into the Lv-sh-circ_0005962 group and Lv-sh-NC group. Tumor volume was observed and recorded once a week following the algorithm ($\text{length} \times \text{width}^2 \times 0.5$), lasting 5 weeks. In the end, the mice were killed, and tumor samples were removed for weighting and further molecular studies.

245

246 **Data analysis**

The data were obtained from at least 3 times independent experiments analysis and conducted using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The survival curve was depicted via the Kaplan-Meier method. The correlation analysis was performed based on Spearman's correlation coefficient. The differences between 2 groups were analyzed by Student's *t*-test or one-way analysis of variance among multiple groups. The data after processing were presented as the mean $\pm$ standard deviation (SD), and $P < 0.05$ was considered to be statistically significant.

254

255 **Result**

256 **High expression of circ_0005962 was observed in NSCLC tissues and cells and** 257 **predicted the low survival rate of NSCLC patients**

The expression of circ_0005962 was detected in NSCLC tissues and cell lines to observe whether circ_0005962 aberrantly regulated in NSCLC. As shown in Figure 1A, the expression of circ_0005962 was significantly higher in tumor tissues (n=45) than that in adjacent normal tissues (n=45). Besides, the expression of circ_0005962 was abundant in A549 and HCC827 cells relative to BEAS-2B cells (Figure 1B). Moreover, the survival curve was depicted utilizing Kaplan-Meier survival rate analysis according to the living status of NSCLC patients, and we found that the survival rate of patients with high expression of circ_0005962 was notably weaker than patients with low circ_0005962 expression (Figure 1C). Additionally, the result of qRT-PCR showed that the expression of circ_0005962 in RNase R+ group decreased a little compared with that in RNase R- group, while the expression of the parental linear mRNA (YWHAZ) was significantly reduced with the treatment of RNase R, indicating that circ_0005962 was resistant to RNase R (Figure 1D). The

271 data indicated that circ_0005962 was aberrantly upregulated in NSCLC.

272

273 **Circ_0005962 knockdown inhibited proliferation, autophagy, and glycolysis but**
 274 **promoted apoptosis in NSCLC cells**

275 The endogenous level of circ_0005962 was knocked down to investigate the role of
 276 circ_0005962 in NSCLC cells. Si-circ_0005962 was inserted into the mature
 277 sequence of circ_0005962 to reduce circ_0005962 expression, si-NC acting as a
 278 control (Figure 2A). Then, the knockdown efficiency was examined by qRT-PCR, and
 279 the result showed that the expression of circ_0005962 in A549 and HCC827 cells
 280 with si-circ_0005962#1, si-circ_0005962#2 and si-circ_0005962#3 transfection was
 281 notably decreased than that with si-NC transfection, and the knockdown efficiency in
 282 the si-circ_0005962#1 group was the highest (Figure 2B). Hence, si-circ_0005962#1
 283 was chosen for the following analyses. The result of CCK-8 assay revealed that the
 284 cell proliferation was prominently suppressed in A549 and HCC827 cells transfected
 285 with si-circ_0005962#1 (Figure 2C). On the contrary, flow cytometry assay presented
 286 that the apoptosis rate in A549 and HCC827 cells with si-circ_0005962#1 transfection
 287 was inversely enhanced (Figure 2D). Besides, the protein levels of autophagy-related
 288 markers were quantified to assess the change of autophagy, and we found that the
 289 levels of Beclin 1 and LC3-II/LC3-I were declined with circ_0005962 knockdown
 290 (Figure 2E and 2F). Moreover, the levels of glucose in culture medium and lactate
 291 production were checked to assess glycolysis progression, and we noticed that the
 292 level of existing glucose in A549 and HCC827 cells with si-circ_0005962#1
 293 transfection was higher than that with si-NC transfection, suggesting that the
 294 consumptive glucose was reduced (Figure 2G). The level of lactate production was
 295 significantly blocked in A549 and HCC827 cells with si-circ_0005962#1 transfection
 296 relative to si-NC (Figure 2H). All data clarified that circ_0005962 knockdown
 297 inhibited proliferation, autophagy, and glycolysis but induced apoptosis in NSCLC
 298 cells.

299

300 **MiR-382-5p was a target of circ_0005962**

301 Generally, circ_0005962 functioned by acting as a ceRNA to modulate the expression
302 of target miRNAs. The putative target miRNAs were predicted by the online tool
303 CircInteractome, and miR-382-5p was one of target miRNAs of circ_0005962 with
304 specific binding sites (Figure 3A). To ascertain the relationship between circ_0005962
305 and miR-382-5p, dual-luciferase reporter assay and RIP assay were performed. The
306 luciferase activity in A549 and HCC827 cells with circ_0005962-wt and miR-382-5p
307 transfection was substantially decreased compared with circ_0005962-wt and NC
308 transfection, while the luciferase activity in A549 and HCC827 cells with
309 circ_0005962-mut and miR-382-5p transfection was no difference compared with
310 circ_0005962-mut and NC transfection (Figure 3B). The RIP analysis detected the
311 higher expression of circ_0005962 and miR-382-5p in the Ago2 pellet of A549 and
312 HCC827 lysate than that in the IgG control (Figure 3C). Moreover, the qRT-PCR
313 analysis exhibited that the expression of miR-382-5p was notably declined with
314 circ_0005962 overexpression but improved with circ_0005962 knockdown in A549
315 and HCC827 cells (Figure 3D). Additionally, the expression of miR-382-5p in
316 NSCLC tumor tissues (n=45) was markedly weaker than that in normal tissues (n=45)
317 (Figure 3E). The expression of miR-382-5p in A549 and HCC827 cells was
318 consistently lower than that in BEAS-2B cells (Figure 3F). Spearman's correlation
319 coefficient revealed that circ_0005962 expression was negatively correlated with
320 miR-382-5p expression in NSCLC tissues (Figure 3G). These analyses maintained
321 that miR-382-5p was a target of circ_0005962, and its expression was regulated by
322 circ_0005962.

323

324 **Inhibition of miR-382-5p reversed the role of circ_0005962 knockdown in** 325 **NSCLC cells**

326 A549 and HCC827 cells were introduced with si-circ_0005962#1, si-NC,
327 si-circ_0005962#1+anti-miR-382-5p and si-circ_0005962#1+anti-NC, respectively.
328 First, the expression of miR-382-5p in these transfected cells was checked, and we
329 found that the expression of miR-382-5p was enhanced in the si-circ_0005962#1 but
330 inhibited in the si-circ_0005962#1+anti-miR-382-5p group (Figure 4A). The cell

proliferation inhibited by si-circ_0005962#1 was recovered in A549 and HCC827 cells with si-circ_0005962#1+anti-miR-382-5p transfection (Figure 4B). The elevated cell apoptosis rate in cells with si-circ_0005962#1 transfection was suppressed in cells with si-circ_0005962#1+anti-miR-382-5p transfection (Figure 4C). The protein levels of Beclin 1 and LC3-II/LC3-I were depleted in A549 and HCC827 cells transfected with si-circ_0005962#1 but restored in cells transfected with si-circ_0005962#1+anti-miR-382-5p (Figure 4D and 4E). The level of existing glucose in medium in the si-circ_0005962#1+anti-miR-382-5p group was declined than that in the si-circ_0005962#1 group (Figure 4F). The level of lactate production inhibited in the si-circ_0005962#1 group was promoted in the si-circ_0005962#1+anti-miR-382-5p group (Figure 4G). These results meant that circ_0005962 knockdown inhibited proliferation, autophagy, and glycolysis but induced apoptosis through enhancing the expression of miR-382-5p.

344

345 **PDK4 was a target of miR-382-5p**

CircRNA-miRNA-mRNA regulatory network is an important mechanism participating in the development of human cancers. The target mRNAs of miR-382-5p were analyzed to observe that whether circ_0005962 functioned following this mechanism. Online bioinformatics tool starbase predicted that PDK4 was one of targets of miR-382-5p with a specific binding site at its 3' UTR (Figure 5A). Besides, miR-382-5p overexpression predominantly inhibited the luciferase activity in A549 and HCC827 cells transfected with PDK4-wt but not PDK4-mut (Figure 5B). Moreover, the enrichment of PDK4 was notably elevated in the miR-382-5p-transfected group compared with that in the NC group after Ago2 RIP, while enrichment of PDK4 after IgG RIP showed no efficacy (Figure 5C). Next, the western blot analysis monitored that the level of PDK4 was weakened in A549 and HCC827 cells with miR-382-5p transfection but elevated in cells with circ_0005962+miR-382-5p transfection (Figure 5D). Also, the expression of PDK4 was detected in NSCLC tumor tissues, and the result presented that the expression of PDK4 at both mRNA and protein levels was abnormally higher in NSCLC tumor

361 tissues (n=45) relative to normal tissues (n=45) (Figure 5E and 5F). Likewise, the
 362 protein level of PDK4 in A549 and HCC827 cells was also enhanced compared to
 363 BEAS-2B cells (Figure 5G). Furthermore, the mRNA level of PDK4 was positively
 364 correlated with circ_0005962 level but negatively correlated with the miR-382-5p
 365 level in NSCLC tumor tissues (Figure 5H and 5I). All data suggested that PDK4 was a
 366 target of miR-382-5p and regulated by miR-382-5p and circ_0005962.

367

368 **PDK4 overexpression abolished the role of miR-382-5p reintroduction in NSCLC** 369 **cells**

370 A549 and HCC827 cells were introduced with miR-382-5p, NC, miR-382-5p+PDK4
 371 and miR-382-5p+vector, respectively. The expression of PDK4 was examined to
 372 assess transfection efficiency, and the result showed that the level of PDK4 was
 373 obviously reduced in cells transfected with miR-382-5p but regained in cells
 374 transfected with miR-382-5p+PDK4 (Figure 6A). Afterwards, the cell proliferation
 375 was inhibited by miR-382-5p reintroduction but promoted by the combination of
 376 miR-382-5p reintroduction and PDK4 overexpression (Figure 6B). The apoptosis rate
 377 was elevated by miR-382-5p reintroduction but restrained by the combination of
 378 miR-382-5p reintroduction and PDK4 overexpression (Figure 6C). Moreover, the
 379 levels of Beclin1 and LC3-II/LC3-I were weakened in A549 and HCC827 cells
 380 transfected with miR-382-5p but rescued in cells transfected with miR-382-5p+PDK4
 381 (Figure 6D and 6E). The existing glucose level was abundant in the miR-382-5p
 382 group but reduced in the miR-382-5p+PDK4 group, suggesting that PDK4
 383 overexpression enhanced the level of glucose consumption inhibited by miR-382-5p
 384 reintroduction (Figure 6F). The level of lactate production was blocked by
 385 miR-382-5p reintroduction but recovered by the combination of miR-382-5p
 386 reintroduction and PDK4 overexpression (Figure 6G). These data hinted that
 387 miR-382-5p attenuated cell proliferation, autophagy, and glycolysis but contributed to
 388 apoptosis through inhibiting the expression of PDK4.

389

390 **Circ_0005962 knockdown inhibited tumor growth *in vivo***

A549 cells with stable Lv-sh-circ_0005962 transfection were subcutaneously injected into the groin of nude mice to determine the role of circ_0005962 *in vivo*. As shown in Figure 7A and 7B, circ_0005962 knockdown remarkably reduced the tumor volume and tumor weight. After injection for 5 weeks, all mice were killed, and the tumors were removed for expression analysis. The expression of circ_0005962 and the mRNA level of PDK4 were noticeably declined in the Lv-sh-circ_0005962 group, while the expression of miR-382-5p was conspicuously strengthened in the Lv-sh-circ_0005962 group (Figure 7C). Besides, the protein level of PDK4 was consistent with its mRNA level. Additionally, the levels of apoptosis-related marker (C-caspase 3) and proliferation-related marker (PCNA) were monitored, and we discovered that the level of C-caspase 3 was reinforced, while the level of PCNA plummeted in the Lv-sh-circ_0005962 group (Figure 7D). Collectively, circ_0005962 knockdown impeded tumor growth *in vivo*.

404

405 Discussion

NSCLC is a severe burden to people's lives, and the prognosis for patients is still unsatisfactory. The responses to existing standard therapies are poor, except for the most localized cancers (Lemjabbar-Alaoui et al., 2015). Hence, more novel mechanisms of NSCLC progression need to be explored so as to develop aimed therapeutic strategies for NSCLC. Here, we investigated the role of circ_0005962 in NSCLC for the first time. Circ_0005962 was observed to be aberrantly overexpressed in NSCLC tissues and cells. The functional analysis concluded that circ_0005962 knockdown attenuated NSCLC progression *in vitro* and *in vivo*. Stepwise identification manifested that circ_0005962 could directly interact with miR-382-5p, leading to an increase of PDK4 expression, thereby contributing to the development of NSCLC. Our study illustrated the importance and carcinogenesis role of circ_0005962 in NSCLC.

CircRNAs were documented to be dysregulated in numerous human cancers and took effects on apoptosis, autophagy, chemoresistance, metastasis and glycolysis (Chi et al., 2019; Kun-Peng et al., 2018; S. Ren et al., 2019; Wei et al., 2019). Up to now,

dozens of NSCLC-related circRNAs were screened and identified, such as circ_100146, CIRC-PRMT5 and circ_0026134 (Chang et al., 2019; L. Chen et al., 2019; Y. Wang, Y. Li, et al., 2019), leading to the malignant progression of NSCLC via acting as oncogenes. On the contrary, certain circRNAs, such as circPTPRA, circ_circ_00059621946 and circSMARCA5 were maintained as tumor suppressors to block NSCLC deterioration (Huang et al., 2019; Y. Wang, H. Li, et al., 2019; Wei et al., 2019). These data suggested the diverse roles of circRNAs in cancer development. Despite the fact that the role of several circRNAs was partly characterized, there were still existing circRNAs lacking functional exploration. A previous study predicted differentially expressed circRNAs in lung adenocarcinoma using Gene Expression Omnibus (GEO) dataset and found that circ_0005962 was highly expressed in lung adenocarcinoma plasma and cells (Liu et al., 2019). In view of this finding, we speculated that dysregulation of circ_0005962 might be associated with the malignant progression of NSCLC. Interestingly, we discovered that circ_0005962 knockdown inhibited cell proliferation, autophagy and glycolysis but accelerated cell apoptosis through *in vitro* analyses. Besides, circ_0005962 knockdown weakened tumor growth in nude mice *in vivo*, suggesting that circ_0005962 was an oncogene at least in NSCLC progression.

The classic action way of circRNAs is as a sponge of miRNAs. In our study, miR-382-5p was identified as a target of circ_0005962. The role of miR-382 in NSCLC gradually became clear to function as a tumor suppressor. For example, miR-382 suppressed proliferation and migration of NSCLC cells by binding to the 3' UTR of LMO3 (D. Chen et al., 2019). Besides, miR-382 was significantly downregulated in NSCLC tissues and cells, and enrichment of miR-382 depleted cell proliferation, migration and invasion through targeting SETD8 (Chen et al., 2017). Consistent with these findings, we also detected that miR-382-5p was weakly expressed in NSCLC cells, and reintroduction of miR-382-5p inhibited proliferation, autophagy and glycolysis of NSCLC cells. Besides, miR-382-5p inhibition reversed the regulatory effects of circ_0005962 knockdown.

Considering the habitual action mode of miRNAs, we further analyzed the

potential target mRNAs of miR-382-5p to establish a detailed action mechanism of circ_0005962 in NSCLC. Among the mRNAs whose levels were increased in A549 and HCC827 cells, one of the most significant was PDK4. Similarly, PDK4 has been reported to be highly expressed in cisplatin-resistant lung adenocarcinoma (Yu et al., 2018). Besides, oncogene LINC00243 contributed to proliferation and glycolysis in NSCLC by positively regulating PDK4 (Feng & Yang, 2019). Consistently, we also noticed that PDK4 was upregulated in NSCLC tissues and cell lines. Moreover, PDK4 overexpression eliminated the role of miR-382-5p reintroduction, leading to the malignant activities of NSCLC cells. These data indicated that PDK4 played a carcinogenic role in NSCLC.

Taken together, the expression of circ_0005962 was increased in NSCLC tissues and cell lines. Knockdown of LINC00243 blocked cell proliferation, autophagy and glycolysis but accelerated cell apoptosis *in vitro* and weakened tumor growth *in vivo*. Besides, circ_0005962 was a sponge of miR-382-5p, and PDK4 was a target of miR-382-5p. Circ_0005962 functioned in NSCLC progression by inducing PDK4 through sponging miR-382-5p. Our results not only corroborate the role of circ_0005962 in NSCLC *in vitro* and *in vivo* but also provide the circ_0005962/miR-382-5p/PDK4 regulatory axis, which may be promising to develop novel therapeutic approaches for NSCLC.

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Disclosure of interest

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483 Author contributions

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625 **Figure legends**

626 **Figure 1. Circ_0005962 was highly expressed in NSCLC tissues and cells.** (A) The
627 expression of circ_0005962 in tumor tissues (n=45) and adjacent normal tissues (n=45)
628 was detected by qRT-PCR. (B) The expression of circ_0005962 in NSCLC cell lines
629 (A549 and HCC827) and normal cells (BEAS-2B) was detected by qRT-PCR. (C) The
630

631 survival curve was depicted according to the Kaplan-Meier plot and analyzed by the
632 log-rank test. (D) The tolerance of circ_0005962 and corresponding linear RNA to
633 RNase was measured according to their mRNA levels. $*P < 0.05$.

634 **Figure 2. Circ_0005962 knockdown inhibited proliferation, autophagy, and**
635 **glycolysis but contributed to apoptosis in NSCLC cells.** (A) The diagram of
636 circ_0005962 knockdown. (B) The efficiency of circ_0005962 knockdown in
637 different transfection lines was detected by qRT-PCR. A549 and HCC827 cells were
638 transfected with si-circ_0005962#1 or si-NC. (C) Cell proliferation was assessed by
639 CCK-8 assay. (D) Cell apoptosis was executed by flow cytometry assay. (E and F)
640 The protein levels of Beclin 1 and LC3-II/LC3-I were quantified by western blot. (G
641 and H) The glycolysis progression was evaluated according to the level of glucose in
642 culture medium and lactate production. $*P < 0.05$.

643 **Figure 3. MiR-382-5p was a target of circ_0005962.** (A) The binding sites between
644 circ_0005962 and miR-382-5p were analyzed by the online tool CircInteractome. (B)
645 The relationship between circ_0005962 and miR-382-5p was verified by
646 dual-luciferase reporter assay. (C) The relationship between circ_0005962 and
647 miR-382-5p was further confirmed by RIP assay. (D) The expression of miR-382-5p
648 in A549 and HCC827 cells transfected with circ_0005962 or si-circ_0005962 was
649 detected by qRT-PCR. (E and F) The expression of miR-382-5p in NSCLC tissues and
650 cell lines was detected by qRT-PCR. (G) The correlation between circ_0005962 and
651 miR-382-5p was analyzed by Spearman's correlation coefficient. $*P < 0.05$.

652 **Figure 4. Inhibition of miR-382-5p reversed the role of circ_0005962 knockdown.**
653 A549 and HCC827 cells were introduced with si-circ_0005962#1, si-NC,
654 si-circ_0005962#1+anti-miR-382-5p or si-circ_0005962#1+anti-NC. (A) The
655 transfection efficiency was examined by qRT-PCR. (B) Cell proliferation was
656 assessed by CCK-8 assay. (C) Cell apoptosis was executed by flow cytometry assay.
657 (D and E) The protein levels of Beclin 1 and LC3-II/LC3-I were quantified by
658 western blot. (F and G) The glycolysis progression was evaluated according to the
659 levels of glucose in culture medium and lactate production. $*P < 0.05$.

660 **Figure 5. PDK4 was a target of miR-382-5p.** (A) The binding sites between PDK4 3'

UTR and miR-382-5p were analyzed by online tool starbase. (B) The relationship between PDK4 and miR-382-5p was verified by dual-luciferase reporter assay. (C) The relationship between PDK4 and miR-382-5p was further confirmed by RIP assay. (D) The expression of PDK4 at the protein level in A549 and HCC827 cells transfected with miR-382-5p or circ_0005962+miR-382-5p was detected by western blot. (E and F) The expression of PDK4 at mRNA and protein levels in NSCLC tissues was detected by qRT-PCR and western blot. (G) The protein level of PDK4 in NSCLC cell lines was detected by western blot. (H and I) The correlation between PDK4 and circ_0005962 or miR-382-5p was analyzed by Spearman's correlation coefficient. * $P < 0.05$.

Figure 6. PDK4 overexpression abrogated the role of miR-382-5p reintroduction.

A549 and HCC827 cells were transfected with miR-382-5p, NC, miR-382-5p+PDK4 or miR-382-5p+vector. (A) The transfection efficiency was checked according to the expression level of PDK4 using western blot. (B) Cell proliferation was assessed by CCK-8 assay. (C) Cell apoptosis was monitored by flow cytometry assay. (D and E) The protein levels of Beclin 1 and LC3-II/LC3-I were quantified by western blot. (F and G) The glycolysis progression was evaluated according to the level of glucose in culture medium and lactate production. * $P < 0.05$.

Figure 7. Circ_0005962 knockdown depleted tumor growth *in vivo*.

(A) The tumor volume was recorded once a week, lasting 5 weeks. (B) The tumor weight was measured after 5 weeks. (C) The expression of circ_0005962, miR-382-5p and PDK4 was examined in excised tumor tissues by qRT-PCR. (D) The protein levels of PDK4, C-caspase 3 and PCNA were quantified by western blot. * $P < 0.05$.

684

A **B** **C** **D**

A **B** **C** **D** **E** **F** **G** **H**

A **B** **C** **D** **E** **F** **G**

A **B** **C** **D** **E** **F** **G**

A

PDK4-wt 3' UTR

hsa-miR-382-5p

PDK4-mut 3' UTR

Target: 5' uugcagugaaggcauCAACUUa 3'

miRNA : 3' gcuuagguggugcuuGUUGAAg 5'

5' uugcagugaaggcauGUUGAAa 3'**B** **C** **D** **E** **F** **G** **H** **I**

A **B** **C** **D** **E** **F** **G**

A **B** **C** **D**