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MiR-101-3p Suppresses Progression of Cervical Squamous Cell Carcinoma by Targeting and Down-Regulating KPNA2

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Abstract

Objective We explored mechanism of microRNA-101-3p/Karyopherin α2 (KPNA2) axis in cervical squamous cell carcinoma. **Methods:** Bioinformatics methods were applied to identify genes for the study. Cell functional assays were implemented to examine the role of the genes in malignant progression of cervical squamous cell carcinoma. Targeting relationship between genes was verified by dual-luciferase assay. **Results:** MicroRNA-101-3p was lowly expressed in cervical squamous cell carcinoma, while KPNA2 was highly expressed. Dual-luciferase assay identified direct targeting relationship between microRNA-101-3p and KPNA2. Functional assays manifested that highly expressed microRNA-101-3p suppressed cervical squamous cell carcinoma cell growth by targeting KPNA2. **Conclusion:** Overall, microRNA-101-3p/KPNA2 axis can play an important part in progression of cervical squamous cell carcinoma.

Keywords

microRNA-101-3p, KPNA2, cervical squamous cell carcinoma, proliferation, migration, invasion

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Introduction

Incidence and mortality of cervical cancer both rank fourth worldwide.[1] Cervical squamous cell carcinoma comprises more than 85% of all cervical cancer types. Surgery is the main treatment method for cervical cancer in early stages with great therapeutic effect, while surgery combined with radiotherapy and chemotherapy is commonly used for cervical cancer in middle and advanced stages generally with poor prognosis. Additionally, a recent study found that more than 30% of cervical cancer cases are diagnosed in middle and advanced stages.[2] Therefore, finding specific molecular markers of cervical squamous cell carcinoma is of great value for early diagnosis and searching into new therapeutic methods.

MicroRNA-101-3p is engaged in regulation of various cancer activities. For instance, lncRNA PTAR promotes epithelial-mesenchymal transition, invasion, and metastasis of serous ovarian cancer by competitively binding microRNA-101-3p to regulate ZEB1.[3] MicroRNA-101-3p inhibits HOX transcript antisense RNA-induced proliferation and invasion of gastric cancer cells via directly targeting SRF.[4] Nevertheless, functions of microRNA-101-3p in cervical squamous cell carcinoma are less studied.

Many clinical studies indicated that Karyopherin α2 (KPNA2) is upregulated in a variety of malignancies.[5,6] Partial biological functions of KPNA2 were discovered in several cancers. An example is that benign breast cancer cell lines with overexpressing KPNA2 can proliferate and migrate like malignant tumors.[7] KPNA2 enhances migration and survival of lung cancer cell lines as well.[8] In addition, knockout of KPNA2 can inhibit proliferation of prostate cancer,[9] hepatocellular carcinoma[10] and ovarian carcinoma.[11] However, there is little information on the regulatory mechanism of KPNA2 in cervical squamous cell carcinoma.

Therefore, we explored interaction of microRNA-101-3p with KPNA2, and discussed their potential mechanism of action in malignant progression of cervical squamous cell carcinoma.

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Methods

Bioinformatics Approaches

Expression profiles of mature microRNAs (Normal: 3, Tumor: 309) and mRNAs (Normal: 3, Tumor: 306) were obtained from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). Target differentially expressed microRNA (DEmicroRNA) (|logFC|>2, padj<0.01) was identified by differential analysis using package "edgeR". With median expression value of the DEmicroRNA in tumor samples as the threshold, high- and low-expression groups were set to perform survival analysis. GTEx expression files along with sample information files, and FPKM data of mRNA along with sample information files were downloaded from UCSC Xena (https://xenabrowser.net/datapages/), respectively. The processed GTEx data were merged with TCGA-CESC data (Normal: 13, Tumor: 306), and then "limma" package was used to conduct differential analysis to obtain differentially expressed mRNAs (DEmRNAs) (|logFC|>2, padj<0.05). StarBase, mirTarBase, mirDIP, and miRDB databases were used to predict target mRNAs of the target DEmicroRNA. An intersection of the above predicted results and DEmRNAs was obtained to obtain the research object.

Cell Cultivation

The information of cell lines was listed in Table 1. RPMI-1640 medium (Invitrogen) + 10% fetal bovine serum (FBS) (Invitrogen) was used for cell culture under the conditions of 37 °C and 95% air/5% CO_2.

Cell Transfection

pcDNA3.1-KPNA2 plasmid vector (oe-KPNA2), and empty pcDNA3.1 vector (oe-NC) were transfected into C33a cells by Lipofectamine 2000 (Invitrogen). Transfected cells were cultured with 5% CO_2 at 37 °C.

qRT-PCR

RNA was extracted and reversely transcribed with TRIzol reagent (Invitrogen) and PrimeScript RT Reagent Kit (Takara Biotechnology Co., Ltd), respectively. SYBR® Premix Ex Taq™ (Takara Biotechnology Co., Ltd) was used for detection on the ABI 7900 Sequence Detection System (Thermo-Fisher Scientific) with primer sequences listing in Table 2. GAPDH and U6 were internal controls for KPNA2 and microRNA-101-3p, respectively. Relative gene expression was analyzed by $2^{-\Delta\Delta Ct}$ method.

Table 1. Information of Cell Lines Used in the Experiment.

Type	Name	Number	Source
Human cervical squamous epithelial cell line	Ect1	CRL-2614	American Type Culture Collection
Human cervical squamous cell carcinoma cell line	Siha	HTB-35	
	Caski	CRM-CRL-1550	
	C33a	HTB-31	
	ME180	HTB-33	
Human renal epithelial cell line	HEK293	CRL-1573	

CCK-8 Assay

Cervical squamous cell carcinoma cells (2×10^3 cells/well) were inoculated into 96-well plates. At 0 h, 24 h, 48 h, 72 h, and 96 h, CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added and cells were cultured at 37 °C for 2 h. The optical density value was read at 490 nm by a microplate reader (Bio-Tek) and survival curves were plotted.

Wound Healing Assay

Transfected cancer cells were seeded into 6-well plates. When cell fusion degree was about 80%, the cell was scratched with a 200 μL sterile pipette tip in each well. After washing with serum-free medium, cells were incubated in medium with 0.5% FBS. Finally, cell migration was observed under a microscope (Axioskop 40, Carl Zeiss AG).

Transwell Invasion Assay

Transwell chamber (Coring Costar) with membrane pore size of 8.0 μm was used for cell invasion assay. Transfected C33a cells were inoculated in the upper chamber which pretreated with Matrigel (BD Biosciences) and filled by serum-free medium, while medium + 10% FBS was added into the lower chamber. After incubation, invaded cells were treated with 4% formaldehyde and 0.1% crystal violet, respectively. The final step was to count cells in five random fields by using a microscope (Olympus Corporation).

Western Blot

After extraction of total proteins, protein concentration was assayed by a bicinchoninic acid kit (Takara). Separated by 10% SDS-PAGE, proteins were then transferred onto a PVDF membrane. After sealing with 5% skim milk, membrane was reacted with corresponding primary antibodies (rabbit anti-KPNA2 [ab170495, 1: 10 000] and rabbit anti-GAPDH [ab181602, 1: 10 000] [internal reference]). Then, the membrane was washed with PBST solution, and sequentially incubated with goat antirabbit IgG (ab6721, 1:2000). Protein bands were examined by ECL-Plus reagent (Millipore). All antibodies used were from Abcam.

Dual-Luciferase Reporter Gene Detection

Wild-type (WT) or mutant (MUT) pmicroRNAGLO-KPNA2-3′UTR (KPNA2-WT/MUT) and microRNA-101-3p

Table 2. Primer Sequences for qRT-PCR.

Gene	Primer sequence (5′→ 3′)	
miR-101-3p	F: GGTCACTAAGGCGGT	R: CAGTCGTTGCGTCGGAGT
U6	F: CTCGCTTCGGCAGCACA	R: ACGCTTCACGAATTTGCGT
KPNA2	F: CTCATAACCATGTCCACCAACG	R: CTCTATTCTGCGACGCCTCAT
GAPDH	F: GACCTGACCTGCCGTCTA	R: AGGAGTGGGTGTCGCTGT

mimic/mimic NC were transfected into HEK293 cells together. Luciferase activity was measured using dual-luciferase reporter gene assay system (Promega) after transfection.

Flow Cytometry

Cell apoptosis was explored by FITC Annexin V/PI apoptosis detection kit (Invitrogen, Thermo-fisher Scientific). Cells were digested by trypsin, centrifuged for 5 min, rinsed with PBS, and resuspended in 500 µl binding buffer. Later, cells were cultivated in FITC Annexin V and PI (100 µg/mL) solution for 15 min and further diluted with 400 µl 1× Annexin binding buffer. Coulter FC500 Flow cytometry (Beckman Coulter) was used to measure cell apoptosis.

Statistical Analysis

SPSS 21.0 (IBM Corp) and GraphPad Prism 6.0 were used for data analysis and results visualization. All the above experiments were performed in triplicate independently. All experimental data accorded with normal distribution, with mean ± standard deviation. *T*-test was utilized to analyze statistical significance between two groups. $P < .05$ represented statistical significance.

Results

MicroRNA-101-3p is Down-Regulated in Cervical Squamous Cell Carcinoma Cells

Differential analysis of microRNA expression of cervical squamous cell carcinoma in TCGA-CESC dataset was conducted (Figure 1A). The result exhibited that microRNA-101-3p expression was at a significant low level in cervical squamous cell carcinoma (Figure 1B). The result of survival analysis indicated that low expression of microRNA-101-3p predicted poor prognosis (Figure 1C). Expression of microRNA-101-3p detected by qRT-PCR was underexpressed in cancer cells (Figure 1D).C33a cell line with the lowest microRNA-101-3p expression among all cancer cell lines were selected for subsequent experiments.

MicroRNA-101-3p Overexpression Restrains Progression of Cervical Squamous Cell Carcinoma Cells

The following experiments were conducted to explore the effects of microRNA-101-3p expression on behaviors of cancer cells. MicroRNA-101-3p expression in C33a cells was significantly increased with overexpression treatment (Figure 2A). Then, we detected cell proliferation of the two groups through CCK-8 assay. It was noted that microRNA-101-3p overexpression remarkably repressed C33a cell proliferation (Figure 2B). Next, Transwell invasion and wound healing assays displayed that overexpression of microRNA-101-3p prominently hampered cell invasion and migration (Figure 2C and D). Flow cytometry result demonstrated that elevated expression of microRNA-101-3p significantly increased apoptosis level of C33a cells (Figure 2E). Thus, overexpressing microRNA-101-3p inhibited malignant behaviors of cervical squamous cell carcinoma cells.

MicroRNA-101-3p Targets and Inhibits KPNA2 in Cervical Squamous Cell Carcinoma Cells

1265 DEmRNAs were obtained from differential analysis (Figure 3A). Meanwhile, intersection of predicted target mRNAs and 645 up-regulated DEmRNAs identified 9 targets of microRNA-101-3p (Figure 3B). Correlation analysis result displayed that the Pearson correlation coefficient of microRNA-101-3p and KPNA2 was the highest in terms of expression (Figure 3C). The expression data of KPNA2 in TCGA-CESC dataset also indicated that KPNA2 was notably highly expressed in cervical squamous cell carcinoma tissue (Figure 3D). Then, KPNA2 protein expression measured by western blot was notably high in cancer cells (Figure 3E). Next, dual-luciferase assay was utilized to verify that microRNA-101-3p targeted KPNA2 (Figure 3F and G). Then, overexpression of microRNA-101-3p remarkably reduced KPNA2 mRNA and protein expression (Figure 3H and I).

MicroRNA-101-3p Inhibits Progression of Cervical Squamous Cell Carcinoma Cells and Promotes Cell Apoptosis by Down-Regulating KPNA2

Then, we transfected C33a cells with microRNA-mimic and oe-KPNA2, and carried out rescue assays for each treatment group. qRT-PCR and western blot results showed that KPNA2 expression in microRNA-NC + oe-KPNA2 group was significantly elevated, and it was recovered under microRNA-101-3p overexpression (Figure 4A and B). The above results further indicated that KPNA2 expression could be suppressed by microRNA-101-3p. CCK8 result showed that overexpression of KPNA2 significantly enhanced proliferative ability of cervical squamous cell carcinoma cells, while

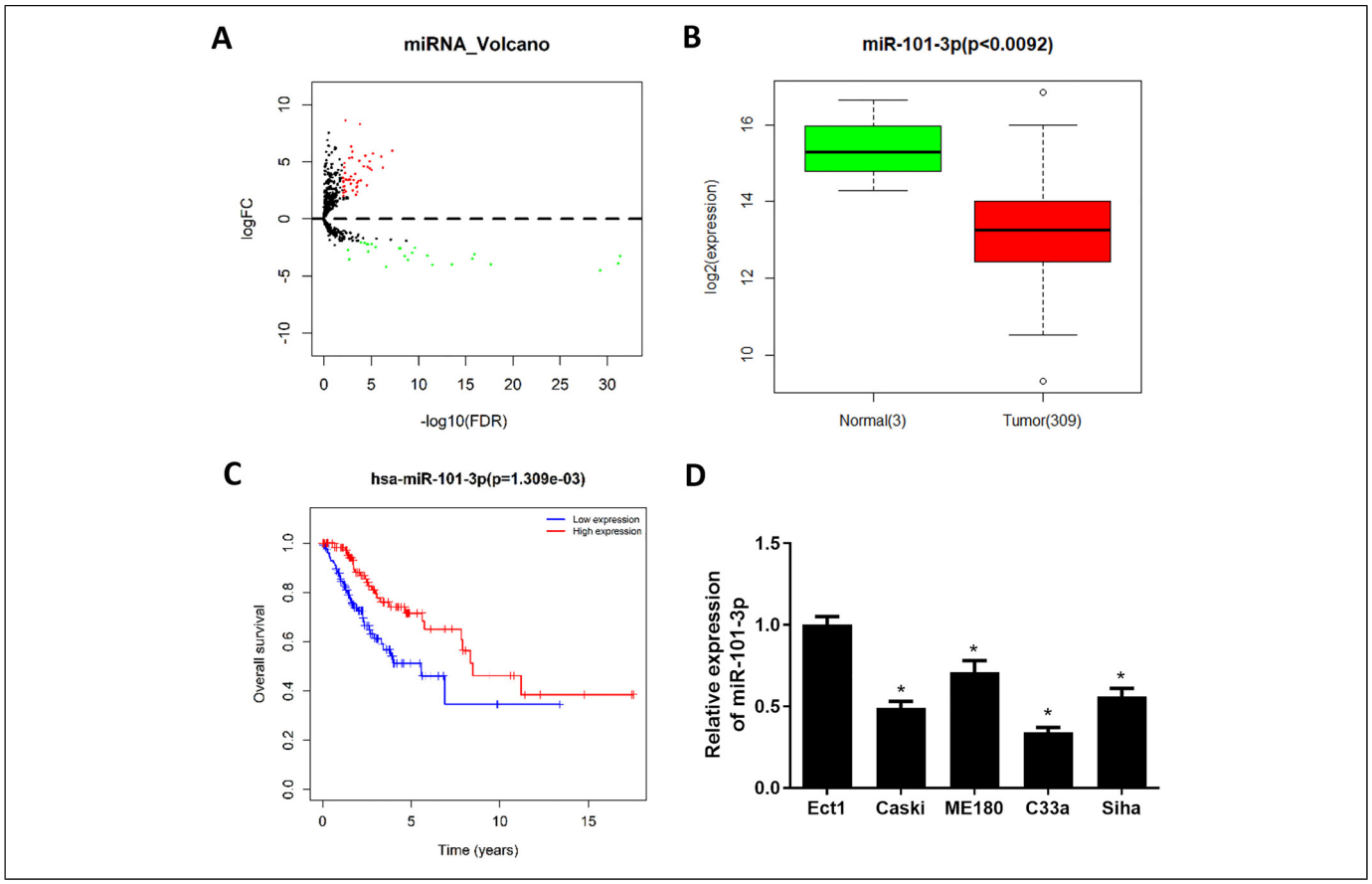

Figure 1. MicroRNA-101-3p is at low expression level in cervical squamous cell carcinoma. (A) Volcano plot of DEmicroRNAs; (B) Box plot of microRNA-101-3p in normal (green) and tumor (red) groups; (C) Survival difference between microRNA-101-3p high-expression group (red curve) and low-expression group (blue curve), with horizontal axis representing time (in years), and vertical axis representing overall survival rate; (D) MicroRNA-101-3p expression in Ect1, Caski, ME180, C33a, and Siha cell lines; *$P < .05$.

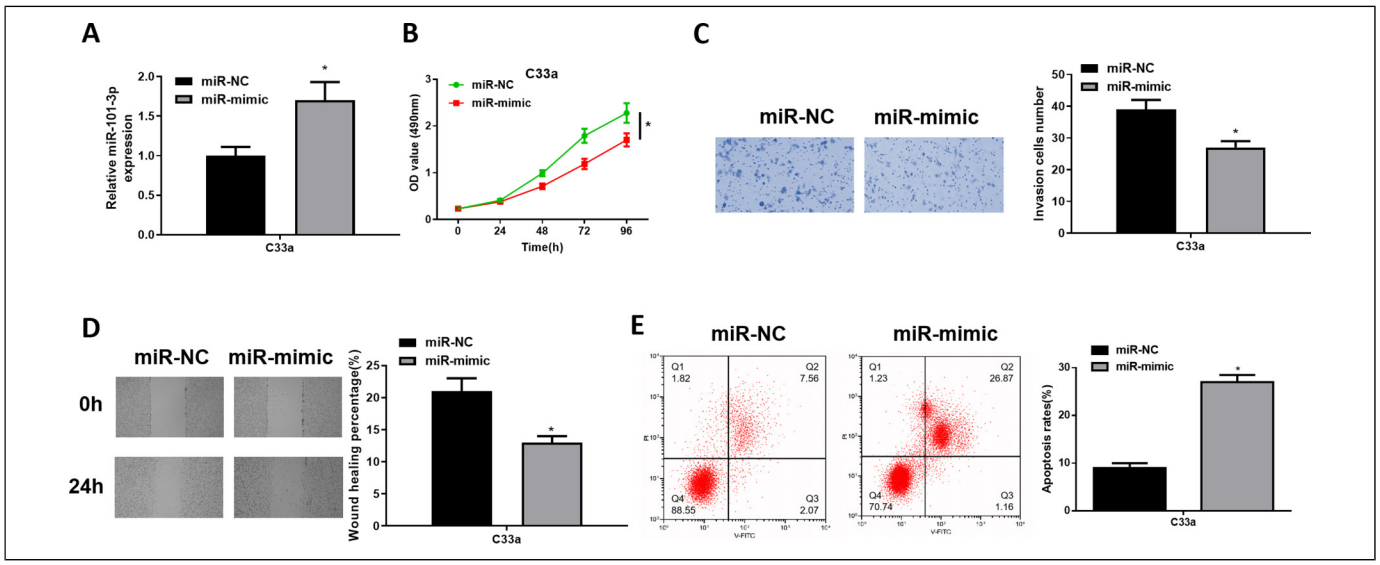

Figure 2. Overexpression of microRNA-101-3p suppresses progression of cervical squamous cell carcinoma cells. (A) MicroRNA-101-3p expression in C33a cells in two groups; (B) C33a cell proliferative ability in different groups; (C) C33a cell invasive ability in transfection groups (100×); (D) Detection of C33a cell migratory ability in different groups (40×); (E) The apoptosis rate of cervical squamous cell carcinoma cells C33a in different groups; *$P < .05$.

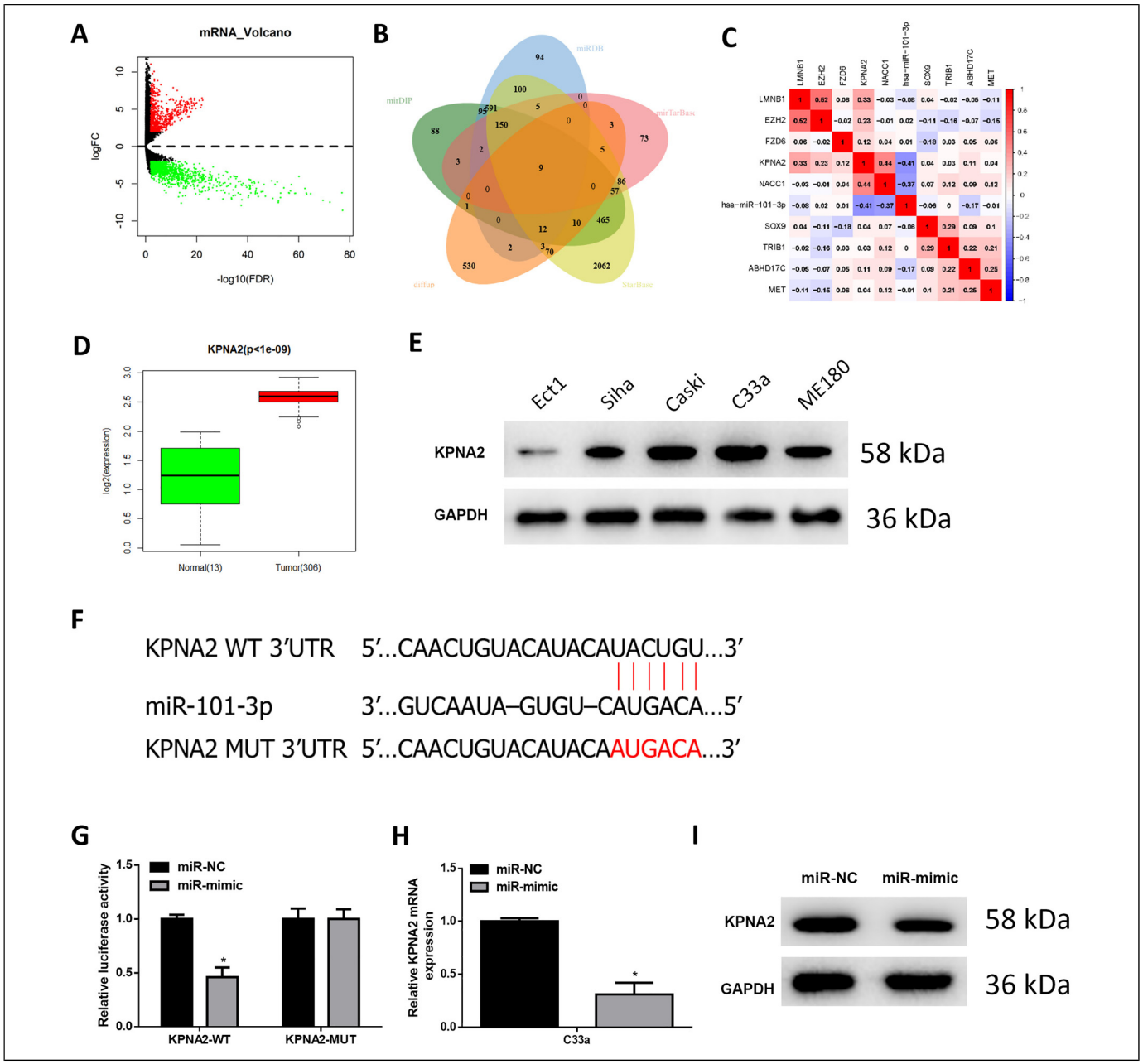

Figure 3. MicroRNA-101-3p inhibits KPNA2 in cervical squamous cell carcinoma cells. (A) Volcano map of DEmRNAs; (B)Venn diagram of intersection of upregulated DEmRNAs and targets of microRNA-101-3p; (C) Pearson correlation analysis between microRNA-101-3p and the predicted genes; (D) Box plot of KPNA2 expression in normal (green) and tumor (red) groups in TCGA-CESC dataset; (E) KPNA2 protein expression in Ect1, Caski, ME180, C33a, and Siha cells; (F) Targeted binding sites between microRNA-101-3p and KPNA2; (G)Binding relationship between microRNA-101-3p and KPNA2; (H and I) The regulation of microRNA-101-3p on KPNA2 was verified by qRT-PCR and western blot; $*p < .05$.

simultaneous overexpressing microRNA-101-3p attenuated this promoting effect (Figure 4C). The results of wound healing and Transwell invasion assays were the same as those of proliferation assay (Figure 4D and E). In addition, flow cytometry results also confirmed that overexpression of KPNA2 could significantly reduce the apoptosis level of C33a cells, while upregulation of microRNA-101-3p reversed this inhibiting effect (Figure 4F). In conclusion, microRNA-101-3p inhibited migration, proliferation, and invasion of cervical squamous cell carcinoma cells and promoted apoptosis by downregulating KPNA2.

Discussion

Though advances in diagnostic technology make it possible for cervical cancer patients to be early diagnosed and treated,[12]

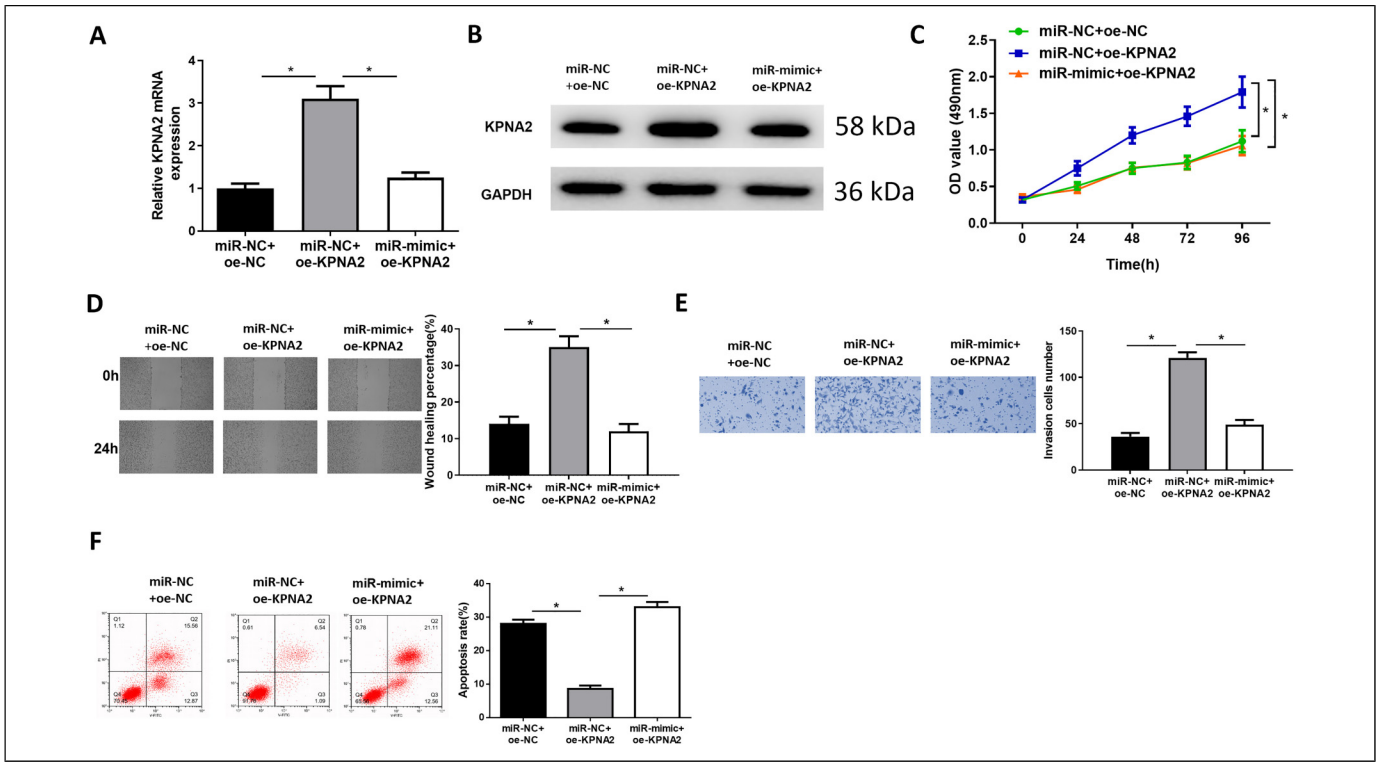

Figure 4. MicroRNA-101-3p restrains cervical squamous cell carcinoma cell growth by targeting KPNA2. (A and B) KPNA2 mRNA and protein expression in microRNA-NC + oe-NC, microRNA-NC + oe-KPNA2, and microRNA-mimic + oe-KPNA2 groups; (C) C33a cell proliferation changes in different groups; (D) Detection of C33a cell migratory ability in different groups (40×); (E) C33a cell invasive ability in different groups (100×); F: C33a cell apoptosis in different groups; $*p < .05$.

mortality of cervical cancer is still quite high at about 50%.[13] Cervical squamous cell carcinoma poses a serious threat to the health of women in both physical and mental states. Therefore, this study was developed to look for biological targets that specifically regulate cervical squamous cell carcinoma.

In recent years, studies found that microRNAs are specifically expressed in multiple cancers, and some microRNAs extensively researched are also used in assisting clinical diagnosis.[14] MicroRNA-101-3p can inhibit progression of various cancers. For instance, transfection of microRNA-101-3p in cells inhibits AMPK expression and inhibits proliferation of triple negative breast cancer cells.[15] MicroRNA-101-3p expression is linked with progression and prognosis of nonsmall cell lung cancer.[16] Wang *et al*[17] demonstrated that microRNA-101-3p can target autotaxin (ATX), while ATX is widely overexpressed in metastatic cancers. First of all, microRNA-101-3p was confirmed to be dramatically down-regulated in cervical squamous cell carcinoma, and overexpression of microRNA-101-3p inhibited cell growth. MicroRNA-101-3p presented as a tumor suppressor, which was consistent with findings of the studies on other cancers.

Initially, based on the data analyzed by bioinformatics, we noted that microRNA-101-3p showed low expression in cervical squamous cell carcinoma, while the expression of KPNA2 was up-regulated. Luciferase reporter gene detection confirmed that KPNA2 was a targeted by microRNA-101-3p in cervical squamous cell carcinoma. Accumulating evidence demonstrated that KPNA2 is important in cancer progression.[6] For example, microRNA-26b suppress proliferation and metastasis of epithelial ovarian cancer by down-regulating KPNA2 and OCT4.[18] MicroRNA-26b targets KPNA2/c-jun pathway to inhibit gastric cancer metastasis.[19] MicroRNA-139 inhibits growth of hepatocellular carcinoma cells by down-regulating KPNA2.[20] Consistent with the above studies, we found that up-regulated microRNA-101-3p significantly down-regulated KPNA2 expression. Importantly, rescue assays manifested that overexpression of microRNA-101-3p could reverse promoting effect of KPNA2 on cervical squamous cell carcinoma cell functions, indicating that KPNA2 had tumor promoting activity in cervical squamous cell carcinoma. In summary, our results suggested that the tumor inhibition mediated by microRNA-101-3p in cervical squamous cell carcinoma was at least partly attributable to the regulation of KPNA2.

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Authors' Note

H. W. contributed to the work, including conception and design, R. X. and contributed to article drafting and revising. B. Y. acquired

the data, performed data analysis, and drafted. H. W. was the guarantor for the article who takes full responsibility for the work.

Declaration of Conflicting Interests

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References

1. Bray F, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394-424. DOI: 10.3322/caac.21492
2. He Y, Zhao H, Li XM, Yin CH, Wu YM. Use of mesothelin as a tumor-associated antigen in cervical squamous cell carcinoma. *Gene*. 2019;690:30-37. DOI: 10.1016/j.gene.2018.12.029
3. Liang H, et al. LncRNA PTAR promotes EMT and invasion-metastasis in serous ovarian cancer by competitively binding miR-101-3p to regulate ZEB1 expression. *Mol Cancer*. 2018;17(1):119. DOI: 10.1186/s12943-018-0870-5
4. Wu X, et al. miR-101-3p suppresses HOX transcript antisense RNA (HOTAIR)-induced proliferation and invasion through directly targeting SRF in gastric carcinoma cells. *Oncol Res*. 2017;25(8):1383-1390. DOI: 10.3727/096504017x14879366402279
5. Tseng SF, Chang CY, Wu KJ, Teng SC. Importin KPNA2 is required for proper nuclear localization and multiple functions of NBS1. *J Biol Chem*. 2005;280(47):39594-39600. DOI: 10.1074/jbc.M508425200
6. Dahl E, et al. Molecular profiling of laser-microdissected matched tumor and normal breast tissue identifies karyopherin alpha2 as a potential novel prognostic marker in breast cancer. *Clin Cancer Res*. 2006;12(13):3950-3960. DOI: 10.1158/1078-0432.Ccr-05-2090
7. Noetzel E, et al. Nuclear transport receptor karyopherin-α2 promotes malignant breast cancer phenotypes in vitro. *Oncogene*. 2012;31(16):2101-2114. DOI: 10.1038/onc.2011.403
8. Wang CI, et al. Importin subunit alpha-2 is identified as a potential biomarker for non-small cell lung cancer by integration of the cancer cell secretome and tissue transcriptome. *Int J Cancer*. 2011;128(10):2364-2372. DOI: 10.1002/ijc.25568
9. Mortezavi A, et al. KPNA2 expression is an independent adverse predictor of biochemical recurrence after radical prostatectomy. *Clin Cancer Res*. 2011;17(5):1111-1121. DOI: 10.1158/1078-0432.Ccr-10-0081
10. Yoshitake K, et al. Importin-α1 as a novel prognostic target for hepatocellular carcinoma. *Ann Surg Oncol*. 2011;18(7):2093-2103. DOI: 10.1245/s10434-011-1569-7
11. Huang L, et al. KPNA2 Promotes cell proliferation and tumorigenicity in epithelial ovarian carcinoma through upregulation of c-Myc and downregulation of FOXO3a. *Cell Death Dis*. 2013;4(8):e745. DOI: 10.1038/cddis.2013.256
12. He X, et al. Prognostic value of C-reactive protein/albumin ratio in predicting overall survival of Chinese cervical cancer patients overall survival: comparison among various inflammation based factors. *J Cancer*. 2018;9(10):1877-1884. DOI: 10.7150/jca.23320
13. Lee KB, Shim SH, Lee JM. Comparison between adjuvant chemotherapy and adjuvant radiotherapy/chemoradiotherapy after radical surgery in patients with cervical cancer: a meta-analysis. *J Gynecol Oncol*. 2018;29(4):e62. DOI: 10.3802/jgo.2018.29.e62
14. Mishra S, Yadav T, Rani V. Exploring miRNA based approaches in cancer diagnostics and therapeutics. *Crit Rev Oncol/Hematol*. 2016;98:12-23. DOI: 10.1016/j.critrevonc.2015.10.003
15. Liu P, et al. mir-101-3p is a key regulator of tumor metabolism in triple negative breast cancer targeting AMPK. *Oncotarget*. 2016;7(23):35188-35198. DOI: 10.18632/oncotarget.9072
16. Lu HM, et al. Prognostic implications of decreased microRNA-101-3p expression in patients with non-small cell lung cancer. *Oncol Lett*. 2018;16(6):7048-7056. DOI: 10.3892/ol.2018.9559
17. Wang Y, Lyu L, Zhang X, Zhang J. Autotaxin is a novel target of microRNA-101-3p. *FEBS Open Bio*. 2019;9(4):707-716. DOI: 10.1002/2211-5463.12608
18. Lin J, et al. MiR-26b/KPNA2 axis inhibits epithelial ovarian carcinoma proliferation and metastasis through downregulating OCT4. *Oncotarget*. 2015;6(27):23793-23806. DOI: 10.18632/oncotarget.4363
19. Tsai MM, et al. MicroRNA-26b inhibits tumor metastasis by targeting the KPNA2/c-jun pathway in human gastric cancer. *Oncotarget*. 2016;7(26):39511-39526. DOI: 10.18632/oncotarget.8629
20. Zan Y, et al. MicroRNA-139 inhibits hepatocellular carcinoma cell growth through down-regulating karyopherin alpha 2. *J Exp Clin Cancer Res*. 2019;38(1):182. DOI: 10.1186/s13046-019-1175-2