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MicroRNA-141-3p promoted the progression of nasopharyngeal carcinoma through targeting DLC1

J.-W. MU[1], X.-Y. ZHOU[2], Q.-J. WANG[3], L.-H. HAN[4], J.-B. JIAO[5]

[1]E.N.T. Department, People's Hospital of Rizhao, Rizhao, China
[2]Department of Clinical Laboratory, Rizhao Hospital of TCM, Rizhao, China
[3]Department of Nephrology, The People's Hospital of Zhangqiu Area, Jinan, China
[4]Department of Health Examination, The People's Hospital of Zhangqiu Area, Jinan, China
[5]Department of Oncology, Jining No. 1 People's Hospital, Jining, China

Abstract. – OBJECTIVE: Previous studies have shown that the function of miR-141 has tissue specificity. However, the role of miR-141-3p has not been reported in nasopharyngeal carcinoma (NPC). Therefore, this study explored the function of miR-141-3p in NPC.

PATIENTS AND METHODS: MiR-141-3p expression in NPC tissues was examined via quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay. Cell Counting Kit-8 (CCK-8) and transwell assays were used to explore the function of miR-141-3p. The relationship between miR-141-3p and DLC1 was verified by Dual-Luciferase assay. Protein expression was observed by immunocytochemical assay and Western blot analysis.

RESULTS: Upregulation of miR-141-3p associated with poor prognosis was detected in NPC patients. Moreover, overexpression of miR-141-3p promoted cell proliferation, migration, and invasion in NPC cells. It was also found that miR-141-3p promoted EMT and activated the mTOR signaling pathway in NPC. Furthermore, DLC1 was indicated as a direct target of miR-141-3p and miR-141-3p negatively correlated with DLC1 expression in NPC. In particular, upregulation of DLC1 could impair the promoted effect of miR-141-3p in NPC.

CONCLUSIONS: MiR-141-3p promotes the progression of NPC by targeting DLC1 and activating the mTOR pathway.

Key Words:
MiR-141-3p, DLC1, Nasopharyngeal carcinoma, mTOR.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignant tumor that originates in the epithelium and glands of the nasopharyngeal mucosa[1]. NPC patients in China account for 80% of NPC patients in the world, while incidence of NPC is higher in Southern China[2]. Patients with NPC are mainly treated with radiation. The 5-year survival rate after radiotherapy is 8%-62%[3]. In addition, local recurrence and distant metastasis are the main causes of the death in NPC patients after radiotherapy[4]. The prognosis of NPC is related to clinical stage and clinical classification[5]. The 5-year survival rate of NPC patients at I-II stage exceeds 60% and that in stage III-IV falls to 20%-40%[6]. However, the stage III-IV NPC patients account for 70%-80%[7]. Therefore, it is necessary to explore effective diagnostic methods for early diagnosis of NPC.

Previous studies have reported that microRNAs (miRNAs) may block the expression of target genes, thereby regulating the progression of diseases and cancers[8]. In particular, many miRNAs have been proposed to regulate the progression of NPC. MiR-130a-3p suppressed cell viability, proliferation, and invasion in NPC by inhibiting CXCL12 expression[9]. Xu et al[10] proposed that miR-93 promoted cell invasion and tumor growth *via* targeting Dab2 in NPC. In addition, many miRNAs have been found to modulate other cellular processes of NPC, such as cell cycle[11], apoptosis[12], and NPC radio-sensitivity[13]. Recently, miR-141-3p was found to be abnormally expressed in various human cancers. For example, miR-141-3p was upregulated in prostate cancer and promoted osteoblastic metastasis[14]. By contrast, it has been determined that miR-141-3p suppressed cell proliferation and promot-

Corresponding Authors: Junbo Jiao, MD; e-mail: n2824110316vv@163.com

ed apoptosis *via* targeting GLI2 in osteosarcoma cells[15]. In addition, miR-141 can be used as a tumor regulator and prognostic biomarker in human glioblastoma[16]. However, the function of miR-141-3p in NPC remains unclear and needs to be clarified.

Deleted in liver cancer-1 (DLC1) shares the Rho GTPase-activating protein (RhoGAP) domain with DLC2 and DLC3[17]. Moreover, DLC1 has been reported to be downregulated in pancreatic ductal adenocarcinoma[18]. Furthermore, abnormal expression of DLC1 was associated with tumor metastasis and poor prognosis in urothelial carcinoma[19]. Song et al[20] also reported that DLC1 was a potential therapeutic target and an independent prognostic marker for hepatocellular carcinoma. Notably, DLC1 can serve as an emerging metastasis suppressor gene[21]. However, the interaction between DLC1 and miR-141-3p has not been investigated in NPC.

In the current study, the function of miR-141-3p in tumorigenesis of NPC was confirmed by affecting the expression of DLC1. This will help develop novel biomarkers to diagnose and treat NPC patients.

Patients and Methods

Clinical Tissues

Forty-two human NPC tissues and normal tissues were collected from The People's Hospital of Rizhao and Jining No. 1 People's Hospital. All NPC patients did not receive any treatment before surgery. The tissues were frozen in liquid nitrogen, and then, stored in a refrigerator at -80°C. Informed consents were obtained from all the patients. This work was approved by the Institutional Ethics Committee of People's Hospital of Rizhao and Jining No. 1 People's Hospital.

Cell Lines Culture

The CNE-1, SUNE-1, C666-1 cell lines, and the normal nasopharyngeal epithelial cell line NP69 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cell lines were then inoculated into the Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) medium containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), and cultured at 37°C with 5% CO_2.

Cell Transfection

MiR-141-3p mimics or inhibitor and negative control (NC) were obtained from GeneCopoeia Mettler-Toledo (Columbus, OH, USA). The pcDNA3.1-DLC1 plasmid and the pcDNA3.1 blank vector were purchased from Promega Corporation (Madison, WI, USA). They were then transfected to C666-1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively, based on the manufactures' protocol.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in NPC tissues and cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The synthesis of complementary deoxyribonucleic acids (cDNAs) was performed using the RevertAid Reverse Transcription kit (Thermo Fisher Scientifc, Waltham, MA, USA). We performed qRT-PCR using SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan) on the ABI 7500 Fast system (Applied Biosystems, Waltham, MA, USA). U6 or GAPDH was used as a control for miR-141-3p or DLC1. Their expressions were calculated by the $2^{-\Delta\Delta ct}$ method. The primers used in our work were as follows: miR-141-3p forward primer: 5'-GTA ACA CTG TCT GGT AAA GAT GG-3', reverse primer: 5'-AGA CTG CAC CTG TCC GG-3'; U6, forward primer: 5'-CTC GCT TCG GCA GCA CA-3', reverse primer: 5'-AAC GCT TCA CGA ATT TGC GT-3'; DLC1 forward primer: 5'-CCG CCT GAG CAT CTA CGA-3', reverse primer: 5'-TTC TCC GAC CAC TGA TTG ACT A-3'; GAPDH forward, 5'-ACA TCG CTC AGA CAC CAT G-3', reverse, 5'-TGT AGT TGA GGT CAA TGA AGG G-3'.

Cell Counting Kit-8 (CCK-8) Assay

CCK-8 assay was performed according to the manufacturer's instructions to measure cell proliferation. Then, 5×10^4 cells were plated and incubated in 96-well plates for 0, 24, 48, and 72 h. They were placed in an incubator containing 5% CO_2 at 37°C. Next, 10 μL of CCK-8 reagent was added to each well for 2 h (Dojindo Molecular Technologies, Kumamoto, Japan). Finally, they were detected using a microplate reader (Molecular Devices, Kumamoto, Japan) at an absorbance of 450 nm.

Transwell Assay

Cell migration and invasion assays were performed using transwell chambers (8-μm pore size membranes). The lower chamber was added with

10% FBS and incubated with 5% CO_2 at 37°C. The upper surface with Matrigel (BD Biosciences, San Jose, CA, USA) was then used for cell invasion. In addition, cell migration assay was performed without Matrigel. C666-1 cells with miR-141-3p mimic or inhibitor were cultured in a serum free upper chamber. After 48 h, the migrated or invaded cells were fixed with methanol and stained with crystal violet. Finally, we calculated the number of cells using a microscope.

Dual-Luciferase Assay

The 3'-untranslated region (3'-UTR) of wild-type or mutant DLC1 was inserted into the pmirGLO Luciferase vector (Promega, Madison, WI, USA) for Luciferase reporter experiments. Then, the above Luciferase vector and miR-141-3p mimics were transfected into C666-1 cells. Finally, Luciferase activity was measured by a Dual-Luciferase assay system (Promega, Madison, WI, USA).

Immunohistochemistry (IHC)

Ovarian tissue sections were dewaxed, hydrated, and washed twice with phosphate-buffered saline (PBS) for 5 min. After blocking with 5% goat serum (diluted in PBS), we incubated the cells with anti-DLC1 antibody for 1-2 h at 37°C. Then, we washed them for three times with PBS for 5 min. Next, we incubated them with the appropriate secondary antibody for 1 h at 37°C. After washing 3 times with PBS, diaminobenzidine (DAB) mixture was used for color development of this section. The sections were washed, counterstained, dehydrated, transparentized, and fixed. Images were captured using a microscope.

Western Blot Analysis

The protein samples were obtained using radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The protein was then separated by 10% dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE), and blocked with 5% skim milk. Next, the protein was transferred into polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland) at room temperature. We incubated the membranes with E-cadherin, N-cadherin, vimentin, DLC1, mTOR, p-mTOR, and GAPDH antibodies overnight at 4°C. After washing, they were incubated with the corresponding secondary antibody for 2 h at room temperature. Finally, protein expression levels were measured by enhanced chemiluminescence (ECL; Pierce, Rockford, IL, USA).

Statistical Analysis

Data are shown as mean ± SD (standard deviation). Statistical Product and Service Solutions (SPSS) 19.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 6 (GraphPad, La Jolla, CA, USA) were used to analyze these data. Differences between the two groups were analyzed using Student's *t*-test. Comparisons between multiple groups were performed using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Survival curves were plotted by Kaplan-Meier analysis, and survival differences were compared using log-rank test. Significant differences were defined as $p<0.05$.

Results

MiR-141-3p Was Upregulated in NPC Tissues

The expression of miR-141-3p was detected in NPC tissues *via* qRT-PCR experiment. A significant upregulation of miR-141-3p was observed in NPC tissues compared to normal tissues (Figure 1A). In addition, miR-141-3p expression was found to be associated with T stage ($p = 0.006$) and clinical stage ($p = 0.042$, Table I). These results suggested that abnormal miR-141-3p expression may be involved in the development of NPC. Besides that, high expression of miR-141-3p was associated with poor prognosis in NPC patients ($p = 0.0476$, Figure 1B). These results indicated that miR-141-3p can predict the prognosis of NPC patients.

MiR-141-3p Promoted Cell Proliferation, Migration, and Invasion in NPC

Then, the expression of miR-141-3p was measured in CNE-1, SUNE-1, C666-1, and NP69 cell lines. Similarly, upregulation of miR-141-3p was examined in the CNE-1, SUNE-1, and C666-1 cell lines *vs.* GES-1 cells (Figure 2A). Next, the miR-141-3p mimic or inhibitor was transfected into C666-1 cells to investigate its role in NPC. Then, miR-141-3p expression levels were observed in those transfected cells through qRT-PCR assay (Figure 2B). After transfection, we performed CCK-8 and transwell assays to investigate the function of miR-141-3p. Overexpression of miR-141-3p promoted cell proliferation in NPC (Figure 2C). By contrast, the knockdown of miR-141-3p suppressed proliferation of C666-1 cells (Figure 2D). In addition, miR-141-3p overexpression also promoted cell migration, while the knockdown of

Table I. Relationship between miR-141-3p expression and clinic-pathological characteristics in NPC patients.

Characteristics	Cases	miR-141-3p High	miR-141-3p Low	*p*-value
Age (years)				0.633
≥50	22	14	8	
<50	20	15	5	
Gender				0.303
Male	24	17	7	
Female	18	12	6	
T stage				0.006*
T1-T2	10	7	3	
T3-T4	32	22	10	
N stage				0.058
N0-N1	15	10	5	
N2-N3	27	19	8	
M stage				0.318
M0	25	18	7	
M1	17	11	6	
Clinical stage				0.042*
I-II	30	22	8	
III-IV	12	7	5	

Statistical analyses were performed by the χ^2-test. *p<0.05 was considered significant.

miR-141-3p inhibited migration of C666-1 cells (Figure 2E). Similarly, the same effect of miR-141-3p on cell invasion was also observed in C666-1 cells (Figure 2F). All these results suggested that miR-141-3p played a carcinogenic role in NPC.

MiR-141-3p Promoted EMT and Activated the mTOR Signaling Pathway in NPC

To further verify the above results, we investigated how miR-141-3p regulates epithelial-mesenchymal transition (EMT) and mammalian targets of the rapamycin (mTOR) signaling pathway in NPC. Western blot analysis showed that the upregulation of miR-141-3p inhibited E-cadherin expression and promoted N-cadherin and Vimentin expressions in C666-1 cells (Figure 3). Downregulation of miR-141-3p was found to have the opposite effect (Figure 3). In addition, we speculate that miR-141-3p can activate the mTOR signaling pathway. Therefore, protein expression of mTOR and p-mTOR was evaluated in C666-1 cells regulated by miR-141-3p mimics or inhibitor. As we predicted, miR-141-3p overexpression was observed to promote p-mTOR expression (Figure 3), while downregulation of miR-141-3p reduced p-mTOR expression (Figure 3). Moreover, the miR-141-3p mimics or inhibitor in NPC does not affect the expression of mTOR. These findings indicated that miR-141-3p may regulate NPC cell metastasis and proliferation by affecting EMT and mTOR signaling pathway.

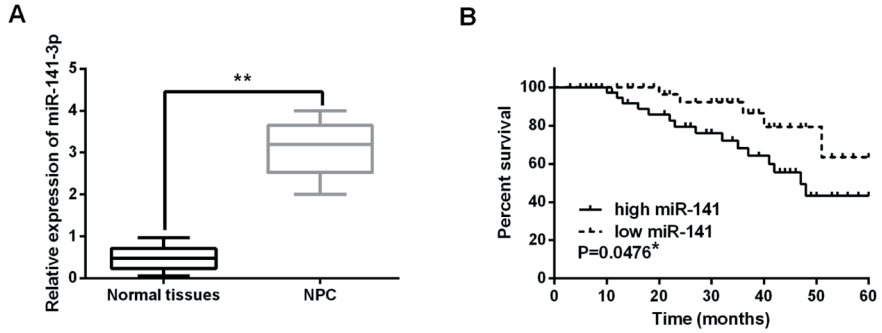

Figure 1. Upregulation of miR-141-3p was identified in NPC tissues. **A,** The expressions of miR-141-3p in NPC tissues detected *via* qRT-PCR. **B,** High miR-141-3p expression was correlated with shorter overall survival of NPC patients. *p<0.05, ** p<0.01.

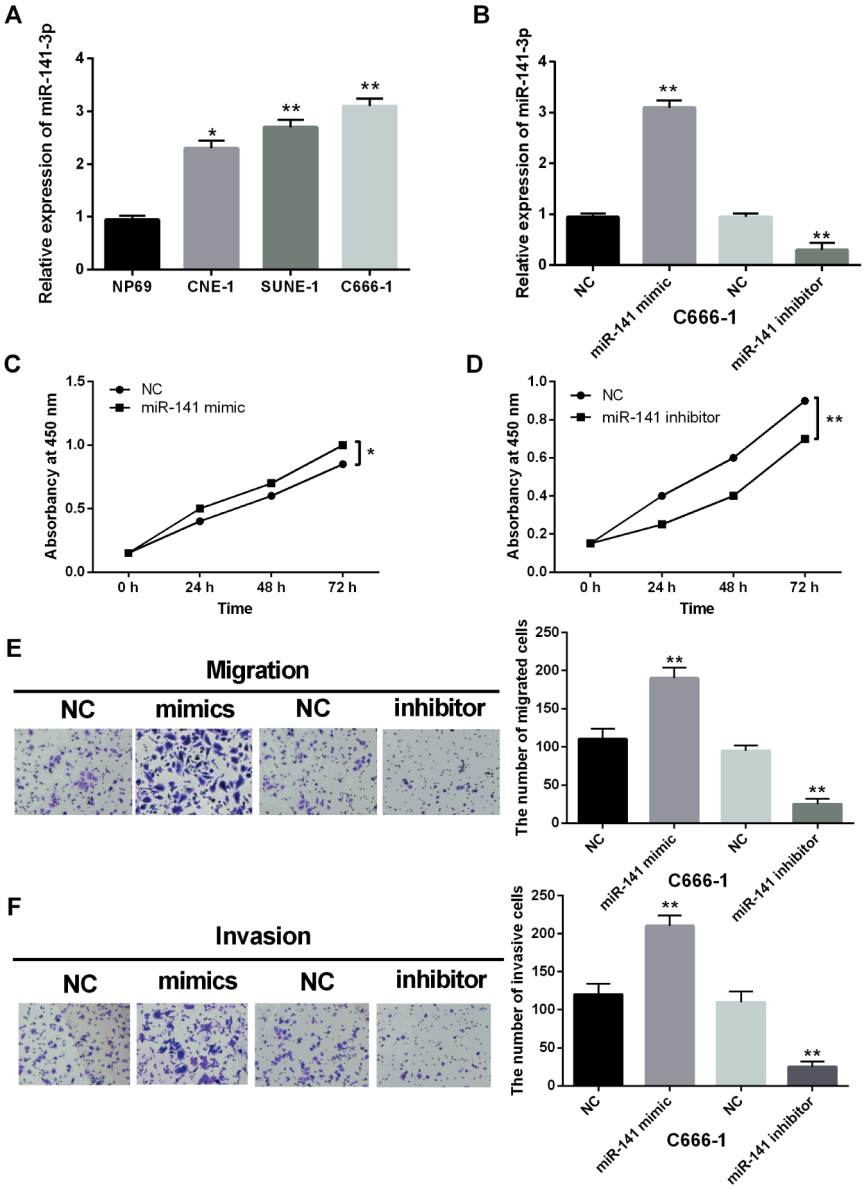

Figure 2. MiR-141-3p promoted cell proliferation, migration and invasion in NPC. **A,** The miR-141-3p expression in CNE-1, SUNE-1, C666-1 and NP69 cell lines. **B,** The expression of miR-141-3p was examined in C666-1 cells with miR-141-3p mimics or inhibitor *via* qRT-PCR. **C-D,** Cell proliferation was measured in cells containing miR-141-3p mimics or inhibitor via MTT assay. **E-F,** Cell migration and invasion analysis in cells containing miR-141-3p mimics or inhibitor was determined by transwell assay (magnification, 200x). * $p<0.05$, ** $p<0.01$.

DLC1 Was a Direct Target of MiR-141-3p in NPC Cells

The downstream targets of miR-141-3p were investigated in NPC. Based on the prediction of TargetScan (http://www.targetscan.org/), we found that DLC1 has a binding site with miR-141-3p (Figure 4A). Then, a Luciferase reporter assay was performed to verify the prediction. The Luciferase activity of Wt-DLC1 was disclosed to be distantly reduced by miR-141-3p mimics. However, it was not found to affect the Luciferase activity of Mut-DLC1 (Figure 4B). Furthermore, DLC1 expression has been identified to be negatively correlated with miR-141-3p expression in NPC tissues ($p<0.01$, $R^2=0.3183$; Figure 4C). In addition, DLC1 expression was detected in C666-1 cells with miR-141-3p mimics or inhibitor. We found that overexpression of miR-141-3p reduced the expression of DLC1 (Figure 4D), while the knockdown of miR-141-3p enhanced expression

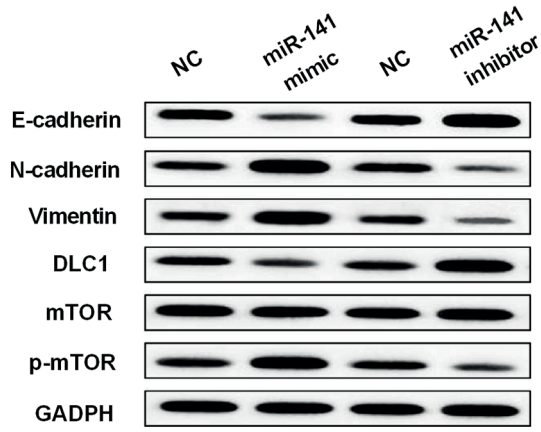

Figure 3. MiR-141-3p promoted EMT and activated the mTOR signaling pathway in NPC. Western blot analysis of E-cadherin, N-cadherin, Vimentin, mTOR, and p-mTOR in C666-1 cells contained miR-141-3p mimics or inhibitor.

of DLC1 in NPC (Figure 4E). Briefly, miR-141-3p directly targets DLC1 that are negatively correlated with each other in NPC cells.

MiR-141-3p Promoted the Progression of Nasopharyngeal Carcinoma through Targeting DLC1

Next, abnormal expression of DLC1 was measured in NPC tissues. IHC experiment suggested that the expression of DLC1 was characterized by positive cytoplasm staining in NPC cells (Figure 5A). It was also suggested that DLC1 expression was lower in NPC tissues than in normal tissues (Figure 5B). Then, miR-141-3p mimics and DLC1 vector were co-transfected into C666-1 cells to explore the interaction between miR-141-3p and DLC1. qRT-PCR assay showed that the decreased expression of DLC1 induced by miR-141-3p mim-

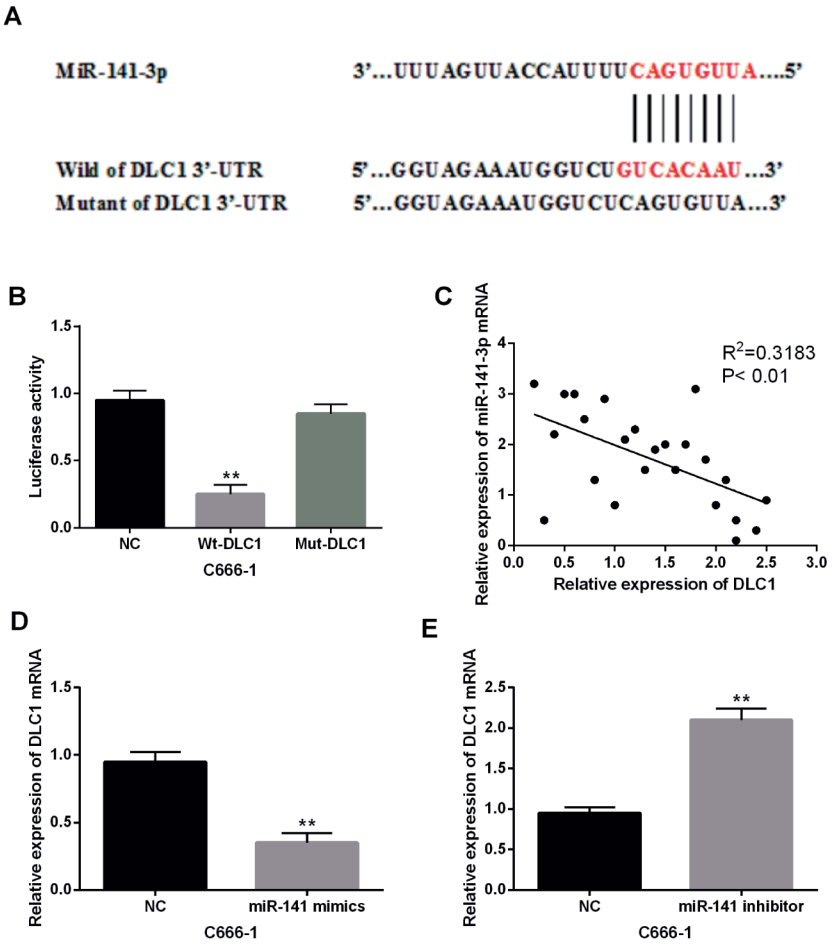

Figure 4. MiR-141-3p directly targets DLC1 in NPC cells. **A,** DLC1 has a binding site with miR-141-3p. **B,** Luciferase reporter assay. **C,** MiR-141-3p had negative correlation with DLC1 in NPC tissues. **D, E,** The expression of DLC1 were observed in C666-1 cells containing miR-141-3p mimics or inhibitor ** p <0.01.

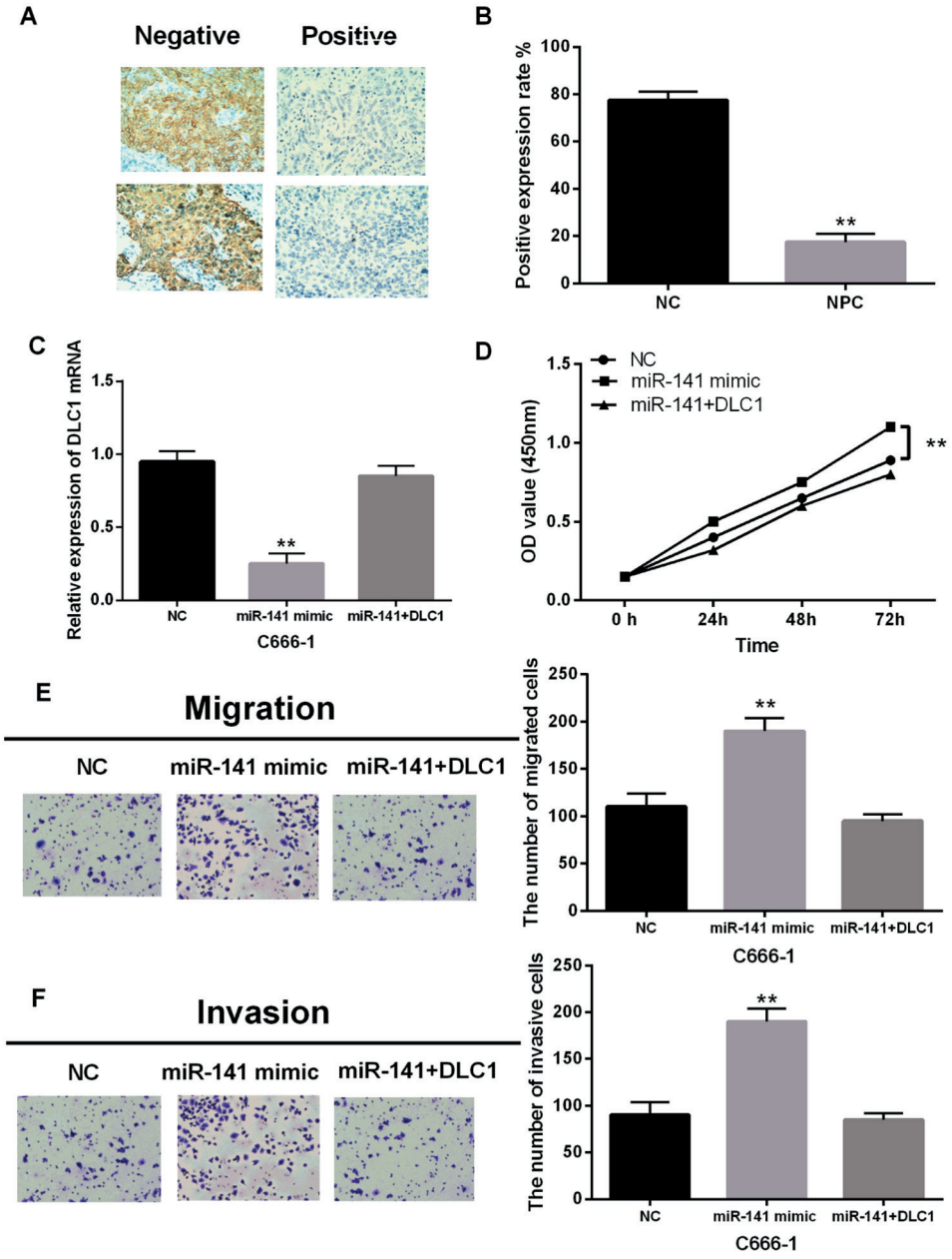

Figure 5. MiR-141-3p promoted the progression of nasopharyngeal carcinoma through targeting DLC1. **A-B,** The protein expression of DLC1 in NPC tissues detected by immunohistochemistry (magnification, 100x). **C,** The expression of DLC1 was measured in C666-1 cells with DLC1 vector and miR-141-3p. **D,** The cell proliferation was measured in C666-1 cells with DLC1 vector and miR-141-3p via MTT. **E-F,** The cell migration and invasion in C666-1 cells with DLC1 vector and miR-141-3p was measured by transwell assay (magnification, 200x). ** $p<0.01$.

ics was recovered by DLC1 vector in C666-1 cells (Figure 5C). Furthermore, DLC1 overexpression impaired the effect of miR-141-3p on cell proliferation in C666-1 cells (Figure 5D). Consistently, the same results were also identified for cell migration (Figure 5E) and invasion (Figure 5F) in NPC. Collectively, miR-141-3p promoted cell proliferation, migration, and invasion by targeting DLC1 in NPC.

Discussion

Recently, many miRNAs have been found to be involved in the process of NPC. Of note, the up-

regulation of miR-93 was observed to enhance cell proliferation by suppressing CDKN1A in NPC[22]. Yan et al[23] proposed that miR-346 promoted cell migration and invasion *via* regulating BRMS1 in NPC. As in the above results, in our study, it was also assessed that upregulation of miR-141-3p promoted cell proliferation, migration, and invasion in NPC. Besides that, the overexpression of miR-141-3p was found to promote EMT and activate the mTOR signaling pathway in NPC. In addition, high miR-141-3p expression was associated with shorter overall survival in NPC patients.

It has been reported that miR-141-3p is involved in the development of many human cancers, such as breast cancer[24], hepatocellular carcinoma[25], and osteosarcoma[26]. Consistent with our results, miR-141 was upregulated in prostate cancer[27]. At the same time, miR-141-3p was also found to promote cell proliferation by suppressing the expression of KLF9 in prostate cancer, similar to our findings in NPC[28]. Furthermore, overexpression of miR-141 was found to promote cell migration and invasion in triple-negative breast cancer cells[29]. In this study, the overexpression of miR-141 in NPC also promoted cell migration and invasion. In addition, miR-141 can be used as a potential diagnostic and prognostic biomarker for ovarian cancer, and low expression of miR-141 predicted good prognosis in ovarian cancer patients[30]. Here, low miR-141-3p expression was also correlated with good prognosis in NPC patients. Furthermore, miR-141-3p directly targets DLC1 and was negatively correlated with DLC1 expression in NPC cells. This is a novelty of our work that has not been reported in previous studies.

MiR-141-3p has been reported to directly target many genes, such as DAPK1[31], ATF5[32], and GAB1[33]. Wu et al[34] revealed that miR-141 can regulate the tumor suppressor DLC1 in colorectal cancer, which is consistent with our findings. The expression of DLC1 was found to be decreased in human cutaneous melanoma[35], and the downregulation of DLC1 was also examined in NPC. Notably, DLC-1 was also found to inhibit proliferation and migration of human NPC cells[36]. In this study, we also found the inhibitory effect of DLC1 on NPC development. Also, miR-106b promoted migration and invasion of colorectal cancer cells by directly targeting DLC1[37]. Similarly, miR-141-3p promoted cell proliferation, migration, and invasion by targeting DLC1 in NPC. Another important novelty of this research is that miR-141-3p acts as an oncogene in NPC by promoting EMT and activating the mTOR signaling pathway.

Conclusions

In this investigation, we demonstrated that upregulation of miR-141-3p was found in NPC, which predicted a poor prognosis in NPC patients. Moreover, miR-141-3p promoted proliferation, migration, and invasion of NPC cells by targeting DLC1. Also, miR-141-3p promoted EMT and activated the mTOR signaling pathway in NPC. These findings will provide new ideas for the diagnosis and treatment of NPC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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