Biochemistry and Biophysics Reports 28 (2021) 101122

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Article

The high expression of miR-31 in lung adenocarcinoma inhibits the malignancy of lung adenocarcinoma tumor stem cells

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ARTICLE INFO

Keywords:
MiR-31
LUAD
CSCs
Tumorigenesis
Metastasis

ABSTRACT

Therapies for lung adenocarcinoma (LUAD) are mainly limited by drug resistance, metastasis or recurrence related to cancer stem cells (CSCs) with high proliferation and self-renewing. This research validated that miR-31 was over-expressed in LUAD by the analysis of generous clinical samples data. And the results of clinical data analysis showed that high expression of miR-31 was more common in patients with worse prognosis. The genes differentially expressed in LUAD tissues compared with normal tissues and A549CD133$^+$ cells (LUAD CSCs) compared with A549 cells were separately screened from Gene Expression Profiling Interactive Analysis and GEO datasets. The target genes that may play a role in the regulation of lung adenocarcinoma was screened by comparison between the differential genes and the target genes of miR-31. The functional enrichment analysis of GO Biological Processes showed that the expression of target genes related to cell proliferation was increased, while the expression of target genes related to cell invasion and metastasis was decreased in LUAD tissues and A549CD133$^+$ cells. The results suggested that miR-31 may have a significant inhibitory effect on the differentiation, invasion, metastasis and adhesion of LUAD CSCs, which was verified *in vivo* and *in vitro* experiments. Knock down of miR-31 accelerated xenograft tumor growth and liver metastasis *in vivo*. Likewise, the carcinogenicity, invasion and metastasis of A549CD133$^+$ CSCs were promoted after miR-31 knockdown. The study validated that miR-31 was up regulated in LUAD and its expression may affect the survival time of patients with lung adenocarcinoma, which indicated that miR-31 may have potential value for diagnosis and prognosis of LUAD. However, the inhibitory effect of miR-31 on tumorigenesis, invasion and metastasis of lung adenocarcinoma CSCs suggested its complexity in the regulation of lung adenocarcinoma, which may be related to its extensive regulation of various target genes.

1. Introduction

Lung cancer attracts extensive attention due to its leading incidence and mortality of all cancers, and lung adenocarcinoma is a subtype with the highest incidence [1–4]. Past decades have seen the inspiring development of treatment. The survival rate of LUAD patients is still not high. It is of great significance to find sensitive and reliable diagnostic markers for LUAD patients as it is difficult to diagnose LUAD early. Meanwhile, LUAD patients are prone to relapse and metastasis after radiotherapy and chemotherapy. Cancer stem cells (CSCs) have been found to play a decisive role in the process of drug resistance [5]. Increasing evidence indicates CSCs present therapeutic resistance phenotype which promote tumor recurrence and metastasis [6–9]. Therefore, looking for effective inhibitors of CSCs may be of great significance in the treatment of LUAD.

CSCs, considered as the initiation of tumors, are a bunch of cells exhibiting stem cell properties in various malignant tumors [10]. To date, a number of preclinical and clinical studies of CSCs targeting agents support it as a promising therapy [11,12]. Decades of scientific discovery have demonstrated that most of signal pathways related to

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https://doi.org/10.1016/j.bbrep.2021.101122
Received 19 July 2021; Received in revised form 26 August 2021; Accepted 27 August 2021
Available online 30 August 2021

stem cell development and differentiation are dysfunctional in human malignancies, which suggests that CSCs play a key role in a variety of malignancies [13–16].

MicroRNA is an endogenous non-coding RNA widely located in cancer-related areas and reading frame, which plays a vital role in the occurrence and development of human cancer [17]. Compared with normal tissue, miRNA express differentially in malignant tumors, which represents the activated status and has predictive and diagnostic value [18]. The abnormal expression of miRNA, usually caused by genetic modification, is very important for the occurrence and development of human malignant tumors, because they both play a role of tumor suppressor and oncogene [19,20]. The potential carcinogenic of miRNA is mainly targeting mRNAs to degrade 3′- UTR of several genes related to normal cell homeostasis, such as cell proliferation, cell cycle regulation, differentiation, development, migration, angiogenesis and apoptosis [21–25].

As a micro-regulator, miR-31 shows complex effect on different cancers and becomes a potential therapeutic target. Previous studies have indicated miR-31 could inhibit the metastasis of colorectal cancer and breast cancer while it participated in the occurrence of lung cancer by down-regulating LATS2 and RRR2R2A [26–29]. This contradictory turning point may lie on the expression or mutation of target genes regulated by miR-31, that is, whether miR-31 is proto-cancer or anti-cancer may depend on the selection of all targeted genes. Given that the regulation of miR-31 on multiple target genes, the physiological regulation of miR-31 may be different due to different expression of target genes in different tissues, cells and pathological states. The expression of target genes should be fully considered when exploring the role of miR-31. But most studies have not focused on this.

In this study, we evaluated the clinical diagnostic and prognostic value of miR-31. The selectivity of miR-31 target genes in physiological regulation of LUAD and LUAD CSCs was also speculated. This study demonstrated the regulatory effect of miR-31 on lung adenocarcinoma stem cells, providing a new research for the regulation of miRNA in tumor and suggesting a potential strategy for the diagnosis, treatment and prognosis of lung adenocarcinoma with miR-31.

2. Materials and methods

2.1. Data collection

The level of miR-31 in lung adenocarcinoma was evaluated by microarray results searched in the GEO database (http://www.ncbi.nlm.nih.gov/geo/). The keywords were (MicroRNA OR “Micro RNA” OR miRNA) AND (Lung OR pulmonary) AND (cancer OR tumor OR neoplasm OR malignancy OR carcinoma OR adenocarcinoma OR AC OR SCC OR NSCLC). The entry type was filtered by “series”, and the “*Homo sapiens*” was chosen as organism. Data that accord with the following criteria can be included in the analysis: (1) patients were diagnosed with lung adenocarcinoma; (2) cancerous and noncancerous tissues were included; (3) the control and malignant groups included at least three samples in each dataset; (4) and the expression of miR-31 were detected and quantified.

2.2. Statistical analysis and comprehensive meta-analysis

After log2-transformed, the expression of miR-31 profiling information was used to calculate the number, mean and standard deviation for each control and experimental group with IBM SPSS Statistics V20.0 software. Stata16.0 software was used for meta-analysis of data. Standardized mean difference was used as the effect value of continuous variables. Cohen’s method was used for standardization method. Heterogeneity index I2 was used to evaluate the heterogeneity of expression level.

2.3. MiR-31 expression data from the cancer genome atlas

YM500v2 (http://ngs.ym.edu.tw/ym500v2/index.php) was used to obtain the detailed information of miR-31 in LUAD and noncancerous samples. The differences in the miR-31 expression in the LUAD samples and the normal controls were calculated. OncoLnc (http://www.oncolnc.org/) was used to link TCGA survival data of lung adenocarcinoma samples to miR-31 expression levels.

2.4. Comprehensive analysis of all targets of miR-31 in LUAD tissues and LUAD CSCs

MiRWALK2.0 was mined to forecast the genes targeted by miR-31. The high-expressed and down-expressed genes in LUAD were acquired through Gene Expression Profiling Interactive Analysis (GEPIA). Gene expression data of A549 and A549CD133$^+$ cells (GSE35603, GPL570) were obtained from GEO datasets and analyzed in Gene Cloud of Biotechnology Information website (GCBI, https://www.gcbi.com.cn/gclib/html/index).

2.5. Functional analysis for promising target genes

The overlapping genes screened by cross comparison were used in the functional analysis. Main signaling pathways involved in these genes were obtained by GO Biological Processes analysis in Metascape (http://metascape.org/gp/).

2.6. Cell lines and cell culture

The human lung adenocarcinoma cell lines A549 and SV40-transformed embryonic kidney cell line HEKA-293 were obtained from the Cell Resource Center of Institute of Basic Medical Sciences, Peking Union Medical College. A549CD133$^+$ cells were CD133 positive cells in A549 cells screened by flow cytometry and cultured in the medium containing DMEM-GLU medium: F-12 medium (1:1) with 1 × B27 NeuroMix (PAA), 2 ng/mL EGF, 2 ng/mL bFGF, 100U/mL penicillin/streptomycin and 2 ML-glutamine for further screening and maintaining cell stemness. The tumorigenicity and metastatic ability of A549CD133$^+$ cells and A549 cells were detected as our previous studies [30].

2.7. Vector construction

The sequence of miR-31 sponge element (GTTAAC AGCTATGCCTCGTCTTGCCT CCGG AGCTATGCCTCGTCTTGCCTCCGG AGCTATGCCTCGTCTTGCCT CCGG AGCTATGCCTCGTCTTGCCTCCGG AGCTATGCCTCGTCTTGCCT CCGG AGCTATGCCTCGTCTTGCCTCCGG AGCTATGCCTCGTCTTGCCT CTCGAG) was inserted into the *Hpa*I and *Xho*I sites in pLL3.7 vector (Shanghai CPG Biotech Co., Ltd.) and the A549CD133+-NC cells and A549CD133$^+$-KD cells were screened by flow cytometry after transfection with the lentivirus as described previously [30]. .

2.8. Xenograft model

BALB/c athymic (NU$^+$/NU$^+$) mice (female, 3–4 weeks old) were randomly divided into two groups. The right sub-axillaries of the mice were subcutaneously injected with equal amount of A549CD133$^+$-NC cells and A549CD133$^+$-KD cells (1.0×10^5) suspended in 100 μL growth medium/Matrigel (1:1, BD) (n = 5). We observed whether there were hard tumor nodules in the axilla every day, and the length (L) and width (W) of the tumors were measured by calipers to calculate the tumor volumes ($V = L \times W^2/2$). Finally, we took the time when we touched the hard tumor mass and the tumor volume reached or exceeded 100 mm^3 as the time for successful tumor extraction. All animal experiments had been comply with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

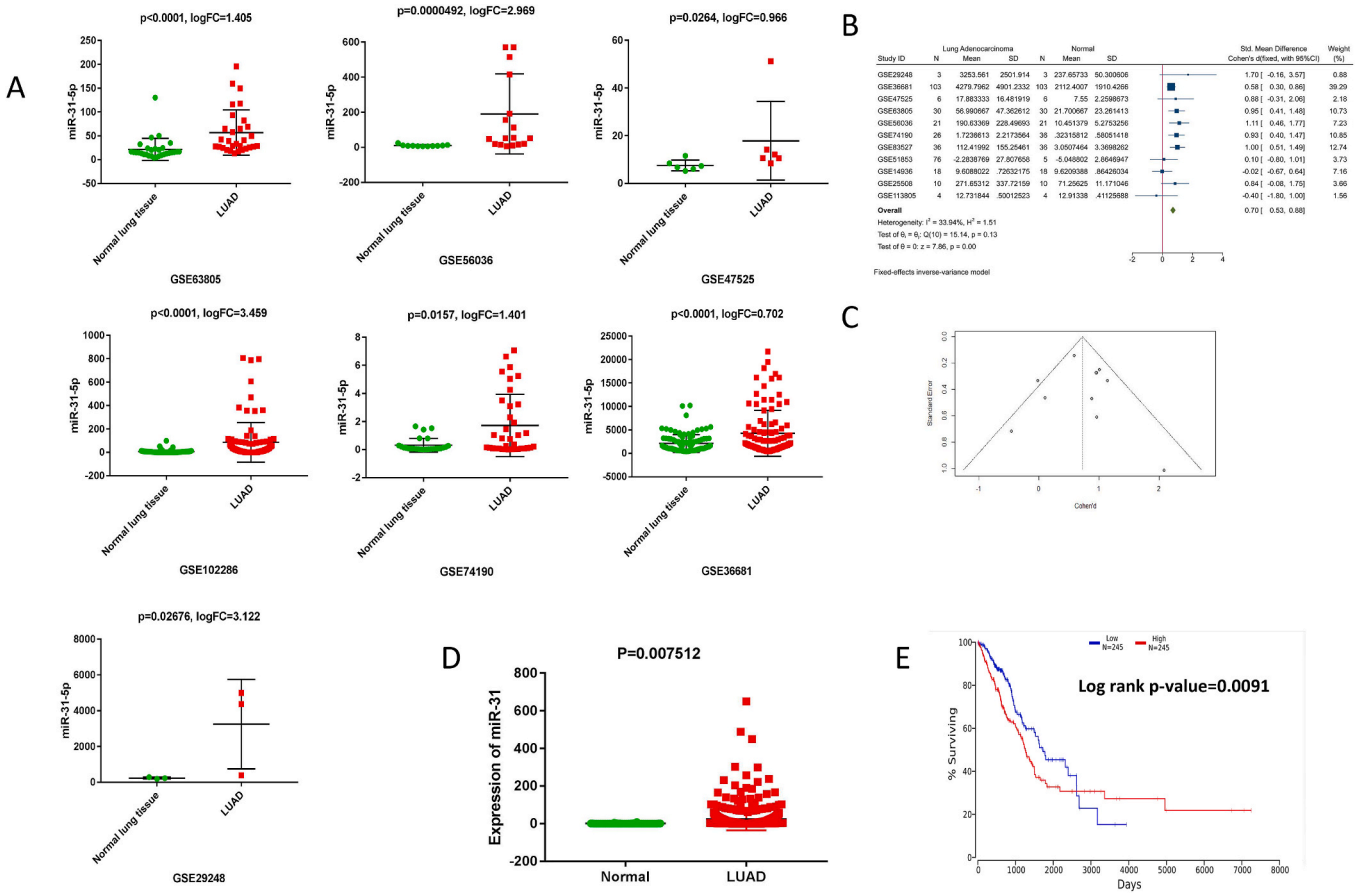


Fig. 1. The expression of microRNA-31 in LUAD according to the clinical data. Up-regulation of microRNA-31 in the LUAD tissues, based on Gene Expression Omnibus datasets (A). (B) The Forest plot of GEO chips. The overall standard mean difference Cohen's d (fixed, with 95% CI) was 0.70 (0.53, 0.86) with heterogeneity ($I^2 = 33.94\%$, p = 0.00); (C) The funnel plot was applied to evaluate the publication bias of GEO chips and the funnel plot was symmetrical. Egger's test was used to analyze the publication bias (t = 2.61, P > 0.05). Up-regulation of microRNA-31 in LUAD tissue samples and normal tissue samples from TCGA was analyzed by the OMCD (p = 0.007512, D). Kaplan–Meier curves for microRNA-31 in lung adenocarcinoma based on TCGA. (p = 0.0091, E).

2.9. H-E stain and immunochemistry

Liver tissues from sacrificed mice of were fixed in 4% paraformaldehyde, paraffin-embed, sectioned and stained with hematoxylin and eosin. For immunofluorescence staining, paraffin sections were incubated with primary antibodies at 4 °C overnight, then with secondary antibodies at room temperature for 1 h following by counterstaining with DAPI in mounting media.

2.10. Transwell assay

Cells were digested into single cells and added to the upper chamber without serum while 600 μL DMEM medium with 10% fetal bovine serum was added to the lower chamber. The surfaces of the Transwell chambers were stained by 0.1% crystal violet, 8–10 field view records were selected randomly under the microscope and IPP software was used to count stained cells.

2.11. Colony formation assay

The A549CD133$^+$-NC and A549CD133$^+$-KD cells at a density of 5000 cells/well were seeded into the 6-well plates with agarose gel medium or serum-free medium. The cells were incubated for 2 weeks then fixed with methanol and scanned under the microscope.

2.12. Statistical analysis

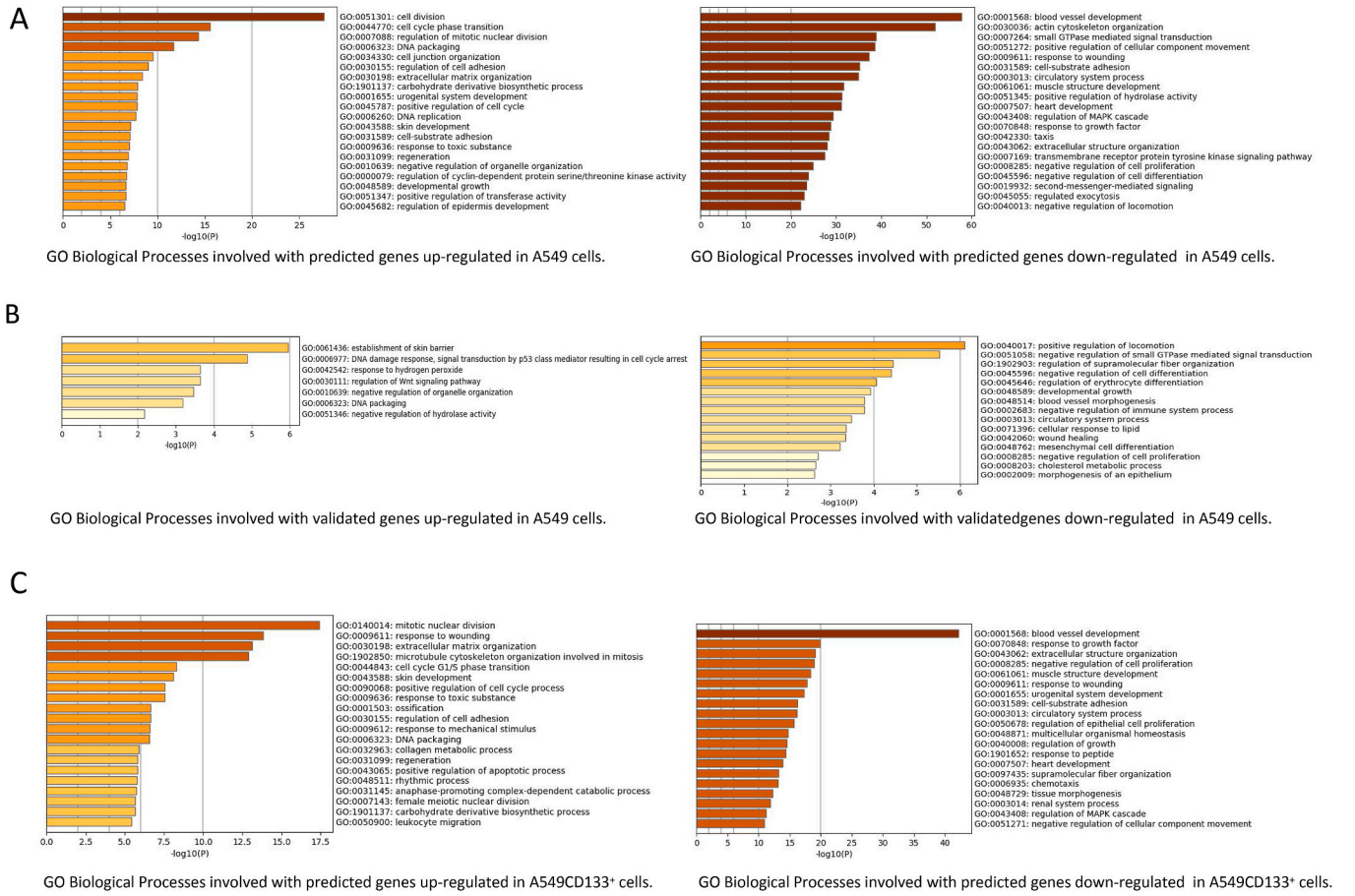
The three independent experiments results expressed as mean ± standard deviation (SD) and were analyzed by SPSS 18.0. Statistical analysis carried out by 2-tailed Student t-test or one-way ANOVA as requested and P value < 0.05 was considered significant (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.0001$).

3. Results

3.1. The expression and clinical value of microRNA-31 in lung adenocarcinoma according to the gene expression analysis of microarrays

A total of 11 GEO databases during 2011–2018 are included for analysis. Among them, the expression of miR-31 in four microarrays (GSE51853, GSE25508, GSE14936 and GSE113805) did not show significant difference between normal tissues and lung adenocarcinoma tissues, while it increased significantly in tumor tissues in the other 7 sets of microarray data (GSE63805, GSE56036, GSE47525, GSE29248, GSE102286, GSE74190 and GSE36681), as Fig. 1A shown.

Subsequently, we tested whether there was heterogeneity in the data of these studies, as shown in Fig. 1B and C. The results indicated that heterogeneity existed but was not significant ($I^2 = 33.94$). Therefore, the fixed effect model was selected for Meta-analysis to calculate the combined research effect. The results showed that the expression of miR-31 in lung adenocarcinoma was significantly higher than that in control group with 95% CI of (0.53, 0.88) (SMD = 0.70: z = 7.86, P = 0.00).



GO Biological Processes involved with predicted genes up-regulated in A549 cells.

GO Biological Processes involved with predicted genes down-regulated in A549 cells.

GO Biological Processes involved with validated genes up-regulated in A549 cells.

GO Biological Processes involved with validatedgenes down-regulated in A549 cells.

GO Biological Processes involved with predicted genes up-regulated in A549CD133^{+} cells.

GO Biological Processes involved with predicted genes down-regulated in A549CD133^{+} cells.

Fig. 2. The main signaling pathways involved in predicted genes with differential expression between normal tissues and LUAD tissues according to GEPIA database by GO Biological Processes analysis in Metascape (A). The main signaling pathways involved in validated genes with differential expression between normal tissues and LUAD tissues according to GEPIA database by GO Biological Processes analysis in Metascape (B). The main signaling pathways involved in predicted target genes differentially expressed in A549CD133^{+} cells compared with A549 cells by GO Biological Processes analysis in Metascape (C).

Egger's test was used to analyze the publication bias. The results showed that $t = 2.61$, $P > 0.05$ in Egger's tests, and the funnel plot was symmetrical. In sum, the results indicate no significant bias for the studies.

The expression of miR-31 in 458 LUAD tissue samples and 46 normal tissue samples from the Cancer Genome Atlas (TCGA) was analyzed by the OncomiR Cancer Database (OMCD) to verify the above results (Fig. 1D). MiR-31 was also significantly increased in lung adenocarcinoma compared with the control group ($p = 0.007512$, Ratio = 27.86). The results analyzed by the Oncolnc online database (Fig. 1E) showed that LUAD patients who exhibited higher expression of miR-31 might have worse outcomes early. However, it should be noted that the trend was opposite in the later stage. Based on the above results, we wonder whether the abnormal expression of miR-31 was one of the cause of malignant aggravation of LUAD, and whether miR-31 can promote the occurrence and metastasis of lung cancer. Therefore, we summarized and analyzed the pathways of miR-31, according to the expression of its target genes.

3.2. *The analysis of target genes and signal pathways regulated by miR-31*

There were 15947 target genes of miR-31 predicted in the miRWalk 2.0 database. Subsequently, 1111 up-regulated genes and 3134 down-regulated genes were obtained from GEPIA database. There were 2019 genes down-regulated, but 684 genes were up-regulated. The main signaling pathways involved in these genes were obtained by GO Biological Processes Analysis (Fig. 2A Left). For the up-regulated genes, the summary pathways were Cell division, Cell cycle phase transition, Regulation of mitotic nuclear division and so on. In brief, up-regulated target genes seemed to be more related to cell proliferation and tumor growth. For the down-regulated genes, the summary pathways were Blood vessel development, Actin cytoskeleton organization, small GTPase mediated signal transduction (Fig. 2A Right). In brief, down-regulated genes seemed to be more related to invasion and metastasis of LUAD.

In order to verify the above analysis results, we obtained the target genes of miR-31 verified in previous studies from miRTarBase and TarBase V7 databasets. Subsequently, we also carried out enrichment analysis. Altogether 704 genes targeted by miR-31 verified in previous studies were summarized, including 47 down-regulated and 23 up-regulated genes. Genes enriched in cell cycle and division were mostly up-regulated (Fig. 2B Left), while genes enriched in cell motility were mostly down-regulated (Fig. 2B Right). This is consistent with the above analysis.

We screened genes differentially expressed in A549CD133^{+} cells compared with A549 cells, including 245 up-regulated genes and 437 down-regulated genes (FC > 2). By cross comparing with all predicted target genes of miR-31, we found that the up-regulated target genes were also more related to cell proliferation such as mitosis (Fig. 2C Left), and the target genes with down-regulation were also more related to cell migration such as angiogenesis, cell movement and adhesion (Fig. 2C Right).

According to the above experimental results, the expression of target genes related to cell proliferation was consistent with that of miR-31; while the expression of target genes related to cell invasion and

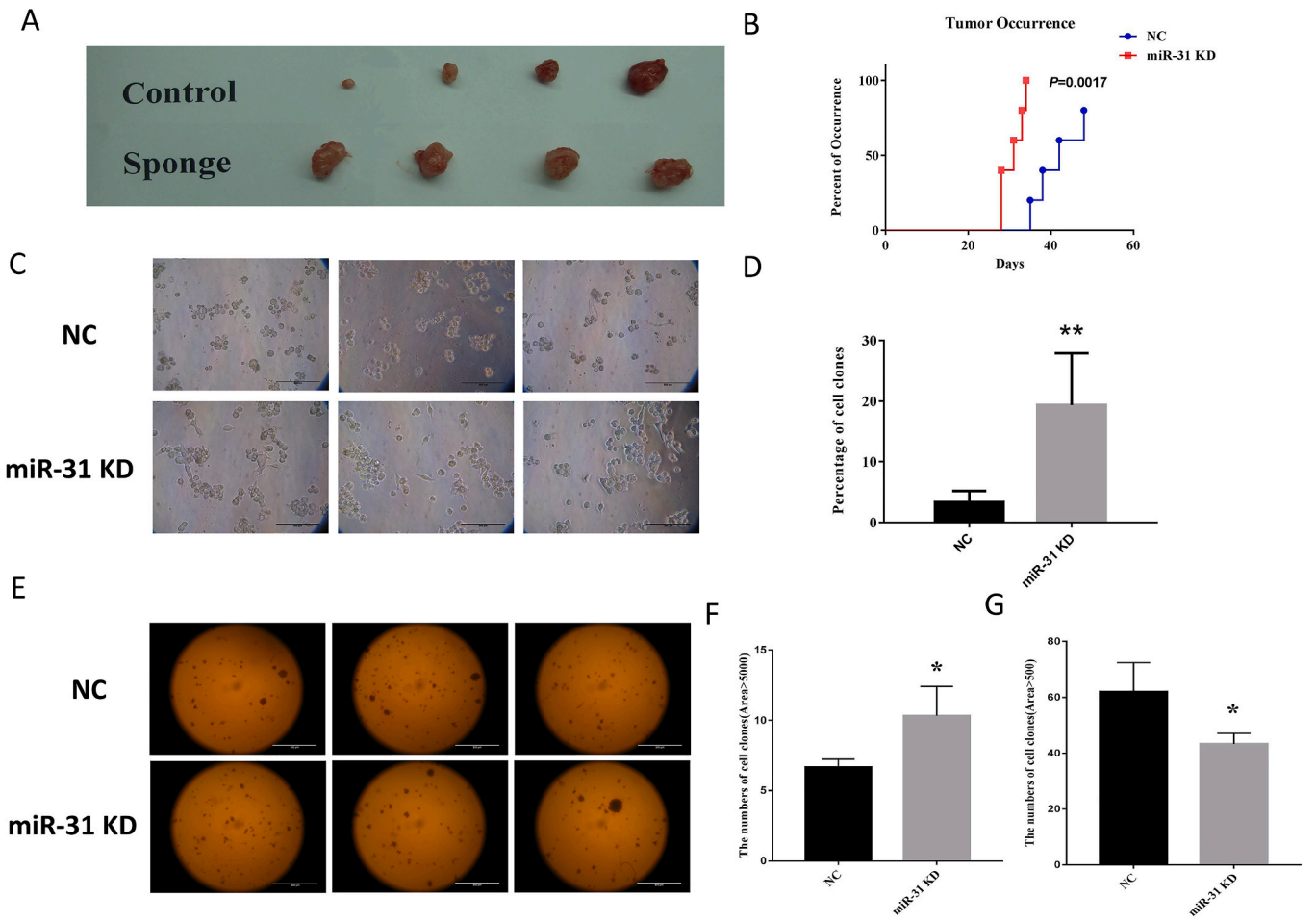


Fig. 3. Effects of miR-31 knockdown on tumorigenesis of A549CD133$^+$ cells *in vivo* and *in vitro*. (A) Tumor tissues of NC mice and miR-31 KD mice. (B) Kaplan–Meier curves for tumorigenesis after the inoculation of A549CD133$^+$ cells. Demonstration (C) and statistical results (D) of the aggregated clone balls formed in the serum-free medium. Demonstration (E) and statistical results (F, G) of the clone sphere formation in the agar. Data are mean ± SD for n = 4 per group. In all the figures, *P < 0.05; **P < 0.01, ***P < 0.005 compared with their controls, Scale bars = 500 μm.

metastasis was opposite to that of miR-31. Consequently, we speculated that miR-31 may have a significant inhibitory effect on the differentiation, invasion, metastasis and adhesion of lung adenocarcinoma stem cells, which was verified by the following experiments.

3.3. Regulation of miR-31 on tumorigenesis and metastasis of LUAD CSCs

We selected A549CD133$^+$ cells with higher expression of miR-31 and higher malignancy to verify the role of miR-31 in the regulation of carcinogenesis, invasion and metastasis of LUAD. The A549CD133$^+$ cells with miR-31 KD showed a faster rate of tumorigenesis compared with the control cells still with a high level of miR-31 ($p < 0.005$, Fig. 3A and Fig. 3B). These results suggested that the up-regulation of miR-31 in LUAD CSCs may play a certain restrictive role in tumorigenesis.

The results of the aggregated clone balls assays showed that the KD cells were more likely to assemble to form clones, while the NC cells were more likely to exist alone ($p < 0.005$, Fig. 3C and D). We then carried out experiments on clone sphere formation (Fig. 3E). The results showed that the percentage of cell clones of A549CD133$^+$-KD group was higher than the control group (Area>5000) (Fig. 3F). However, further analysis of the clone area showed that the clones number of A549CD133$^+$-KD group was lower than the control group when we limited the clone area>500 (Fig. 3G). Together, these results indicated that the cells became more concentrated and cloned more quickly, while less clones formed after miR-31 was down-regulated.

Moreover, we found that the livers of the mice inoculated with A549CD133$^+$ cells appeared white nodules similar to tumors, and the suspected inoculated tumor cells showed the potential of liver metastasis. We checked the liver tissues of the mice to identify the effect of miR-31 knockdown on the number of these nodules induced by CSCs and confirmed the origin of tumor cells in these nodules. Compared with NC cells, miR-31 KD cells showed stronger liver metastasis by observing the livers of experimental mice (Fig. 4A). As the H-E staining result showed, miR-31 KD mice presented multiple liver metastasis (Fig. 4B), which originated from the GFP-labeled A549CD133$^+$ cells by immunofluorescence tracing (Fig. 4C). Similarly, the transwell assays were carried out to evaluate the invasion and metastasis ability of A549CD133$^+$ cells with or without matrix. The number of miR-31 KD cells passing through membrane was about 36% higher than that of NC cells (Fig. 4D), while the number of miR-31 KD cells passing through basement membrane was about three times higher than NC cells (Fig. 4E). These results indicated that knockdown of miR-31 promotes the migration, invasion and metastasis of CSCs.

4. Discussion

Although many studies have found that the expression of miR-31 in lung cancer tissues is increased, the expression of miR-31 in lung adenocarcinoma tissues, lung adenocarcinoma tumor stem cells and its correlation with the development of lung adenocarcinoma have not been fully elucidated. In this study, we confirmed that the expression of miR-31 in LUAD. And the high expression of miR-31 was found closely

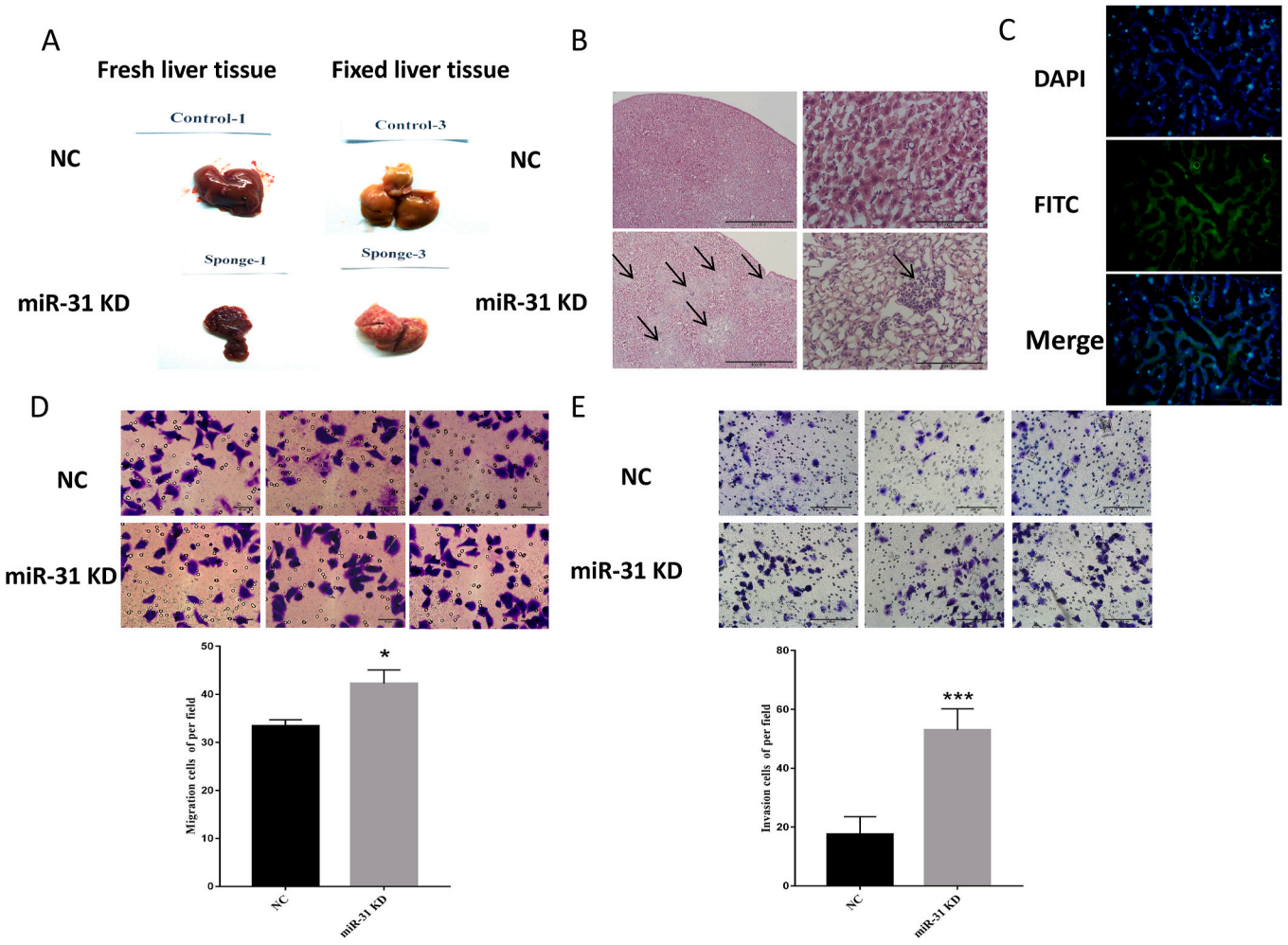


Fig. 4. Effects of miR-31 knockdown on invasion and metastasis of A549CD133$^+$ cells *in vivo* and *in vitro*. (A) Liver tissues of NC mice and miR-31 KD mice. (B) The pictures of liver tissue sections after HE staining, Scale bars = 500 μm (Left) and 100 μm (Right). (C) Representative frozen section images of liver tissues. A549CD133$^+$ cells with e-GFP (green), DAPI (blue) and merged image. Scale bars = 100 μm. Transwell assays were used to assay the migration (D, Scale bars = 100 μm) and invasion (E, Scale bars = 200 μm) of NC cells and miR-31 KD cells. Data are mean ± SD for n = 4 per group. In all the figures, *P < 0.05; **P < 0.01, ***P < 0.005 compared with their controls. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

related to the prognosis of LUAD. All these results showed that the high level of miR-31 may be one of the indicators for the advanced progress and the poor prognosis of lung adenocarcinoma. MiR-31 has been reported in many previous studies, but it is not consistent. It has been reported to have both cancer promoting and cancer suppressive effects [31]. It is well known that the specific regulatory role of microRNA depends on its target genes regulated. MicroRNAs inhibit the expression of specific genes mainly via complementary binding with the 3′-UTR end or CDS region of their target gene mRNAs to degrade mRNAs or inhibit the translation of mRNAs. This mechanism enables miRNAs to target multiple genes and be regulated by multiple genes. Therefore, the effect of microRNA on specific tissues is the product of its comprehensive regulation of all target genes. Many of the target genes regulated by miR-31 were effective molecules involved in tumorigenesis and development, including both proto-oncogene and tumor-suppressor gene in different tissues. Therefore, the regulatory effect of miR-31 on LUAD was not necessarily promoted, but may depend on its selective regulation of its target genes. Different from other researches which focus only on a single or several target genes, we analyzed the expression of all predicted and validated target genes of miR-31. Combined with the invasive advantages of CSCs, it is further suggested that the high expression of miR-31 may be closely related to the invasion and metastasis of LUAD.

In our previous study, we screened A549CD133$^+$ cells with stronger proliferation and metastasis potential by CSCs marker CD133 [32]. Most studies have shown that metastatic cells expressed CSC phenotype, and CSCs also showed high metastatic potential [33]. The CD133$^+$ LUAD cell group showed a high degree of metastasis [34]. This study indicated that high level of miR-31 was conducive to tumor aggregation and adhesion, which was consistent with our hypothesis based on the analysis of miR-31's target genes. Namely, miR-31 can inhibit the tumorigenesis, invasion and metastasis of lung adenocarcinoma cells and their cancer stem cells by down-regulating a variety of target genes involved in cell adhesion, invasion and metastasis.

It is worth noting that our results seem to be different from some results of miR-31 as a carcinogen and promoting the occurrence of lung cancer in the past [34–36]. We speculate that its selective regulation of target genes may be affected by many factors. On the one hand, the affinity between miRNA and target gene is an important factor. At three different levels of miR-17-92 (TKO, WT and TG), the target genes showed different sensitivity to miRNA inhibition, and only a small number of target genes were actually inhibited by a given concentration of miRNA. Only a small number of miRNA binding sites were occupied by miRNA molecules in a given time [37]. On the other hand, the expression level difference of the target gene may also affect the effect of miRNAs. The conserved human miRNAs selectively target genes with

moderate expression level, but ignore high expression target genes which were coexpressed with the miRNAs [38]. We speculated that many cell division related genes in LUAD cells may be highly expressed and co-expressed with miR-31. To sum up, the expression of miRNA may be affected by many factors such as the type of diseases, tissues, etc., and its regulation on target genes is also selective, which is easily affected by diseases, co-expression genes, mutation and so on. The role of each microRNA in each disease is discussed with full and detailed analysis and verification.

Declaration of competing interest

All authors declare that they have no actual or potential conflicts of interest.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (Grant No. 81903935) and by the 203-Fundamental Research Funds for the Central Universities (2020-JYB–2013-003).

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