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MiR-647 inhibits proliferation and improves apoptosis in cisplatin-treated non-small cell lung cancer via down-regulating *IGF2* expression

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ABSTRACT: The purpose of this study was to identify the correlation between *MiR-647* and *insulin like growth factor 2(IGF2)* in non-small cell lung cancer (NSCLC) and their effect on cell proliferation and apoptosis. In the study, A549 cell line was selected as NSCLC cell. The pcDNA3.1-CMV-*MiR-647*-EGFP and the pcDNA3.1-CMV-*IGF2*-mCherry were designed for the overexpression of *MiR-647* and *IGF2* respectively; while the pAAV-mCherry-shRNA-*MiR-647* and the pAAV-EGFP-shRNA-*IGF2* were respectively for *MiR-647* and *IGF2* knockdown in cisplatin-treated A549 cell line. Ki-67 and caspase-3 were performed to reflect cell proliferation and apoptosis in cisplatin-treated A549 cells. According to the results, *MiR-647* enhanced apoptosis and cell death of cisplatin-treated NSCLC cells *in vitro*;, and in the cells after cisplatin treatment and *MiR-647* overexpression, a significant decrease of *IGF2* expression was presented. Furthermore, by plasmid transfection in cisplatin-treated A549 cells, we found that *IGF2* silence and *MiR-647* overexpression could synergistically promote cell apoptosis and stop NSCLC cell growth. In conclusion, *MiR-647* and *IGF2* have opposite effect on NSCLC cell proliferation and apoptosis; that is, *MiR-647* can improve NSCLC cell apoptosis via down-regulating *IGF2* expression.

KEYWORDS: *MiR-647*, cell proliferation, apoptosis, non-small cell lung cancer, *IGF2*

INTRODUCTION

Lung cancer, especially non-small cell lung cancer (NSCLC), has been proved to be one of the leading causes for human death worldwide. NSCLC is a typical cell type of lung cancer, and pathologically accounts for 85% of all the lung cancer types [1]. Thanks for early diagnosis and early therapy of NSCLC via high-sensitive image, biopsy, and cytological examination, we now are able to apply chemotherapy drugs to treat NSCLC, thus avoiding surgery, postoperative complications, and bad adaptation of patients after surgery [2–4]. However, the 5-year or 10-year survival rate of NSCLC patients treated with chemotherapy drugs is still poor. Many NSCLC cannot be inhibited well because of unexpected proliferation and abnormal invasive pattern, which make physicians confuse and struggle [5]. As the growth and the apoptosis characteristics is closely related to NSCLC patient's prognosis after chemotherapy, more researches are required to explore the growth mechanism of NSCLC cells after chemotherapy.

MicroRNAs (miRNAs) are important non-coding small RNAs involved in many biological processes, especially gene expression inhibition. Such inhibition can be achieved by the binding of miRNAs to the 3′-untranslated region (UTR) of regulated mRNAs and, therefore, negatively regulating the expression level of target genes [6–8]. Many cancer-related researches have focused on various miRNAs as they are correlated with the growth and the apoptosis of multiple cancers by down-regulating target gene to a certain expression level [9–11]. Among these miRNAs, *MiR-647* has been confirmed to inhibit cancer growth and invasion and serve as a useful biomarker for prognosis prediction in many kinds of cancers including gastric cancer, ovarian cancer, as well as NSCLC [12, 13]. Overexpression of *MiR-647* has a positive correlation with a better prognosis in these cancers. Nonetheless, whether *MiR-647* still has any function in NSCLC cell treated with chemotherapy drugs is unknown. This question is important because if *MiR-647* has a similar inhibiting effect on cancer cells treated with chemotherapy drugs, *MiR-647* can be further used as a biomarker for detecting the prognosis of chemotherapy treated patients.

miRNAs usually regulate the expression of downstream target genes. Hence, genes regulated by *MiR-647* should be further detected for better understanding the mechanism of its function. According to existing studies, some genes have close relation with the function of *MiR-647*, such as *focal adhesion kinase* (*FAK*), *insulin like growth factor 2 (IGF2), nuclear factor I/X (NFIX), matrix metallopeptidase 12 (MMP12)*, and *TNF receptor associated factor 2 (TRAF2)* [13]. These genes have been reported in gastric cancer and NSCLC without treatment of chemotherapy drugs [14, 15]. Therefore, they were selected as candidates to explore their correlation with *MiR-647* in chemotherapy-treated NSCLC in the present study.

MATERIALS AND METHODS

NSCLC cell line culture and grouping

Typical NSCLC cell line, called A549 cell line (86012804) and derived from human lung carcinoma,

was purchased from Merck KGaA (Darmstadt, Germany) and maintained in Dulbecco's Modified Eagle Medium (DMEM/F12) (Merck KGaA) containing 10% fetal bovine serum (Thermo Fisher Scientific, Massachusetts, USA) in cell incubator at 37 °C under 5% CO_2. After 3 days of culture, the cells were used for subsequent experiments. As for grouping, the cultured cells were divided generally into two parts. The first part was used to observe the changes of cell number and cell proliferation after treatment of cisplatin (PHR1624, Sigma-Aldrich, MO, USA) or/and *MiR-647*. The cells were divided into three groups: untreated group, cisplatin group, and cisplatin+*MiR-647* overexpression group with three dishes per group. The second part was used to explore the correlation between *MiR-647* and *IGF2* in A549 cells and their effect on cell proliferation and apoptosis. The cultured cells were divided into four groups, namely, *MiR-647*+++ group (*MiR-647* overexpression), *sh-MiR-647* group (*MiR-647* silence), *MiR-647*+++/*IGF2*+++ group (*MiR-647* overexpression + *IGF2* overexpression), and *MiR-647*+++/*sh-IGF2* group (*MiR-647* overexpression + *IGF2* silence) with three dishes per group.

Plasmid construction and transfection

pcDNA3.1-CMV-*MiR-647*-EGFP and pcDNA3.1-CMV-*IGF2*-mCherry were designed for *MiR-647* and *IGF2* overexpression in cisplatin-treated A549 cell line, respectively, while pAAV-mCherry-shRNA-*MiR-647* and pAAV-EGFP-shRNA-*IGF2* were for *MiR-647* and *IGF2* silence, respectively. These plasmids were transfected into cultured A549 cells by Lipofectamine 2000 following the cell grouping in the previous section, and the treated cells were collected 30 h after transfection.

Candidate gene selection and amplification in A549 cells after *MiR-647* overexpression

To understand the mechanism of *MiR-647* in A549 cell proliferation and apoptosis, we selected some candidate genes after *MiR-647* overexpression in A549 cells through literature retrieval. Our selected candidates all had some pathological correlation with *MiR-647* in cancer growth and development, showing up-regulation or down-regulation in A549 cells after *MiR-647* treatment and resulting in invasion and metastasis of cancer cells. The selected candidate genes were *FAK*, *IGF2*, *NFIX*, *MMP12*, and *TRAF2*. Subsequently these candidate genes were amplified via qPCR after performing RNA extraction by RiboPure™ RNA Isolation Kit (AM1924, Thermo Fisher Scientific) and cDNA synthesis by TaqMan™ Advanced miRNA cDNA Synthesis Kit (A28007, Thermo Fisher Scientific). The primer information was shown in Table S1. Through the amplification, we evaluated their relative expression level in A549 cells with *MiR-647* overexpression, thus confirming the downstream targets which might be regulated by *MiR-647* to affect A549 cell growth and proliferation.

A549 cell proliferation evaluation

The proliferation ability of cultured A549 cells was evaluated with the following specific steps. We first imaged the cultured cell under differential interference microscope and observed the cell status including morphology, tangling, survival status etc. Then the proliferative cells were labeled with immunofluorescence staining of Ki-67 with anti-Ki-67 (ab16667, Abcam, Cambridge, USA) and Goat polyclonal Secondary Antibody to Rabbit IgG - H&L (Alexa Fluor® 488, Cambridge, MA, USA) *in vitro*. Both microscopic images and Ki-67 staining images were quantified via averaging the positive cells from 10 high power fields (40×).

A549 cell apoptosis evaluation

Next we evaluated the apoptosis status of A549 cells. Specifically, anti-caspase3 (ab32150, Abcam) immunofluorescence staining was performed to label the apoptosis cells with Goat polyclonal Secondary Antibody to Rabbit IgG - H&L (Alexa Fluor® 594). After that, the positive cells from 10 high power fields (40×) were averaged for quantitative evaluation.

RESULTS

Proliferation and apoptosis of A549 cells treated with cisplatin

Cisplatin treated A549 cells presented a significant decrease of cell number, as shown in the differential interference microscope image, the Ki-67 staining, and the quantitative statistics (Fig. 1A-B). Such decrease illustrated that cisplatin was effective in NSCLC cell *in vitro*. On the contrary, caspase-3-labeled images showed more apoptotic cells in the cisplatin reated group than the untreated group, reflecting bad proliferation of cisplatin-treated NSCLC cells (Fig. 1C). Hence, cisplatin was able to inhibit cell growth and proliferation of NSCLC cell *in vitro*, and this drug could be applied for our further experiments.

Proliferation and apoptosis of cisplatin treated-A549 cells with or without *MiR-647* overexpression

After exposure to cisplatin, the A549 cells were transfected with plasmids to overexpress *MiR-647* in the cells, and cell proliferation and apoptosis abilities were detected again. The results demonstrated that the cisplatin-treated NSCLC cells became sparser after overexpressing *MiR-647* and had less Ki-67 labeling cells than the non-treated cells (Fig. 2A-B). Besides, these A549 cells with *MiR-647* overexpression showed more apoptosis and cell death (Fig. 2C). Collectively, *MiR-647* was able to enhance NSCLC cell apoptosis and cell death *in vitro* after cisplatin treatment.

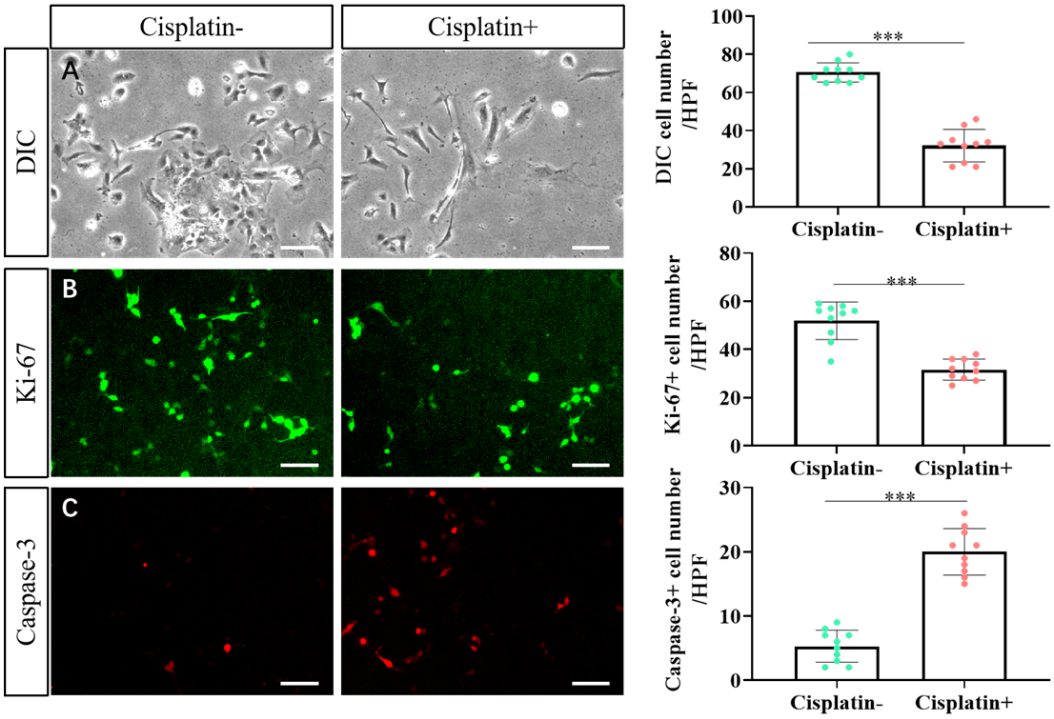

Fig. 1 Proliferation and apoptosis of A549 cells treated and untreated with cisplatin, and average number of positive cells in each group. DIC image, (A); Ki-67 labeling, (B); caspase-3 labeling, (C). *** $p < 0.001$. Scale bar: 50 μm. DIC, differential interference microscope.

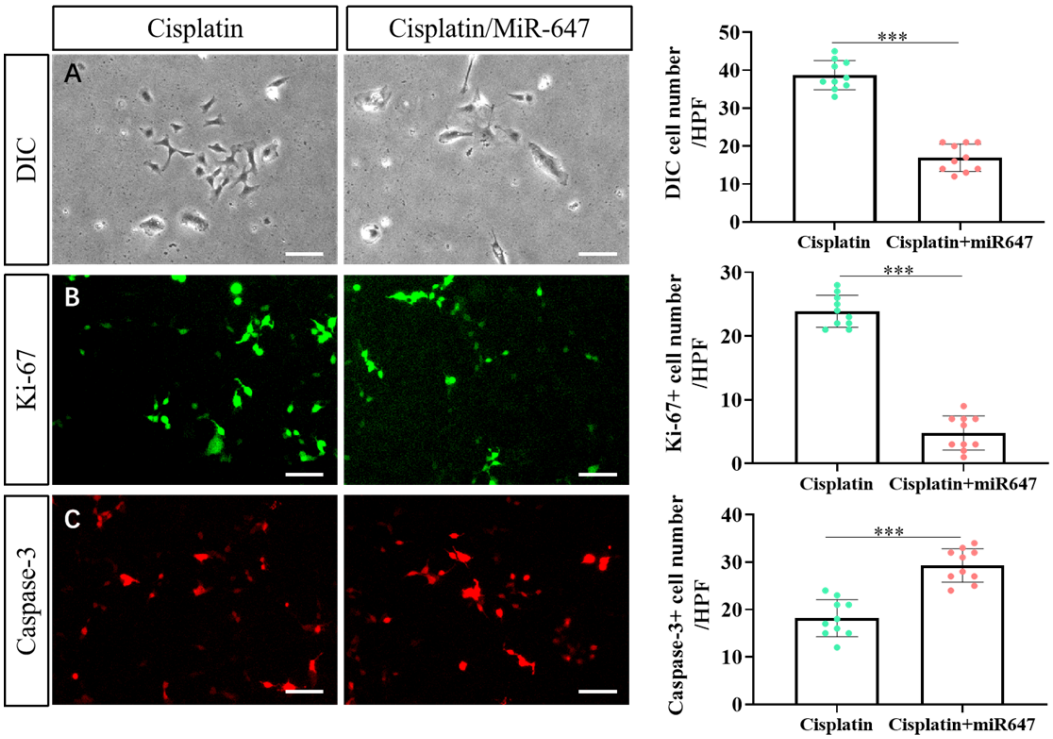

Fig. 2 Proliferation and apoptosis of cisplatin-treated A549 cells with or without *MiR-647* overexpression and average number of positive cells in each group. DIC image, (A); Ki-67 labeling, (B); caspase-3 labeling, (C). *** $p < 0.001$. Scale bar: 50 μm. DIC, differential interference microscope.

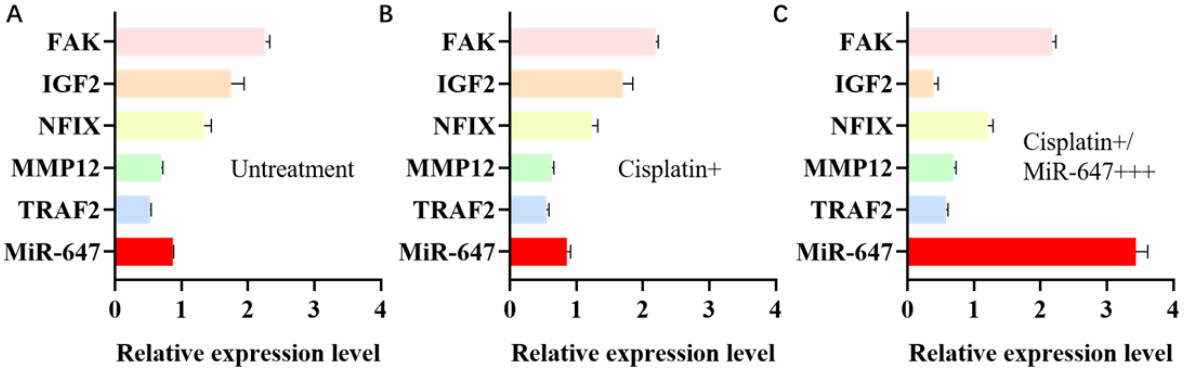

Fig. 3 Amplification of *MiR-647*-regulated candidate genes in A549 cell with *MiR-647* overexpression. Unteated A549 cells, (A); cisplatin-treated A549 cells, (B); cisplatin-treated A549 cells with *MiR-647* overexpression, (C).

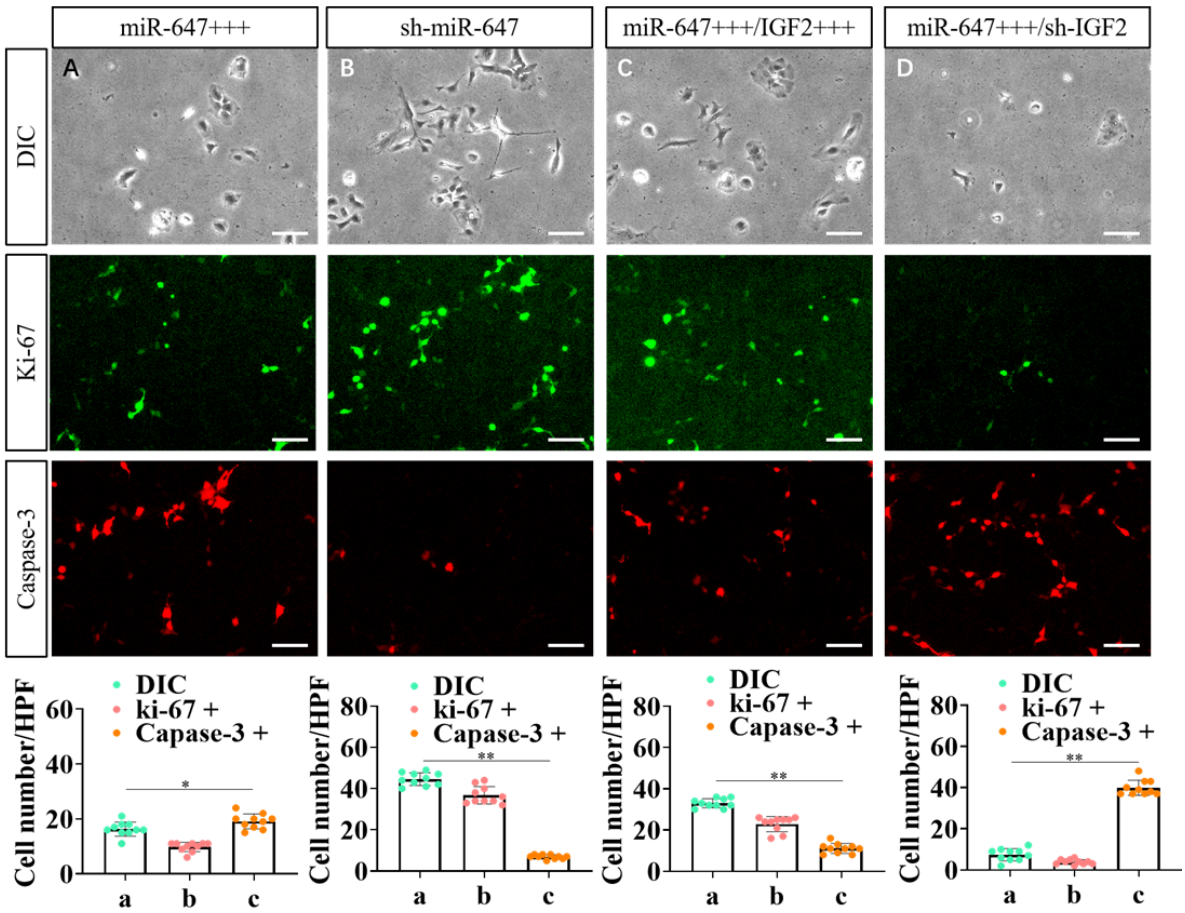

Fig. 4 Correlation between *MiR-647* and *IGF2* in cisplatin-treated A549 cells and their effect on cell proliferation and apoptosis and their quantitative statistics. DIC image, Ki-67 labeling, and caspase-3 labeling of cisplatin-treated A549 cells with MiR-647 overexpression, (A); cisplatin-treated A549 cells with *MiR-647* knockdown, (B); cisplatin-treated A549 cells with *MiR-647* overexpression and *IGF2* overpexression, (C); and cisplatin-treated A549 cells with *MiR-647* overexpression and *IGF2* knockdown, (D), $^{*}p < 0.05$, $^{**}p < 0.01$. Scale bar: 50 μm. DIC, differential interference microscope.

MiR-647-regulated candidate gene amplification in A549 cell with *MiR-647* overexpression

There were no significant changes in the expression levels of the candidate genes in both the untreated and the cisplatin groups. (Fig. 3A-B). However, after overexpressing *MiR-647* (red bar in Fig. 3C) in cisplatin-treated A549 cells, *IGF2* expression (orange bar in Fig. 3C) exhibited a significant decrease, while little changes were observed in the other four. According to this test, we detected *IGF2* as the downstream target gene, which was possibly regulated by *MiR-647* directly to affect the proliferation and apoptosis in cisplatin-treated NSCLC cells *in vitro*.

Correlation between *MiR-647* and *IGF2* and their effect on proliferation and apoptosis of cisplatin-treated A549 cells

In cisplatin-treated A549 cells, *MiR-647* overexpression could inhibit cell proliferation and improve cell apoptosis (Fig. 4A), while *MiR-647* knock down had the total opposite phenotype (Fig. 4B). However, after overexpressing *IGF2* in cisplatin-treated A549 cell with *MiR-647* overexpression, NSCLC cell had more growth trend and less cell death (Fig. 4C), suggesting the increase of *IGF2* expression caused the reduction in the inhibitory effect of *MiR-647* on NSCLC cell proliferation. On the contrary, *IGF2* knockdown could synergistically enhance cell apoptosis and stop NSCLC cell growth with *MiR-647* overexpression (Fig. 4D). In conclusion, *MiR-647* and *IGF2* had opposite effects on NSCLC cell proliferation and apoptosis, i.e., *MiR-647* improved NSCLC cell apoptosis via down-regulating *IGF2* expression.

DISCUSSION

MiR-647 has been regarded as a biomarker for some cancers including gastric cancer, prostate cancer, and ovarian cancer because of its tight correlation with recurrence, invasion, and metastases of these tumors [7]. In NSCLC, *MiR-647* could induce cell apoptosis and inhibit cell proliferation despite few relative reports [16, 17]. Our study focused on the function of *MiR-647* in cisplatin-treated NSCLC cells. We aimed to find a predictive biomarker for NSCLC patients treated with chemotherapy in order to provide better therapy strategies to them.

MiR-647 was proved to have not only the capacity to inhibit NSCLC cell growth and invasion in cell experiments but also a positive correlation with good clinical prognosis. Additionally, it showed positive effect on cell apoptosis in cisplatin treated-NSCLC cells. With overexpressing *MiR-647*, apoptosis and cell death presented in cultured NSCLC cells increased, indicating *MiR-647* could enhance NSCLC cell apoptosis and cell death in cisplatin-treated cells. This result is of great clinical significance because it predicts that we may further use *MiR-647* as an effective prognostic biomarker for patients after chemotherapy.

We further studied the mechanism of *MiR-647* in maintaining its function in NSCLC cell apoptosis and narrowed down to a gene called *IGF2*. A marked decrease of *IGF2* expression was identified after overexpressing *MiR-647* while the other candidates had very small changes. As a result, we confirmed that *MiR-647* could improve NSCLC cell apoptosis via down-regulating *IGF2* expression. *IGF2*, a kind of peptide associated with mitosis, can be expressed widely in the body. This gene has a tight relation with cell proliferation and differentiation especially during embryonic stage. *IGF2* had been found to improve embryonic development; it presents in a decrease level after birth and maintains a low serum level in adults [18–20]. Growing number of reports demonstrated that a high serum level of *IGF2* often occurs in various cancers; such as liver cancer, breast cancer, colon carcinoma, and ovarian cancer, and has a negative correlation with patients' clinical prognosis [21, 22]. It has also been reported that *IGF2* could up-regulate tumor proliferation and cancer development, indicating its contribution to bad prognosis [23]. Our study clarified IGF2 as a downstream target of *MiR-647*, which reasonably explained why *MiR-647* could inhibit NSCLC cell growth.

CONCLUSION

In this study, we illustrated the correlation between *MiR-647* and *IGF2* in cisplatin-treated NSCLC cells. We also found that *MiR-647* could improve NSCLC cell apoptosis via down-regulating *IGF2* expression *in vitro*. This result had great clinical significance for *MiR-647* being a potentially effective prognostic biomarker for patients after chemotherapy.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2022.099.

REFERENCES

1. Fang L, Cai J, Chen B, Wu S, Li R, Xu X, Yang Y, Guan H, et al (2015) Aberrantly expressed mir-582-3p maintains lung cancer stem cell-like traits by activating wnt/β-catenin signalling. *Nat Commun* **6**, 8640–8654.
2. Chang A (2011) Chemotherapy, chemoresistance and the changing treatment landscape for nsclc. *Lung Cancer* **71**, 3–10.
3. Ge Y, Yan D, Deng H, Chen W, An G (2015) Novel molecular regulators of tumor necrosis factor-related apoptosis-inducing ligand (trail)-induced apoptosis in nsclc cells. *Clin Lab* **61**, 1855–1863.
4. Gudala S, Khan U, Kanungo N, Bandaru S, Hussain T, Parihar M, Nayarisseri A, Mundluru HP (2015) Identification and pharmacological analysis of high efficacy small molecule inhibitors of egf-egfr interactions in clinical treatment of non-small cell lung carcinoma: a computational approach. *Asian Pac J Cancer Prev* **16**, 8191–8196.

5. Stefano A, Russo G, Ippolito M, Cosentino S, Murè G, Baldari S, Sabini MG, Sardina D, et al (2016) Evaluation of erlotinib treatment response in non-small cell lung cancer using metabolic and anatomic criteria. *Q J Nucl Med Mol Imaging* **60**, 264–273.
6. Farazi TA, Hoell JI, Morozov P, Tuschl T (2013) Micrornas in human cancer. In: Schmitz U, Wolkenhauer O, Vera J (eds) *MicroRNA Cancer Regulation. Advances in Experimental Medicine and Biology*, vol 774, Springer, Dordrecht, pp 1–20.
7. Rawlings-Goss RA, Campbell MC, Tishkoff SA (2014) Global population-specific variation in mirna associated with cancer risk and clinical biomarkers. *BMC Med Genomics* **7**, 53–66.
8. Chen M, Zhao F, Wang Z, Lang H, Zhang K, Zhang T, Wang R, Shi P, et al (2020) Low-expression of microRNA-203a in mid-gestational human fetal keratinocytes contributes to cutaneous scarless wound healing by targeting Tenascin-C. *ScienceAsia* **46**, 133–141.
9. Park JH, Shin C (2014) Microrna-directed cleavage of targets: mechanism and experimental approaches. *BMB Rep* **47**, 417–423.
10. Wang C-C, Huang K-T, Chen K-D, Hsu L-W, Lin C-C, Chiu K-W (2021) Hepatic mir-122 expression correlated with il-28b genetic polymorphisms in hepatocellular carcinoma patients with living donor liver transplantation. *ScienceAsia* **47**, 578–584.
11. Graves P, Zeng Y (2012) Biogenesis of mammalian microrna: a global view. *Genomics Proteomics Bioinformatics* **10**, 239–245.
12. Kim YW, Kim EY, Jeon D, Liu JL, Kim HS, Choi JW, Ahn WS (2014) Differential microrna expression signatures and cell type-specific association with taxol resistance in ovarian cancer cells. *Drug Des Devel Ther* **8**, 293–314.
13. Cao W, Wei W, Zhan Z, Xie D, Xie Y, Xiao Q (2017) Role of *MiR-647* in human gastric cancer suppression. *Oncol Rep* **37**, 1401–1411.
14. Heng YH, Zhou B, Harris L, Harvey T, Smith A, Horne E, Martynoga B, Andersen J, et al (2015) *NFIX* regulates proliferation and migration within the murine svz neurogenic niche. *Cereb Cortex* **25**, 3758–3778.
15. Zhang YS, Chen T, Cai YJ, Dong J, Bai F, Gao X, Tian L, Duan N, et al (2018) Microrna-647 promotes the therapeutic effectiveness of argon-helium cryoablation and inhibits cell proliferation through targeting *TRAF2* via the nf-κb signaling pathway in non-small cell lung cancer. *Onco Targets Ther* **11**, 6777–6784.
16. Yang B, Jing C, Wang J, Guo X, Chen Y, Xu R, Peng L, Liu J, et al (2014) Identification of micrornas associated with lymphangiogenesis in human gastric cancer. *Clin Transl Oncol* **16**, 374–379.
17. Bell JL, Haak AJ, Wade SM, Sun Y, Neubig RR, Larsen SD (2013) Design and synthesis of tag-free photoprobes for the identification of the molecular target for ccg-1423, a novel inhibitor of the rho/mkl1/srf signaling pathway. *Beilstein J Org Chem* **9**, 966–973.
18. Xu WW, Li B, Guan XY, Chung SK, Wang Y, Yip YL, Law SY, Chan KT, et al (2017) Cancer cell-secreted *IGF2* instigates fibroblasts and bone marrow-derived vascular progenitor cells to promote cancer progression. *Nat Commun* **8**, 14399.
19. Yu Y, Fu L, Wang S, Jin Y, Han S, Chu P, Lu J, Guo Y, et al (2017) Investigation of *IGF2*, igfbp2 and p63 proteins in rhabdomyosarcoma tumors. *Growth Horm IGF Res* **33**, 17–22.
20. Barroca V, Lewandowski D, Jaracz-Ros A, Hardouin SN (2017) Paternal insulin-like growth factor 2 (*IGF2*) regulates stem cell activity during adulthood. *EBioMedicine* **15**, 150–162.
21. Raynaud-Simon A (2003) Levels of plasma insulin-like growth factor i (igf i), igf ii, igf binding proteins, type 1 igf receptor and growth hormone binding protein in community-dwelling elderly subjects with no malnutrition and no inflammation. *J Nutr Health Aging* **7**, 267–273.
22. Liu L, Greenberg S, Russell SM, Nicoll CS (1989) Effects of insulin-like growth factors i and ii on growth and differentiation of transplanted rat embryos and fetal tissues. *Endocrinology* **124**, 3077–3082.
23. Kim SY, Toretsky JA, Scher D, Helman LJ (2009) The role of igf-1r in pediatric malignancies. *Oncologist* **14**, 83–91.

Appendix A. Supplementary data

Table S1 Primer information for qPCR.

Gene	Primer ($5' \rightarrow 3'$)
MiR-647	F: GUGGCUGCACUCACUUCCUUC
	R: CTCAACTGGTGTCGTGGA
FAK	F: GGATTCGGTCACACTGGCTACTACGGAG
	R: CATATCAACCCCACACATCTGGCGCA
IGF2	F: GAGCCAGCTGCCCCA
	R: CGGAGGGGCTCAG
NFIX	F: GTCTAAACTTTCACTTTCAC
	R: AGGCCGGAGGAGCCGAGCCGG
MMP12	F: TGCAGGCCACTGCTTCTGGAGCTC
	R: AAGTTTCTTCTAATACTGCTCC
TRAF2	F: GAAACCAGCTTCCCTCGGAGGCT
	R: TTCCCCTGGGGACAGCACCA
β-actin	F: CTACGTCGCCCTGGACTTCGAGC
	R: GATGGAGCCGCCGATCCACACGG
U6	F: TGCGGGTGCTCGCTTCGGCAGC
	R: CCAGTGCAGGGTCCGAGGT