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Authors: Yizhi Liu, Xing Feng, Shuhong Kang, Feng Lv, Yunfeng Ni and Hua Wu

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CircRIP2 promotes NSCLC progression by sponging for miR-671-5p to regulate FOXM1 expression

Yizhi Liu¹, #, Xing Feng², #, Shuhong Kang³, #, Feng Lv³, #, Yunfeng Ni³, *, Hua Wu⁴, *

¹ Department of Medical Oncology, Shaanxi Provincial Cancer Hospital, Xi'an, 710068, Shaanxi, China
² Nursing Department, Shaanxi Provincial Cancer Hospital, Xi'an, 710068, Shaanxi, China
³ Department of Thoracic Surgery, Tangdu Hospital, Air Force Medical University, Xi'an 710038, PR China
⁴ Department of Respiratory Medicine, Shaanxi Provincial People's Hospital, Xi'an, 710068, Shaanxi, China

# Co-first authors
*Corresponding authors

Yunfeng Ni, Department of Thoracic Surgery, Tangdu Hospital, Air Force Medical University, Xi'an 710038, PR China, E-mail: niyunfng@fmmu.edu.cn;
Hua Wu, Department of Respiratory Medicine, Shaanxi Provincial People's Hospital, Xi'an, 710068, Shaanxi, China, E-mail: wuhspph@163.com

Abstract

Lot of attention had been paid to the role of circular RNAs (circRNAs) in carcinogenesis recently. However, knowledge about circRNAs in NSCLC development is far from satisfactory. In this study, we aimed to provide a novel insight into the circRIP2 in NSCLC development. We used NSCLC tissues, as well as cell lines to elucidate the expression and location of circRIP2 in NSCLC. We also established the circRIP2 overexpression cells A549-circRIP2 and repression cells HCC827-shcircRIP2 for further functional and mechanism studies. The protumorigenic role of circRIP2 was tested by using CCK-8, BrdU and transwell assays. The interaction between circRIP2 and miR-671-5p were validated by luciferase reporter assay, RIP assay, as well as RNA pull down assay. We showed circRIP2 is differentially expressed NSCLC, and acted as a predictor for overall survival (OS) and disease-free survival (DFS). CircRIP2 promoted NSCLC progression by acting as a miRNA sponge for miR-671-5p, thus facilitating its target gene FOXM1 expression. Targeting circRIP2 could be potentially beneficial for NSCLC patients in the future.

Key words: NSCLC, circRIP2, miR-671-5p, FOXM1.

Introduction

Lung cancer is known worldwide as one of the fatal malignancies with high risk of cancer-related death (Baker et al., 2017). Non-small cell lung cancer (NSCLC) is the major histological subtype of lung cancer, which accounts for over 80% of all patients diagnosed with lung cancer (Wang et al., 2020). It is agreed that chemotherapy and radiotherapy showed certain positive effects on NSCLC patients (Baldini et al., 2020; Corrao et al., 2020; Yossi et al., 2020). However, most of NSCLC patients still die due to the poor prognosis. More seriously, although thousands of molecules have been studied and have shown associations with NSCLC, the key regulators involved in the progression of NSCLC are still unclear. So, novel regulators are urgently needed to elucidate the potential mechanisms of the molecules in NSCLC.
Circular RNAs (circRNAs) are a class of multifunctional non-coding RNAs with closed-loop structures. Evidence has shown that circRNAs possess many biological functions, especially acting as a microRNA (miRNA) sponge to regulate its activity (Hansen et al., 2013; Memczak et al., 2013). Recently, many researchers are starting to pay attention to the role of circRNAs in cancer development. For example, it is reported that in gastric cancer, circLARP4 regulated LASTS1 expression by inhibiting miR-424-5p expression (Zhang et al., 2017). Previous studies also indicated that circITCH promoted bladder cancer by sponging for miR-17 and miR-224, thus regulating p21 and PTEN expression (Yang et al., 2018). It is documented that in pancreatic ductal adenocarcinoma, circZMYM2 sponged miR335-5p and promoted the expression of JMJD2C (An et al., 2018). Several circRNAs have been proposed to be important in the progression of lung cancer, such as circFOXM1, circABCB10 and circAKT3 (Xu et al., 2020; Yu et al., 2020; Zheng et al., 2020). However, the importance of certain circRNAs in NSCLC is still not fully understood.

In this study, we identified that circRIP2 is differentially expressed in NSCLC, and acted as a predictor for overall survival (OS) and disease-free survival (DFS). CircRIP2 promoted NSCLC progression by acting as a miRNA sponge for miR-671-5p, thus facilitating its target gene FOXM1 expression. Targeting circRIP2 could be potentially beneficial for NSCLC patients in the future.

Materials and methods

NSCLC specimen
NSCLC specimens were obtained during surgery, and the collection followed a standardized procedure. All the specimens were free of chemotherapy and radiotherapy, and the written-informed consents were signed by all the patients enrolled in this study. The histological identification was established by HE stains before experimental use. The human specimen study was proved by the Ethics Committee of Shaanxi Provincial People's Hospital. The expression of target molecules was evaluated by qRT-PCR, and the relationship between the target molecules and overall survival of NSCLC patients were calculated.

Cell lines and cell treatment
Immortalized lung epithelial cell line 16HBE and NSCLC cell lines A549, H460 and HCC827 used in this study were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Cells were cultured in DMEM medium with 10% fetal bovine serum and were tested for mycoplasma infection. CircRIP2 overexpressing cells A549-circRIP2 and suppression cells HCC827-shcirc RIP2 were generated for functional study of circRIP2 by using lentiviral transfection. Lentiviral infection in the HCC827 and A549 cells were conducted according to the protocol of the Genechem Recombinant Lentivirus Operation Manual provided by Genechem Corporation. Stable overexpressed circRIP2 cells were constructed with vector “plenty-ciR-GFP-T2A-puro”. The exploration of miR-671-5p functions was performed by transfecting miR-671-5p mimic or inhibitor into NSCLS cells. All experimental procedures were performed according to the manufacturer’s protocols. Sequences of miR-671-5p mimics and inhibitor were listed as follows: The miR-671-5p mimic: 5’-AGGAAGCCUGGAGGGCUGGAG-3’, mimic negative control: 5’-
RNA extraction and quantitative reverse transcription PCR (qRT-PCR)
The total RNA was isolated from NSCLC tissues and whole-cell lysate by using Trizol reagent. The gene expression level was tested by qRT-PCR assay. The reverse transcription of miRNA was established by Mir-X™ miRNA First-Strand Synthesis Kit (Takara, Japan), U6 was used as an internal reference. The reverse transcription of other types of total RNA was performed by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), GAPDH was used as an internal reference. qRT-PCR was performed by SYBR Premix Ex TaqII (TaKaRa, Japan). Primer sequences were listed as follows: miR-671-5p forward, 5’ - ACACTCCAGCTGGGAGGAAGCCCTGGAGGGG - 3’; miR-671-5p reverse, 5’ - CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGCCTCCAG - 3’; U6 forward, 5’ - CTGCTTCCGAGCACA - 3’; U6 reverse, 5’- AACGCTTCAGAATTTGCCTCTCGG - 3’; circRIP2 forward, 5’ - CACCATCAAGTTCGTTTGCT - 3’; circRIP2 reverse, 5’ - GGCTGGTAGTGGCAGTGATT - 3’; FOXM1 forward, 5’ - CGTCGGCCACTGATTCTC AAA - 3’; FOXM1 reverse, 5’ - GCCAGGGATCTCTTAGGTTC - 3’; GAPDH forward, 5’ - GGAGCGAGATCCCTCCAAAAT - 3’; GAPDH reverse, 5’-GGCTGTTGTCATACTTCTCATGGG - 3’.

Protein extraction and Western blot
The protein extraction was established by treating the cells with RIPA buffer and protease and phosphatase inhibitors, followed by centrifugation at 12000rpm for 15min. The supernatants were collected and tested with BCA assay kit (Beyotime, China). The protein level of target genes were evaluated by SDS-PAGE, and analyzed by Bio-Rad system. Antibodies used for Western blot are listed as follows: anti-GADPH (1:1000; ab8245; abcam); anti-FOXM1 (1:1000; ab207298; abcam); anti-PCNA (1:1000; ab92552; abcam).

RNA immunoprecipitation (RIP) assay
RIP assay was evaluated by Magna RIP™ RNA-binding protein immunoprecipitation kit (Millipore, USA) according to the manufacturer’s protocols. Briefly, cells were treated with equal volume of RIP lysis buffer after washing with PBS. Then the magnetic beads incubated with 5μg antibody against argonaute 2 (AGO2) for 30 min at room temperature (RT) to collect antibody-coated beads. Immunoprecipitation was performed by incubating cell lysate with the antibody-coated beads overnight at 4 °C. After washing with RIP wash buffer, the immunoprecipitates were treated with Proteinase K for digestion. Finally, the RNA was purified and the enrichment of RNAs with target proteins were analyzed by qRT-PCR.

Fluorescent in situ hybridization (FISH) assay
FISH probe for circRIP2 was synthesized by Gene pharm (Suzhou, China). FISH assay was evaluated by a FISH kit (Ribobio, China). A549 and HCC827 cells were fixed with 4% formaldehyde. The cells were incubated in a hybridization buffer mixed with the FISH probes after pepsin treatment and ethanol dehydration. Finally, 6-diamidino-2-phenylindole (DAPI) was
used for nuclear staining and the results were visualized by fluorescence microscope.

**RNA pull-down assay**
RNA pull-down assay was investigated by using Pierce Magnetic RNA-Protein Pull-Down Kit (ThermoFisher Scientific, USA). Then the biotin-labelled oligonucleotide probes specifically against circ- RIP2 (Ribobio; China), and streptavidin-coupled dynabeads (Invitrogen, USA) were used for capturing the circ- RIP2-miRNA complexes. Then the circ- RIP2-miRNA complexes were isolated and detected with RT-qPCR.

**Luciferase reporter assay**
Circ- RIP2 3' UTR containing the wild-type (WT) or mutated miR-1827 binding sites were generated and inserted into the pmirGLO vector. Then, miR-671-5p mimic or its control, circ- RIP2-WT vector or circ-RIP2-mutate vector were co-transfected into indicated NSCLC cells, and the luciferase activity was measured and analyzed.

**Cell proliferation and migration assays**
Cell proliferation assays were determined by CCK8 assay kit (Dojingdo Molecular Technologies, Japan), and BrdU assay kit (Millipore, USA). CCK8 assay was applied in 1*10^5 cells in triplicate. Cells were seeded into 96-well plates and incubated for 5 days. The cell viability was examined everyday using the CCK-8 solution and measured at 490 nm. BrdU assay was applied in 5*10^3 cells in triplicate. Cells were seeded into 96-well plates, and incubated for 24 hours. The cell proliferation rate was examined by adding the BrdU assay solution and measured at 490 nm. Cell migration assay was evaluated by transwell test in 8μm-size pore membrane Boyden chambers (Corning, USA). Transwell test was applied in 1*10^4 cells in triplicate. Cells were seeded into the upper chambers and cultured for 24h. The migration rates were examined and quantified by microscope.

**Statistical analysis**
Statistical analysis used in this study was analyzed by GraphPad Prism 7. Student's t-test and Pearson’s correlation were used as appropriate. Data are presented as mean ± SD. p-value (two-sided) less than 0.05 was considered statistically significant.

**Results**

**Characterization of circRIP2 in NSCLC patients and cell lines**
To elucidate the role of circRIP2 in NSCLC, we collected 50 pairs of NSCLC tissues and their adjacent tissues from surgery patients, and tested the expression of circRIP2 by qRT-PCR. The results indicated that overexpression of circRIP2 is a common phenomenon in NSCLC (Figure. 1A). The higher expression of circRIP2 is associated with tumor size, nodule metastasis and AJCC stage (Supplementary Table.1). Based on the follow-up information of the NSCLC patients, we found that higher circRIP2 expression indicated relatively poorer overall survival (OS) and disease-free survival (DFS) (Figure. 1B and 1C). Consistently, elevated levels of circRIP2 were found in NSCLC cell lines compared with lung epithelial cell line 16HBE (Figure. 1D). The localization of circRIP2 was mainly found in cytoplasm in HCC827 cells examined by FISH assay (Figure. 1E). These findings demonstrated the importance of circRIP2 in NSCLC.
CircRIP2 promotes cell growth and migration of NSCLC
Since circRIP2 may be important in the progression of NSCLC, we next elucidated the functions of circRIP2 by employing the lentivirus to generate circRIP2 overexpression cells A549-circRIP2 and repression cells HCC827-shcircRIP2 (Figure 2A). Analyzed by CCK-8 assay, we found that knockdown of circRIP2 resulted in inhibited NSCLC cell growth, while upregulation of circRIP2 was able to promote NSCLC cell growth (Figure. 2B). Consistently, the BrdU assay showed the same results in A549-circRIP2 and HCC827-shcircRIP2 cells (Figure. 2C). The migration ability was observed by transwell test, and the data indicated that knockdown of circRIP2 resulted in slowed NSCLC cell migration, while upregulation of circRIP2 could be able to accelerate NSCLC cell migration (Figure. 2D). In vivo experiments further validated the pro-tumorigenic role of circRIP2 in NSCLC. A549-circRIP2 cells showed larger tumor size, as well as more lung metastasis nodules compared with the corresponding control cells (Figure. 2E). These data supported that circRIP2 acted as a tumor promoter in NSCLC growth and migration.

CircRIP2 facilitated NSCLC progression by interacting with miR-671-5p
Since most of cytoplasmic circRNAs act as “miRNA sponges” in regulating carcinogenesis, and cytoplasm localization of circRIP2 was found in NSCLC cells, we speculate that circRIP2 exerts its tumor promoter role in NSCLC by interacting with certain miRNAs (Wu et al., 2018). Therefore, we analyzed certain miRNAs known to be regulated by circRIP2. Interestingly, we found that miR-671-5p may functioned as the target of circRIP2. Then, we analyzed the relation and interaction between miR-671-5p and circRIP2 in NSCLS tissues and cells. Data from NSCLC tissue samples showed miR-671-5p expression was often silenced in NSCLC tissues compared with adjacent controls (Figure. 3A). What’s more, miR-671-5p expression was correlated with circRIP2 expression (Figure. 3B). The dysregulation of miR-671-5p was further validated in A549-circRIP2 and HCC827-shcircRIP2 NSCLC cell lines, respectively (Figure. 3C). Luciferase reporter assay showed the luciferase activity of circRIP2-WT, but not circRIP2-mutant cells could mitigated when the cells co-transfected with miR-671-5p mimic (Figure. 3D). Along with the RNA pull-down and the RIP assay data (Figure. 3E and 3F), we confirmed that circRIP2 directly interacts with miR-671-5p.

CircRIP2 played an oncogenic role in NSCLC via miR-671-5p/FOXM1 axis
As for circRNAs acting as “miRNA sponges” in carcinogenesis by regulating its target gene expression, we further detected the target genes related with miR-671-5p. Based on luciferase reporter assay, we found that FOXM1 is a direct target of miR-671-5p (Figure. 4A). We also looked for the role of FOXM1 in NSCLC patients, and found that overexpression of FOXM1 was negatively related to the prognosis of NSCLC (Figure. 4B). Besides, FOXM1 expression level was positively correlated with circRIP2 levels (Figure. 4B). Consistently, the dysregulation of FOXM1 was further validated in A549-circRIP2 and HCC827-shcircRIP2 NSCLC cell lines, respectively (Figure. 4C). Furthermore, a miR-671-5p inhibitor was able to attenuate the effect of inhibition of circRIP2 (Figure. 4D-4F). These data further demonstrated the regulatory network of circRIP2/miR-671-5p/FOXM1 axis.
Discussion

In the current study, we identified that circRIP2 is differentially expressed in NSCLC patients and cell lines. The in-depth validation studies demonstrated that increased expression of circRIP2 is associated with OS and DFS of NSCLC patients. Loss/gain-of-function studies confirmed the critical roles of circRIP2 in NSCLC development by promoting cell growth and migration. Mechanistically, circRIP2 functioned as miR-671-5p sponge to regulate its gene FOXM1 expression. We speculate that targeting circRIP2 could be potentially beneficial for NSCLC patients in the future.

Many studies have attached importance to the role of circRNAs in cancer development (Liang et al., 2019). Previous studies had reported that multiple circRNAs are differentially expressed in NSCLC, and regulate cell growth, invasion, migration, as well as metastasis, such as circ-ACACA, circ-FOXM1 and circ-ABCB10 (Wu et al., 2020; Yu et al., 2020; Zheng et al., 2020). Because of their extremely stable circular structure, much emphasis were placed on their potential application as biomarkers. However, the detailed functions of circRNAs in NSCLC are still very elusive. We demonstrated that circRIP2 is highly expressed in NSCLC tissues and was able to act as a predictor of OS and DFS. Thus, circRIP2 serves as a tumor promoter at a clinical level. Latterly, we performed cellular studies, and demonstrated that circRIP2 gained a tumor promotion role in NSCLC proliferation. Since the results showed cytoplasm localization of circRIP2, we speculate that circRIP2 might exert its tumor promotive role in NSCLC by interacting with certain miRNAs. To test our hypothesis, we checked certain miRNAs known to be regulated by circRIP2 and found that miR-671-5p was able to function as the target of circRIP2 in NSCLC cells. MiR-671-5p is a well-known tumor suppression miRNA in many types of malignancies by inhibiting cell growth, invasion, migration and promoting cell apoptosis (Chen et al., 2019; Xin et al., 2019; Chi et al., 2020; Lin et al., 2020). MiR-671-5p has been identified as a novel asbestos-related molecule in lung cancer (Nymark et al., 2011). To further prove circRIP2 could interact with miR-671-5p in NSCLC cells, we evaluated the miR-671-5p expression level in NSCLC tissues and showed that miR-671-5p level was negatively correlated with circRIP2 expression. In addition, the downregulation of miR-671-5p was further validated in A549-circRIP2 and the upregulation of miR-671-5p HCC827-shcircRIP2 NSCLC cell lines, suggesting that circRIP2 may interact with miR-671-5p. Based on this hypothesis, we performed luciferase reporter assay and demonstrated that the luciferase activity of circRIP2-WT, but not circRIP2-mutant cells was mitigated when the cells co-transfected with miR-671-5p mimic. Similarly, the RNA pull-down and the RIP assay data further confirmed the that circRIP2 directly interacts with miR-671-5p. As for circRNAs usually carried out its function by sponging for miRNAs in carcinogenesis by regulating its target gene expression, we further detected the target gene known to be related with miR-671-5p. Using luciferase reporter assay, we found that FOXM1 is a direct target of miR-671-5p. Upregulation of FOXM1 is a hall mark of tumors, and FOXM1 was able to regulate tumor progression through so many signaling pathways (Borhani and Gartel, 2020). We also sought the role of FOXM1 in NSCLC patients, and found that overexpression of FOXM1 was negatively correlated to the prognosis of NSCLC and was positively correlated with circRIP2 levels. Consistently, a miR-671-5p inhibitor could attenuate the anti-proliferation and anti-migration effect of inhibition of circRIP2. These data further demonstrated the regulatory network of circRIP2/miR-671-5p/FOXM1 axis in NSCLC.

In conclusion, in this study we reported the significant function of circRIP2 in NSCLC
proliferation and migration. We also unveiled the potential mechanisms of circRIP2 in NSCLC progression by regulating circRIP2/miR-671-5p/FOXM1 axis. We speculate that targeting circRIP2 could be potentially beneficial for NSCLC patients in the near future.

Abbreviations
NSCLC, non-small cell lung cancer; circRNA, circular RNA; miRNA, microRNA; OS, overall survival; DFS, disease-free survival; qRT-PCR, quantitative reverse transcription PCR; AGO2, argonaute 2; RT, room temperature; DAPI, 6-diamidino-2-phenylindole; WT, wild-type.

Conflicts of Interest
The authors declare no competing interests.

Acknowledgments
We thank all patients who have participated in this study. We thank the members of our group for useful discussion of the work.

References
Figure legends

Figure 1. CircRIP2 was highly expressed in NSCLC, and negatively correlated with OS and DFS. (A). Determination of circRIP2 expression in NSCLC tissues and adjacent tissues by RT-qPCR. Data are mean ± SD, n=30; (B). The association between circRIP2 and OS by Kaplan-Meier curve; (C). The association between circRIP2 and DFS by Kaplan-Meier curve; (D). Determination of circRIP2 expression in NSCLC cell lines and lung epithelial cell line 16HBE by RT-qPCR. Data are mean ± SD. *p <0.05, **p <0.01; (E). Examination localization of circRIP2 by FISH assay. Scale bars, 10μm.

Figure 2. CircRIP2 promotes cell growth and migration of NSCLC. (A). Analysis of circRIP2 expression in A549 cells transfected with control vector or circRIP2 overexpression plasmid, or in HCC827 cells transfected with the shRNAs. Data are mean ± SD. **p <0.01, ***p <0.001; (B). Analysis the effect of circRIP2 on cell viability by CCK-8 assay. Data are mean ± SD. *p <0.05; (C). Analysis the effect of circRIP2 on cell proliferation by BrdU assay. Data are mean ± SD. **p <0.01; (D). Quantification of migration cells tested by transwell assay. Data are mean ± SD. *p <0.05; (E). Images of cell migration by transwell assay. Scale bars, 10μm; (F). Images of metastasis by in vivo assay. Scale bars, 20μm.

Figure 3. CircRIP2 exerts its tumor promoter role by interacting with miR-671-5p. (A). Detection miR-671-5p expression in NSCLC tissues and adjacent tissues by RT-qPCR. Data are mean ± SD, n=30; (B). Correlation of circRIP2 and miR-671-5p levels in NSCLC patients. n=50; (C). Detection miR-671-5p expression in A549-circRIP2 cells, HCC827-shcircRIP2 cells and their corresponding controls. Data are mean ± SD. *p <0.05; (D). The luciferase activity of Luc-circRIP2-wt or Luc-circRIP2-mutant in HCC827 cells co-transfected with miR-671-5p mimics. Data are mean ± SD. **p <0.01; (E). RNA pull down assay for the degree of circRIP2 and miR-671-5p with circRIP2 probe.
Data are mean ± SD. **p <0.01, ***p <0.001; (F). RIP assay for the degree of circRIP2 in NSCLC cells expressing Flag-AGO2 or Flag-GFP. Data are mean ± SD. **p <0.01, ***p <0.001.

**Figure 4. CircRIP2 played an oncogenic role in NSCLC via miR-671-5p/FOXM1 axis.** (A). The luciferase activity of Luc-FOXM1-wt or Luc- FOXM1-mutant in HCC827 cells co-transfected with miR-671-5p mimics. Data are mean ± SD. ***p <0.001; (B). Detection FOXM1 expression in NSCLC tissues and adjacent tissues by RT-qPCR. Data are mean ± SD, n=30; Correlation of circRIP2 and FOXM1 levels in NSCLC patients. n=30; (C). Detection FOXM1 expression in A549-circRIP2 cells, HCC827-shcircRIP2 cells and their corresponding controls. Data are mean ± SD. **p <0.01, ***p <0.001; (D and E). Detection the effect of inhibition of circRIP2 by co-transfected with miR-671-5p inhibitor. Data are mean ± SD. *p <0.05, **p <0.01, ***p <0.001; (F). Images of cell migration by transwell assay. Scale bars, 10μm.
A. Relative circRIP2 level in NSCLC tissues compared to adjacent tissues, with a significant difference indicated by $p<0.05$.

B. Survival analysis showing overall survival with circRIP2 high and low groups, with a significant difference indicated by $p=0.014$.

C. Disease-free survival analysis with circRIP2 high and low groups, with a significant difference indicated by $p=0.016$.

D. Bar graph comparing circRIP2 levels in different cell lines (16HBE, A549, H460, HCC827), with a significant difference indicated by $p=0.016$.

E. Immunofluorescence images showing DAPI staining (left) and merge of circRIP2 (right) with a bar scale.
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(A) Graph showing relative circRIP2 level in A549-vector and A549-circRIP2 compared to HCC827-control and HCC827-shcircRIP2.

(B) Graphs showing absorbance over days for A549-circRIP2 and A549-vector compared to HCC827-shcircRIP2 and HCC827-control.

(C) Bar charts showing absorbance and number of cells per field for A549-vector and A549-circRIP2 compared to HCC827-control and HCC827-shcircRIP2.

(D) Images showing A549-vector and HCC827-control.

(E) Images showing tumor number for A549-vector and A549-circRIP2 compared to HCC827-control.