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MiR-101-3p targets KPNA2 to inhibit the progression of lung squamous cell carcinoma cell lines

Liangliang Dong[1], Hanliang Jiang[1], Ting Qiu[1], Yiming Xu[1], Enguo Chen[1], Aihua Huang[2] and Kejing Ying[1]

[1]Department of Pulmonary and Critical Care Medicine and [2]Department of Pathology, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang Province, PR China

Summary. We herein discuss the impacts of miR-101-3p on the tumorigenesis-related cell behaviors in lung squamous cell carcinoma (LUSC) by repressing KPNA2. TCGA database was utilized to measure miR-101-3p and KPNA2 levels in LUSC tissues and cells. The interaction of miR-101-3p and KPNA2-3'UTR was determined by dual luciferase assay. Western blot evaluated the protein level of KPNA2. MiR-101-3p was under-expressed in LUSC cells while KPNA2 was overexpressed. Western blot confirmed the impact of KPNA2 expression on cancer cell progression. The negative regulatory impact of miR-101-3p on KPNA2 was also verified. *In vitro* cell function assays revealed the suppressing effect of high miR-101-3p expression on cell invasion, migration and viability, as well as its promoting effect on apoptosis. Up-regulated miR-101-3p weakened the promoting effect of overexpressed KPNA2 on LUSC malignant progression. To conclude, miR-101-3p repressed viability, invasion, and migration, and facilitated cell apoptosis in LUSC by suppressing KPNA2.

Key words: Lung squamous cell carcinoma, miR-101-3p, KPNA2, Proliferation, Migration, Invasion, Apoptosis

Introduction

Lung cancer is considered a fatal disease in the world (Jemal et al., 2011), and 85% of cases are non-small cell lung cancer (NSCLC) (Xu et al., 2018), with lung squamous cell carcinoma (LUSC) being a common subtype comprising more than 30% of NSCLC (Zappa and Mousa, 2016). Due to the late diagnosis of LUSC, complicated pathogenic factors and unclear biological mechanisms, the 5-year survival of patients is unsatisfactory (Kenfield et al., 2008). The current therapeutic strategies including immune therapy for LUSC patients are much limited, as far less has been revealed on molecular mechanisms of LUSC development (Anichini et al., 2020). Therefore, much more basic research on this topic is urgently needed.

In recent years, there has been an application of gene expression characteristics in LUSC diagnosis and treatment, laying a foundation for prognostic improvement and treatment research for different individuals in the future (Raponi et al., 2006). MicroRNAs (miRNAs) are small non-coding RNAs that can play vital regulatory roles by targeting mRNAs for cleavage or translational inhibition (Bartel, 2004). Evidence has exhibited dysregulated miRNAs in many types of malignancies such as colon cancer (Bandres et al., 2006), breast cancer (Xiao et al., 2019), gastric cancer (Wu et al., 2019) and NSCLC (Du et al., 2018). In LUSC, several miRNAs are identified to be abnormally expressed. For instance, miR-218 is significantly lowly expressed in LUSC cells, with cell invasion and migration suppressed by targeting TPD52 (Kumamoto et al., 2016). MiR-558 level in tumor samples is significantly lower than in non-tumor samples, and is related to tumor staging and lymph node infiltration (Qian et al., 2016). MiR-206 is prominently downregulated in LUSC tissue, inhibiting the progression of cancer cells by suppressing MET and EGFR (Mataki et al., 2015). These all indicate dysregulated miRNAs in the development of many diseases, including LUSC, but more work is required to find out the functional role of miR-101-3p in LUSC.

Nuclear globulin α2, Karyopherin α2 (KPNA2, also importin α1), a part of the Karyopherin inhibitor family, consists of 529 amino acids and is about 58 kDa (Radu et al., 1995; Weis et al., 1996). KPNA2 and importin-β can synergistically mediate target proteins, and complete a large number of nuclear transfers through the nuclear pore complex under the guidance of nuclear localization signals. Many studies correlate KPNA2 to malignant tumors. As reported by Dahl et al. (2006), upregulated

Corresponding Author: Kejing Ying, Department of Pulmonary and Critical Care Medicine, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, No. 3 Eastern Qingchun Road, Hangzhou, Zhejiang Province, 310016, PR China. e-mail: ykjsrrsh@zju.edu.cn
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KPNA2 is coupled to high recurrence and poor prognosis of breast cancer patients. There are also reports of elevated KPNA2 expression in other malignant tumors such as endometrial cancer, hepatocellular carcinoma, and prostate cancer (Grupp et al., 2014; Ikenberg et al., 2014; Yang et al., 2017). Studies also found that KPNA2 also promotes tumor progression and formation via cell apoptosis, proliferation, immune response, and differentiation. For instance, KPNA2 facilitates cell proliferation and tumorigenicity of epithelial ovarian cancer by overexpressing c-Myc and down-regulating FOXO3a (Huang et al., 2013). NF-κB/p65 signaling pathway activation by KPNA2 enhances malignant behavior of melanoma cells (Yang et al., 2020). As an inhibitory protein of nuclear export, KPNA2 can also make the subcellular localization of key DDR proteins abnormal, leading to poor prognosis of LUSC (Alshareeda et al., 2015). Moreover, KPNA2 knockout can reduce migratory and proliferative capabilities, with overexpressed KPNA2 increasing the malignant features of liver cancer cells (Guo et al., 2019). However, the exact mechanism of KPNA2 underlying LUSC development is unclear and remains to be explored.

We herein explored that miR-101-3p level was prominently reduced in LUSC tissue and cells, and overexpressed miR-101-3p significantly repressed tumor migration and proliferation *in vitro*. Further, miR-101-3p targeted KPNA2 mRNA and manipulated KPNA2 level. In addition, we obtained through the rescue experiment that miR-101-3p repressed viability, invasion, and migration, and promoted cell apoptosis by repressing KPNA2 level. Our findings revealed that miR-101-3p might be a novel biomarker for LUSC treatment.

Materials and methods

Bioinformatics approach

MiRNA (adjacent tissue samples: 45; tumor samples: 473) and mRNA (adjacent normal tissue samples: 49; tumor samples: 497) expression data in LUSC were sourced from TCGA database (https://portal.gdc.cancer.gov/). Differential expression analysis and survival analysis of target miRNA were introduced. R package edgeR processed the differential expression analysis of mRNAs (|logFC|>2, FDR<0.05). Four databases, miRDB (http://mirdb.org/), miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/index.php), mirDIP (http://ophid.utoronto.ca/mirDIP/index.jsp#r), and starBase (http://starbase.sysu.edu.cn/) predicted the possible downstream target genes of miR-101-3p, and then the obtained genes were subjected to intersection with the differentially up-regulated mRNAs obtained by the differential analysis in the database to obtain the candidate target genes. Finally, the final mRNA was determined by correlation analysis.

Table 1. RT-qPCR primer sequences.

Gene	Sequence (5'→ 3')
miR-101-3p	F: GGTCACTAAGGCGGT R: CAGTCGTTGCGTCGGAGT
U6	F: CTGGTTAGTACTTGGACGGGAGAC R: GTGCAGGGTCCGAGGT
KPNA2	F: CTCATAACCATGTCCACCAACG R: CTCTATTCTGCGACGCCTCAT
GAPDH	F: CTCCTCCTGTTCGACAGTCAGC R: CCCAATACGACCAAATCCGTT

Cell culture

In this study, the cell lines used were normal lung epithelial cell line BEAS-2B (BNCC359274) and four LUSC cell lines SK-MES-1 (BNCC100167), NCI-H2170 (BNCC101664), NCI-H1703 (BNCC101663), and NCI-H520 (BNCC234249). All the cell lines were manufactured by BeNa Culture Collection (BNCC, China) and cultivated in DMEM with 10% fetal bovine serum (FBS) in an environment of 37°C and 5% CO_2.

Cell transfection

Cells in logarithmic growth phase were plated with 6-well plates for culture till 80% confluency. Transfection was done by using Lipofectamine 2000 transfection kit (InvitrogenUSA). pcDNA3.1-KPNA2 plasmid (oe-KPNA2)/pcDNA3.1 plasmid (oe-NC) and miR-101-3p mimic (miR-mimic)/mimic NC (miR-NC) were transfected into SK-MES-1 cells and the cells were cultivated under 37°C and 5% CO_2.

RT-qPCR

Shown in Table 1 are the primer sequences involved. Trizol reagent (Invitrogen, USA) isolated RNA from LUSC cells. Hairpin-it miRNAsqPCR quantitative kit (GenePharma, China) was the tool to examine the expression levels of miR-101-3p and U6 (internal reference). The reverse transcriptions for KPNA2 and GAPDH mRNA were achieved using RT Master Mix for qPCR II (MedChemExpress, USA). The SYBR Green PCR quantitative kit (Qiagen, Germany) was employed to detect KPNA2 mRNA level with GAPDH serving as internal reference. $2^{-\Delta\Delta Ct}$ method finished the calculation.

CCK-8 analysis

After 8h of transfection, LUSC cell line SK-MES-1 (2.5×10^3 cells/well) was placed in a 96-well plate and cultivated at 37°C, 5% CO_2. Then, they were cultivated for 0, 24, 48, 72 and 96h. At each time point, 10 μL of CCK8 reagent (Dojindo, Japan) was introduced to each well for 1h of cultivation at 37°C. Then, the optical density at 450 nm was observed with a microplate reader.

Wound healing test

12h after transfection, SK-MES-1 cells were plated onto a 6-well plate (4×10^5 cells). When the cells were at about 90% confluency, a 200 μL sterile pipette tip scratched a linear cut on monolayer cells and the exfoliated cells were rinsed away. The culture medium was changed in a suitable environment for 24h of cell growth. Cell migration at 0h and 24h was photographed to yield the scratch healing rate.

Transwell assay for cell invasion

Transwell inserts (Corning) with a pore size of 8 μm were implemented. The upper chamber was coated with Matrigel matrix before 1×10^5 cells were seeded (serum-free medium filled). Following 24h of cultivation at 37°C, cells remaining in the upper counterpart were removed, while cells that moved were subjected to a 30 min of fixation in methanol and a 20 min staining in 0.5% crystal violet. Cells were washed with PBS was followed by drying. 5 random fields were picked with a microscope to calculate cell numbers and be photographed.

Western blot assay

Pierce BCA Protein Assay Kit (Pierce, USA) was employed for the determination of protein concentration. Proteins were moved to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA) following 10% SDS-PAGE. After being blocked in TBST containing 5% skim milk, the membrane was cultivated with primary antibodies (anti-KPNA2: 1:1000, ab84440, Abcam, UK; anti-GAPDH: 1:10000, ab181602, Abcam, UK). Then, the secondary antibody goat anti-rabbit IgG H&L (HRP) (ab205718, Abcam, UK) was introduced. ECL system (Life Technology) was used for signal detection. GAPDH was considered as an internal reference.

Dual-luciferase assay

SK-MES-1 cells (2×10^5 cells/well) were grown in 96-well plates. The mutant luciferase reporter vector (KPNA2-MUT) was co-transfected with miR-101-3p mimic or mimic NC into SK-MES-1, also the wild-type luciferase reporter vector (KPNA2-WT) were co-transfected with miR-101-3p mimic or mimic NC in the cell. Dual-Luciferase Reporter Assay System (Promega) quantified firefly and renilla luciferase activities.

Flow cytometry

The cells were digested and underwent a 2 h fixation at 4°C with 70% ethanol. The cells were then subjected to 3 washes with PBS, resuspended with propidium iodide (40%, sigma, USA) and RNase A, and cultivated in the dark at 37°C for 30 minutes. Apoptotic analysis was accomplished with FITC Annexin V Apoptosis Detection Kit (BD Biosciences, USA) and the BD FACS Celesta Flow Cytometer (BD Biosciences). The cells were divided into live, dead, early and late apoptotic cells to be compared under different experimental conditions for the percentages of apoptotic cells.

Statistical analysis

Data underwent SPSS 22 (SPSS Inc, USA) for analysis, and were graphed with GraphPad Prism 6.0 software (GraphPad software, USA). Values were expressed in the form of mean ± standard deviation (SD). Statistical difference between two groups was determined by Student's t test. $P<0.05$ indicated a significant difference.

Results

Downregulated miR-101-3p in LUSC cells

30 lowly-expressed and 112 highly-expressed miRNAs were screened based on TCGA-LUSC dataset by a differential expression analysis (Fig. 1A), of which low miR-101-3p level was prominent in LUSC tissue (Fig. 1B). Studies showed that this miRNA might be an oncogene to promote tumor proliferation and metastasis of NSCLC (Wan et al., 2018), and it can also be down-regulated in cancer cells to affect tumor occurrence and progression (Lu et al., 2018). Therefore, we chose miR-101-3p as a research object. Four LUSC and one normal cell lines were involved, revealing remarkably low miR-101-3p level in LUSC cell lines (Fig. 1C), implying lowly-expressed miR-101-3p in LUSC, with the most remarkable difference between BEAS-2B and SK-MES-1, which was qualified for subsequent experiments.

Overexpressed miR-101-3p represses LUSC cells progression

Mimic NC and miR-101-3p mimic were transfected with SK-MES-1 cell line, respectively, and miR-101-3p level of the transfected cell line was detected by RT-qPCR (Fig. 2A). In addition, we employed CCK-8 to detect the impact of miR-101-3p expression on cell proliferation, and observed a notable decrease in viability in the miR-101-3p mimic group (Fig. 2B), and miR-101-3p mimic had similar effects on cell invasion and migration (Fig. 2C,D). In contrast, flow cytometry suggested a striking facilitation on cell apoptosis in miR-101-3p mimic group (Fig. 2E). In summary, these results indicated that miR-101-3p had the ability to repress LUSC cells progression *in vitro*.

MiR-101-3p in LUSC cells represses KPNA2 expression

Next, we further studied the manipulation downstream of miR-101-3p in LUSC cells. 10 target

mRNAs were obtained via bioinformatics analysis (Fig. 3A). Correlation analysis suggested that KPNA2 had the highest negative correlation with miR-101-3p (Fig. 3B). In addition, KPNA2 was significantly up-regulated in LUSC tissue (Fig. 3C). Bioinformatics analysis suggested a binding site between miR-101-3p and KPNA2 (Fig. 3D). Then, overexpressed miR-101-3p markedly abated the luciferase activity of the wild type gene, with the mutant type intact, indicating that miR-101-3p directly bound to KPNA2 3'UTR (Fig. 3E). Besides, transfection of miR-101-3p mimics caused a notable decrease in KPNA2 mRNA and protein levels (Fig. 3F,G). To conclude, KPNA2 was a direct target of and negatively manipulated by miR-101-3p.

MiR-101-3p represses LUSC cells progression by targeting KPNA2

From previous experiments, we had proved that KPNA2 directly manipulated miR-101-3p, leading us to propose a new hypothesis: miR-101-3p repressed cell progression by directly containing KPNA2 level. Given that, we co-transfected SK-MES-1 cells with miR-101-3p mimic and oe-KPNA2, with miR-NC and oe-KPNA2, and with miR-NC and oe-NC (control group). KPNA2 mRNA and protein levels of the miR-NC+oe-KPNA2 group were remarkably higher over the control group. But those of the miR-101-3p mimic+oe-KPNA2 group had no prominent difference with the control group (Fig. 4A,B), suggesting KPNA2 down-regulation by miR-101-3p. Then, the results of cell proliferation exhibited that the proliferative ability of miR-NC+oe-KPNA2 transfected cells was enhanced over the control group, with no prominent difference observed in the proliferative ability of miR-101-3p mimic+oe-KPNA2 group (Fig. 4C). Meanwhile, the invasion and migration of cells were also enhanced in the miR-NC+oe-KPNA2 group over the control group. However, miR-101-3p and KPNA2 overexpression obviously suppressed such a facilitating effect of KPNA2 (Fig. 4D,E). Besides, flow cytometry also proved that in comparison with the control group, the apoptosis rate in the miR-NC+oe-KPNA2 group was reduced, while overexpression of miR-101-3p attenuated the inhibitory effect of KPNA2 overexpression on apoptosis of SK-MES-1 cells (Fig. 4F). To conclude, miR-101-3p directly targeted KPNA2 to repress the progression and facilitate SK-MES-1 cell apoptosis.

Discussion

With the characteristics of high morbidity and mortality, LUSC is the most common subtype of lung cancer (Gao et al., 2019). Nevertheless, the anti-tumor therapy of LUSC and the basic molecular mechanism of tumorigenesis are still the main clinical challenges. In the malignant progression of LUSC, there are many reports on the abnormal expression of miRNA, and this abnormal expression has a significant influence (Olbromski et al., 2016). For example, miR-486-5p (Yang et al., 2019), miR-144 (Uchida et al., 2019), and miR-541 (Xu et al., 2018) can all be tumor suppressor factors in LUSC. With prominently low level, these genes can abate the malignant progression of LUSC cells. miR-101-3p was qualified for observation of biological functions and specific mechanism in LUSC cells progression.

We firstly predicted the downregulated miR-101-3p in LUSC tissue by bioinformatics analysis, and proved it in LUSC cell lines, implying the close relation of miR-101-3p with LUSC. We also learned from the assays that

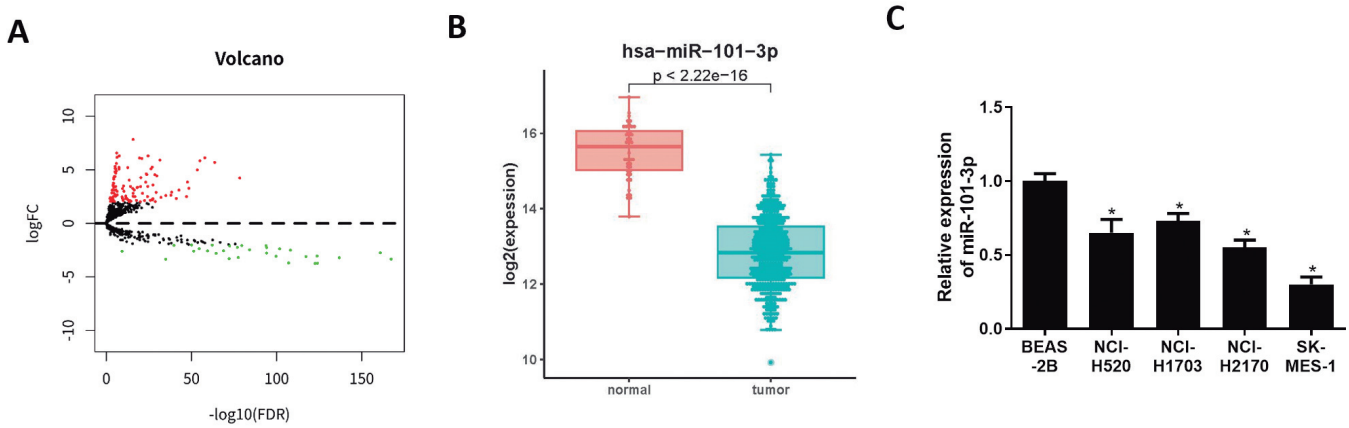

Fig. 1. MiR-101-3p is lowly expressed in LUSC cells. **A.** Volcano plot of differentially expressed miRNAs in LUSC; red: genes of up-regulated expression; green: genes of down-regulated expression. **B.** MiR-101-3p expression in LUSC tissue was significantly lower than that in adjacent normal tissue in TCGA. **C.** The expression of miR-101-3p in four human LUSC cell lines NCI-H520, NCI-H1703, NCI-H2170 and SK-MES-1 detected by RT-qPCR was significantly lower than that in normal cell line BEAS-2B. **P*<0.05.

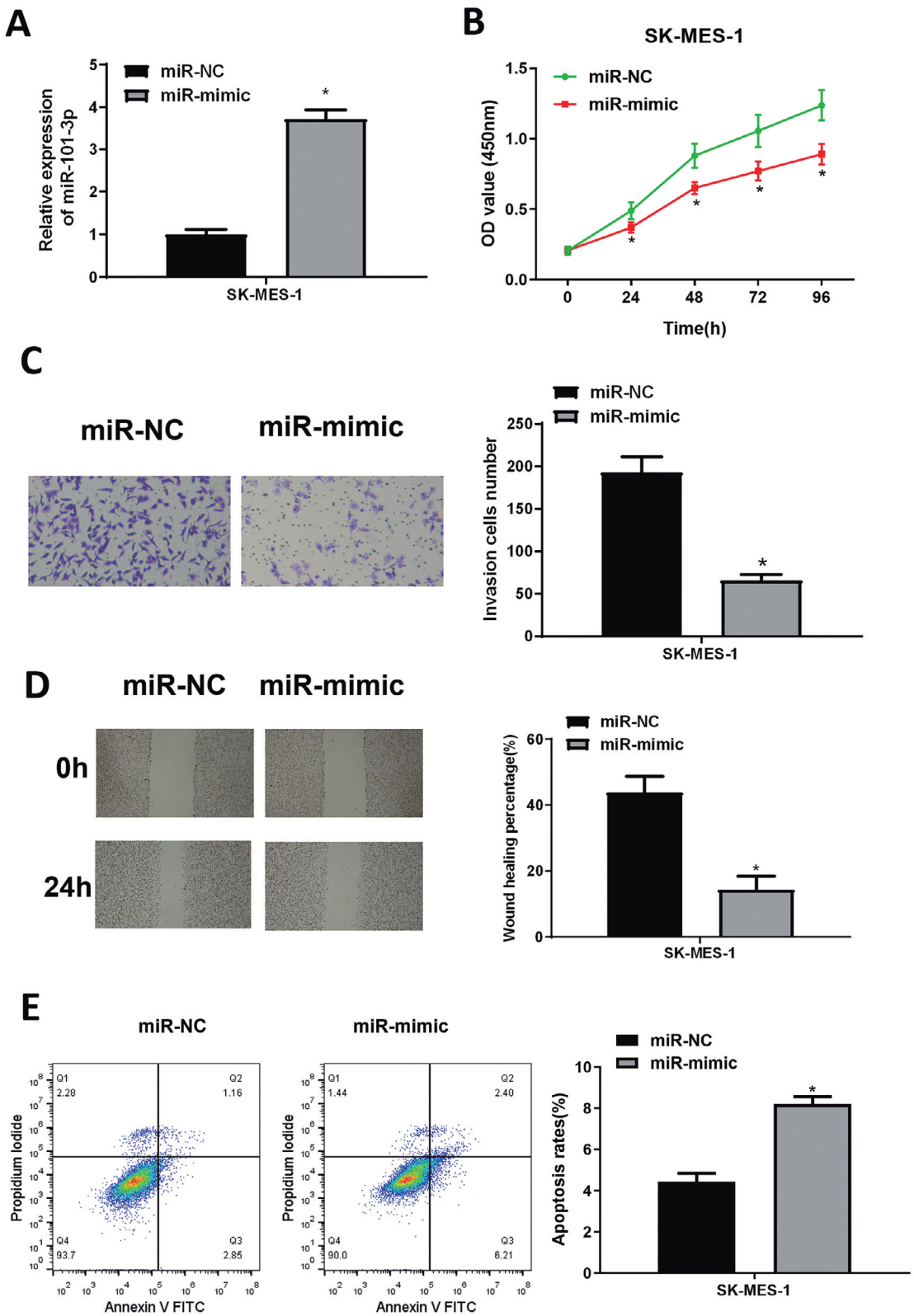

Fig. 2. Overexpression of miR-101-3p inhibits the progression of LUSC cells. **A.** Transfection efficiency of miR-101-3p mimic in LUSC cells detected by RT-qPCR. **B.** CCK8 was performed to determine cell viability. **C, D.** The inhibition of miR-101-3p mimic on invasion and migration of SK-MES-1 cells detected by Transwell invasion test and wound healing test. **E.** The promoting effect of miR-101-3p on cell apoptosis detected by flow cytometry. **P*<0.05.

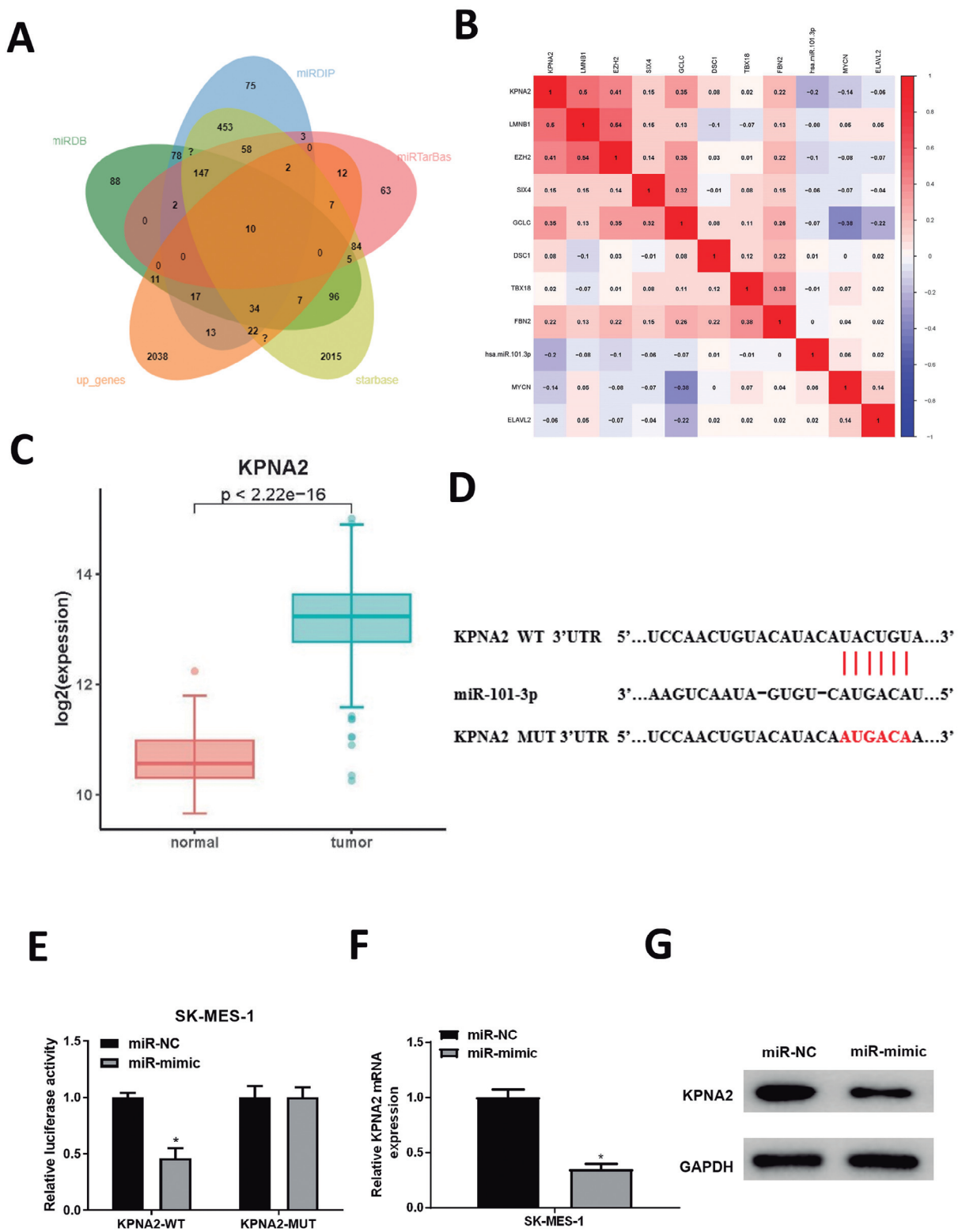

Fig. 3. MiR-101-3p in LUSC cells targets to inhibit KPNA2 expression. **A.** Venn diagram of the intersection of the target genes predicted by mirDIP, miRTarBase, miRDB and starBase databases and the differentially up-regulated mRNAs. **B.** Heat map of the correlation analysis between miR-101-3p and the 10 candidate target genes. **C.** The box plot of the expression of KPNA2 in adjacent normal tissue and cancer tissue in TCGA. **D.** Bioinformatics prediction of the targeted binding sites of miR-101-3p on KPNA2 mRNA 3'UTR. **E.** The luciferase activity of SK-MES-1 cells in different transfection groups detected by dual luciferase assay. **F, G.** RT-qPCR and Western blot detection of KPNA2 mRNA and protein expression in SK-MES-1 cells from different treatment groups. *P<0.05.

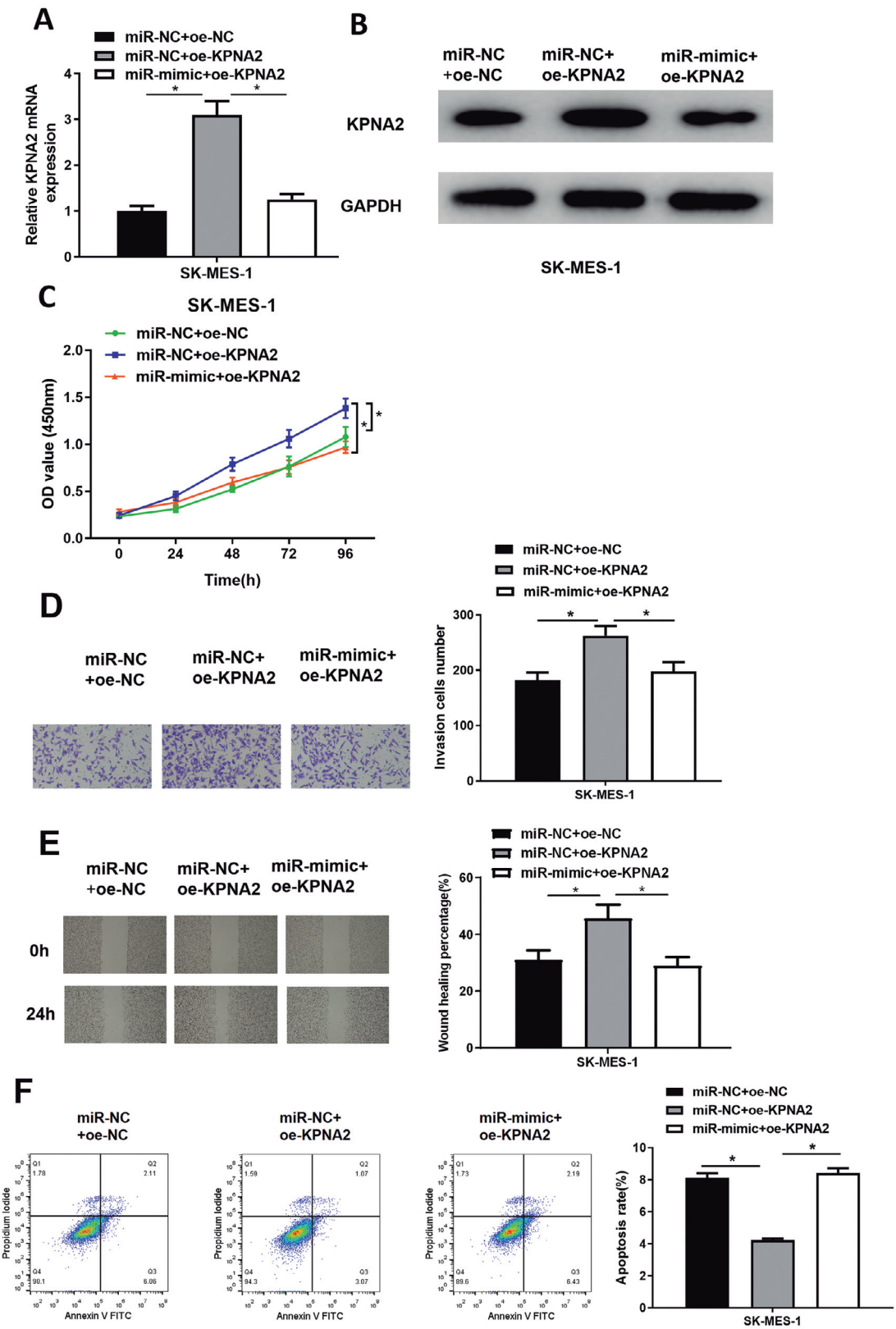

Fig. 4. MiR-101-3p targets KPNA2 to inhibit the progression of LUSC cells. **A, B.** The expression levels of KPNA2 mRNA and protein in transfected LUSC cells SK-MES-1 (miR-NC+oe-NC, miR-NC+oe-KPNA2, and miR-mimic+oe-KPNA2). **C.** The proliferative ability of SK-MES-1 cells detected by CCK-8 test. **D.** The invasive ability of SK-MES-1 cells detected by Transwell assay (100×). **E.** The migratory ability of SK-MES-1 cells detected by wound healing test (40×). **F.** Apoptosis level of SK-MES-1 cells detected by flow cytometry. **P*<0.05.

miR-101-3p mimics remarkably suppressed malignant progression, while enhancing cell apoptosis. Previous studies confirmed the impact of miR-101-3p on the occurrence of multiple cancers. For example, by directly targeting SRF, miR-101-3p inhibits the proliferation and invasion of gastric cancer cells (Wu et al., 2017); miR-101-3p level is prominently reduced and blocks PI3K/AKT signaling via MALAT-1, thus inhibiting the metastasis and growth of NSCLC cells (Zhang et al., 2017b). However, miR-101-3p can also be a cancer-promoting factor. For instance, miR-101-3p facilitates breast cancer progression by directly targeting Med19 and manipulating the EGFR/MEK/ERK signaling (Zhang et al., 2019). Here, the bioinformatics analysis and experimental results illustrated together that miR-101-3p expression was decreased in LUSC cells and that miR-101-3 played the functions of tumor suppressor gene in LUSC.

In other types of malignant tumors, KPNA2 is also reported to be elevated and under regulation of miRNAs. For example, the innate immune response induced by enterovirus 71 can be down-regulated by miR-302 through targeting KPNA2 (Peng, et al., 2018). MiR-26 is lowly expressed in breast cancer and can directly target CHD1, GREB1 and KPNA2 to repress the proliferation and metastasis of breast cancer cells (Tan et al., 2014). MiR-26b targets the KPNA2/c-jun pathway to abate gastric cancer cells progression (Tsai et al., 2016). Wip1 and KPNA2 can modulate tumor metastasis-related factors Twist and E-cadherin, thus affecting the progression of colorectal cancer cells (Wang et al., 2019). In our research, KPNA2 was significantly upregulated in LUSC cells and directly repressed by miR-101-3p. Therefore, we speculated that in LUSC cells, miR-101-3p facilitated the degradation of KPNA2 mRNA or contained the translation of KPNA2 by coupling with KPNA2 3'UTR. CCK8, wound healing and Transwell assays verified the suppressive impact of miR-101-3p as well as the promoting effect of KPNA2 on viability, migration and invasion of LUSC cells, and miR-101-3p overexpression abated the promoting effect of KPNA2. In summary, miR-101-3p and KPNA2 were considered to be both involved in LUSC progression.

We confirmed that miR-101-3p directly manipulated KPNA2, thus inducing cell apoptosis and inhibiting the metastasis of LUSC cells. Our findings provided *in vitro* experimental support for the knowledge of down-regulated miR-101-3p or up-regulated KPNA2 in LUSC. MiR-101-3p and KPNA2 both played a significant role in LUSC malignant progression, implying the potential role of miR-101-3p/KPNA2 for LUSC treatment. Although we have a new understanding of miR-101-3p in the malignant progression of LUSC, it is necessary to determine other targets of this miRNA. In addition, other mechanisms that cause miR-101-3p downregulation, such as promoter methylation (Shao et al., 2018) or interaction with long non-coding RNAs (Zhang et al., 2017a), still need to be clarified in future studies.

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Ethics approval and consent to participate. Not applicable.

Consent for publication. All authors consent to submit the manuscript for publication.

Availability of data and materials. The data used to support the findings of this study are included within the article. The data and materials in the current study are available from the corresponding author on reasonable request.

Conflict of Interest Statement. The authors declare that they have no potential conflicts of interest.

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Authors' contributions. LL contributed to the study design. KJ conducted the literature search. HL and TQ acquired the data. YM wrote the article. EG performed data analysis. AH drafted. LL and KJ revised the article and gave the final approval of the version to be submitted. All authors read and approved the final manuscript.

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