

Original Research Article

Depletion of circ_0001313 suppresses the carcinogenesis of laryngeal carcinoma through miR-512-5p/HMGA1 pathway

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Abstract

Purpose: To probe the influence of circ_0001313 on the occurrence and development of laryngeal carcinoma and the potential molecular mechanism.

Methods: 27 cases of laryngeal carcinoma attending health facility from January 2017 to September 2020 were obtained. The contents of circ_0001313, miR-512-5p and HMGA1 in the tissues were detected. Laryngeal carcinoma TU686 cells were randomly divided into different group via assigned transfection, and cell growth and mobility were evaluated. The investigation of the relationship was then determined.

Results: Laryngeal carcinoma tissues and TU686 cell lines showed high expressed circ_0001313 and HMGA1, as well as low expressed miR-512-5p. Circ_0001313 deficiency or miR-512-5p overexpression weakened the survival, migration and invasiveness of TU686 cells. Circ_0001313 sponged miR-512-5p, which bound to HMGA1. In TU686 cells, the content of miR-512-5p was markedly increased with circ_0001313 depletion, and HMGA1 protein level was decreased with miR-512-5p upregulation. The deficiency of miR-512-5p weakened the influence of circ_0001313 depletion on the growth and mobility of TU686 cells.

Conclusion: Interference of circ_0001313 could impair the migration, proliferation, and invasiveness of laryngeal carcinoma cells and promote cell apoptosis by regulating miR-512-5p/HMGA1 axis.

Keywords: circ_0001313, laryngeal carcinoma, miR-512-5p, proliferation, HMGA1, apoptosis

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INTRODUCTION

Laryngeal carcinoma is a malignancy derived from head and neck. Due to the limitations of current treatment methods, recurrence and metastasis lead to poor prognosis of patients [1,

2]. Molecular targeted therapy has exhibited great clinical success in multiple cancers, as it can effectively and selectively kill tumor cells and reduce the damage to normal cells, and hence can be used as the research subject for laryngeal carcinoma therapy [3]. Accumulating proofs

substantiated that the malignant development of a variety of human malignancies was linked with the deregulated circular RNAs (circRNAs), which were deemed as promising therapeutic target in cancers [4]. Also known as circCCDC66, hsa_circ_0001313 is a new-found circRNA. Colon cancer tissues and cells exhibited highly enrichment of circ_0001313, and circ_0001313 deficiency weakens the growth of colon cancer cells [5]. Circ_0001313 lack has been reported to reduce the radioresistance of colon cancer cells via declining the content of miR-338-3p in a target manner [6]. CircCCDC66 accelerated the invasiveness and growth of gastric cancer by sponging miR-1238-3p [7]. Deficiency of circCCDC66 expression decreased the proliferative, invasive and migratory capacities of NSCLC cells [8]. However, the functions of circ_0001313 on the progression of laryngeal carcinoma and the possible mechanism are still unclear.

In this project, online biological tools were adopted to explore the targets of circ_0001313 and the complementary sequences of miR-512-5p on circ_0001313 or HMGA1. It was evidenced that miR-512-5p declined the ETS1 level in NSCLC cells, and then impaired cancer cells survival [9]. Moreover, miR-512-5p reduced HNSCC cell growth [10]. As reported, laryngeal carcinoma showed higher HMGA1 levels [11, 12]. Depletion of HMGA1 in gastric cancer cells restrained cell proliferative and invaded capacities [13]. Whereas, miR-512-5p functions in laryngeal carcinoma development, and whether it is attributed to targeting HMGA1 are confusing. Additionally, it is unclear whether circ_0001313 regulates miR-512-5p. Therefore, the current study focused on probing the impact of circ_0001313 on laryngeal carcinoma carcinogenesis, as well as the involvement of miR-512-5p and HMGA1.

METHODS

Collection of clinical samples

The current assay was ethically permitted by the Ethic Committee of The First Affiliated Hospital, Medical College, Xiamen University. Tumor tissues and adjacent normal tissues were resected by surgery in 27 cases of laryngeal carcinoma patients at The First Affiliated Hospital, Medical College, Xiamen University from January 2017 to September 2020. All participators afforded written informed consent.

Reagent

Laryngeal carcinoma cell line TU686 was

commercially obtained from ATCC (Manassas, VA, USA); HyClone (Logan, UT, USA) provided RPMI-1640 medium; Invitrogen (Carlsbad, CA, USA) provided TRIzol Reagent; Reverse transcription kit and qRT-PCR kit were obtained from TaKaRa (Shiga, Japan); Protein extraction kit was purchased from ChunduBio (Wuhan, China); MTT and apoptosis detection kits were bought from Dojindo (Kumamoto, Japan); Transwell chambers and Matrigel were purchased from Corning (Corning, NY, USA); BioAssay Systems (USA) provided the kit for bindings analysis.

Cell transfection

SiRNA specially for circ_0001313 (si-circ_0001313), miR-512-5p mimics and the controls were respectively introduced into TU686 cells. Additionally, si-circ_0001313 and miR-512-5p inhibitor (anti-miR-512-5p) were co-introduced into TU686 cells.

QRT-PCR assay

The isolation of total RNA from cancer tissues, matched adjacent normal tissues and TU686 cells in all groups was performed utilizing TRIzol reagent. cDNAs were generated at following condition: 42°C for 1 h, 95°C for 5 min. Subsequently, qRT-PCR was implemented in line with the manufacturer's instruction. Then, fold changes were calculated via $2^{-\Delta\Delta C_t}$ approach [14], with the internal control of GAPDH (for circ_0001313) or U6 (for miR-512-5p). The Forward (F) and Reverse (R) Primers were supplied by Sangon Inc. (Shanghai, China): circ_0001313, 5'-CTGGAGCACCTTTTCAGTGC-3' and 5'-TGCTCCTCCTCTGATCTGTCA-3'; GAPDH, 5'-ATTGTTGCCATCAATGACCC-3' and 5'-AGTAGAGGCAGGGATGATGT-3'; miR-512-5p, 5'-CCTGGCCAATATGGTGAAACC-3' and 5'-TGCCATGGAGCGATCATCAG-3'; U6, 5'-CGCAAGGATGACACG-3' and 5'-GAGCAGGCTGGAGAA-3'.

Western blot analysis

RIPA lysis was applied to obtain total protein. Total protein (30 µg) was subjected to SDS-PAGE, followed by transferring onto the PVDF membranes. After blockage with 5% skim milk, primary antibodies anti-HMGA1 or anti-GAPDH were utilized to incubate with the blocked membranes. Tris Buffered Saline Tween (TBST) was then used for washing. Next, secondary incubation was conducted with HRP-labeled antibodies for 1 h at 37 °C. Protein signal was developed and analyzed.

Colony formation assay

TU686 cells derived from each group were sowed in 6-well plates and maintained at 37°C for 14-21 days until visible colonies generated. After discarding culture medium, colonies were rinsed with PBS for twice, dyed with gissam dye for 10 min, rinsed again and dried in air. Then colonies were counted under microscope.

MTT assay

For evaluation of cell viability, TU686 cells at log phase were cultured for 1, 2 or 3 days, then 20 μ L MTT was pipetted into every well of 96-well plates. At 4 h post reaction, we added DMSO to each well, which were shaken for 10 min. After discarding the liquid, the OD value at 490 nm was tested.

Flow cytometry

Collected cells were suspended in PBS solution. Then 1×10^6 cells were suspended in 500 μ L binding buffer with 5 μ L Annexin V-FITC and 5 μ L PI, consecutively. Apoptotic TU686 cells were identified via a flow cytometer.

Wound healing assay

To evaluate the migratory capability of TU686 cells, transfected cells were cultured at 37°C until cell confluence reached 80%. Then a bioclean pipette (200 μ L) was exploited to create a scratch throughout the cell monolayer. After washing away the detached cells, cells were maintained at 37°C for 24 h. The images at 0 h and 24 h post scratch-making were captured, followed by the measurement of cell migration distance.

Transwell assay

The 24-well plate was placed on flat ice and then were gently move into the Transwell chamber

using sterile tweezers. Taking an appropriate amount of RPMI-1640 medium into Tramwell chamber to the chamber moist, then diluted Matrigel were spread onto the Tramwell cell polycarbonate film at a volume of 35 μ L. Then TU686 cells were transferred into the upper chambers containing RPMI-1640 medium without serum, with complete medium in lower chambers. Invaded cells were counted utilizing a microscope at 24 h after Giemsa staining for 45 min.

Dual-luciferase reporter assay

The sequences of circ_0001313 and HMGA1 3' UTR contained miR-512-5p binding sites or mutant sequences in binding sites was cloned into pmirGLO plasmid (GenePharma, Shanghai, China). Until cell confluence reached 80%, constructed luciferase report vectors were introduced into TU686 cells in combination with miR-512-5p or miR-NC. Luciferase activity was evaluated at 48 h, and the activity of renal luciferase was regarded as an internal reference.

Statistical analysis

All data in this project were expressed as mean \pm standard deviation and processed using SPSS 20.0 software. Student's *t*-test was implemented for comparison. *P* < 0.05 implied the significant difference.

RESULTS

The expression of circ_0001313, miR-512-5p and HMGA1 in laryngeal carcinoma

As shown in Figure 1, circ_0001313 expression and protein level of HMGA1 in laryngeal carcinoma were obviously higher than those in normal tissues. Inversely, laryngeal carcinoma tissues relative to normal tissues exhibited low expressed miR-512-5p expression.

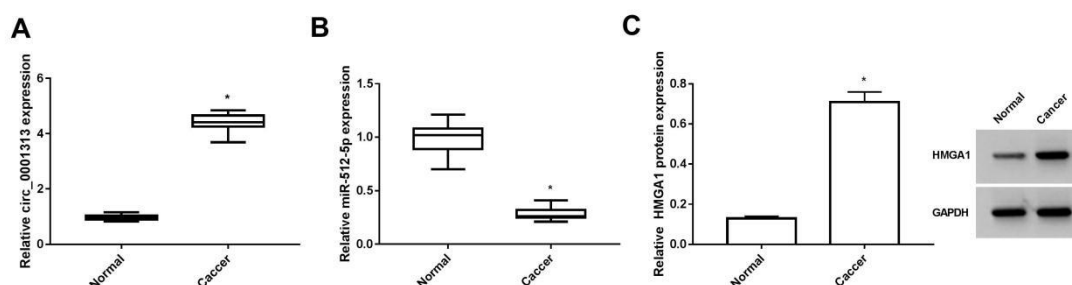


Figure 1: Detection of the expression of circ_0001313, miR-512-5p and HMGA1
 A: QRT-PCR assay for the circ_0001313 expression in laryngeal carcinoma tissues and normal tissues. B: QRT-PCR assay for the miR-512-5p expression in laryngeal carcinoma tissues and normal tissues. C: Western blot assay for the HMGA1 protein expression in laryngeal carcinoma tissues and normal tissues. **P*<0.05.

Effects of circ_0001313 depletion on TU686 cell oncogenic phenotypes

Circ_0001313 deficiency caused the remarkably reduced circ_0001313 expression and HMGA1 protein level, as well as enhanced miR-512-5p expression in TU686 cells (Figure 2A-C). Circ_0001313 lack markedly impaired clonogenicity in TU686 cells which was disclosed

by colony formation assay (Figure 2D). Following MTT assay uncovered the decreased cell viability of TU686 cells transfected with si-circ_0001313 (Figure 2E). Additionally, knockdown of circ_0001313 increased apoptosis rate of TU686 cells (Figure 2F). In addition, circ_0001313 knockdown repressed the migratory and invasive abilities of TU686 cells (Figure 3)

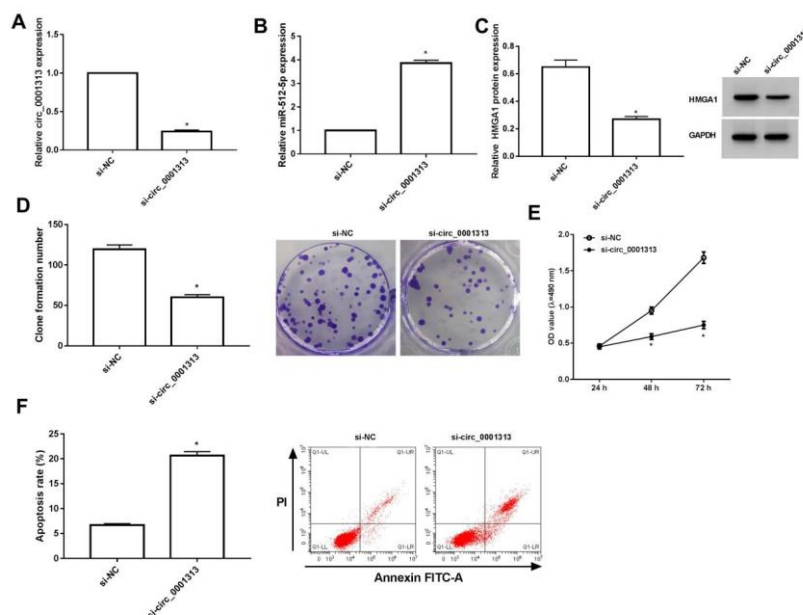


Figure 2: Effect of interference with circ_0001313 on the proliferation and apoptosis of TU686 cells. TU686 cells were transfected with si-NC or si-circ_0001313. A: QRT-PCR assay for the circ_0001313 expression in transfected cells. B: QRT-PCR assay for the miR-512-5p expression in transfected cells. C: Western blot assay for the HMGA1 protein expression in transfected cells. D: Colony formation assay for the clonogenicity in transfected cells. E: MTT assay for the cell viability of transfected cells. F: Flow cytometry for the apoptosis of transfected cells. * $P < 0.05$.

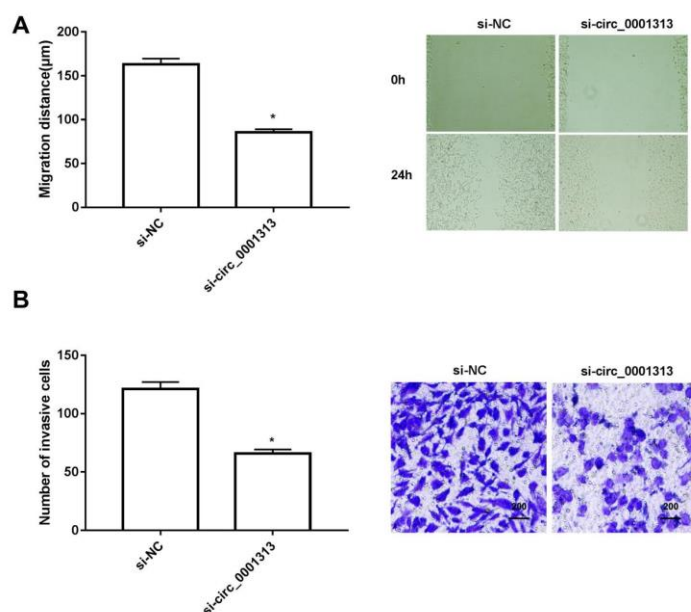


Figure 3: Effect of circ_0001313 depletion on the migration and invasion of TU686 cells. TU686 cells were transfected with si-NC or si-circ_0001313. A: Wound healing assay for the migration in transfected cells. B: Transwell assay for the invasion in transfected cells. * $P < 0.05$.

Target analysis among miR-512-5p, HMGA1 and circ_0001313

Circular RNA Interactome and Targetscan were adopted to predict the underlying network of circ_0001313, and the complementary sequences of miR-512-5p on circ_0001313 and HMGA1 were identified (Figure 4A). Furthermore, the luciferase activity of WT-circ_0001313 or WT-HMGA1 in miR-512-5p-overexpressed TU686 cells was significantly decreased, whereas, little change was observed in MUT-circ_0001313 or MUT-HMGA1 group (Figure 4B).

Effects of miR-512-5p on the malignant biological behaviors of TU686 cells

As depicted in Figure 5B and D, miR-512-5p content obviously increased after miR-512-5p mimic introduction in TU686 cells, while

repressed HMGA1 protein level was discovered after introduction. MiR-512-5p content rise efficiently decreased clonogenicity in TU686 cells (Figure 5A). In addition, miR-512-5p mimic elevated apoptotic rate of TU686 cells (Figure 5C). Meanwhile, the accumulation of miR-512-5p weakened migration and invasiveness of TU686 cells (Figure 5E-F). Moreover, upregulation of miR-512-5p decreased cell viability of TU686 cells, which was disclosed by MTT assay (Figure 5G).

Co-effect of circ_0001313 interference and miR-512-5p inhibition on TU686 cells

Results revealed that si-circ_0001313-mediated the reduced cell viability, expression level of HMGA1 protein, enhanced miR-512-5p content, repressed clonogenicity, migration and invasiveness of TU686 cells were all attenuated by miR-512-5p inhibitor (Figure 6 and Figure 7).

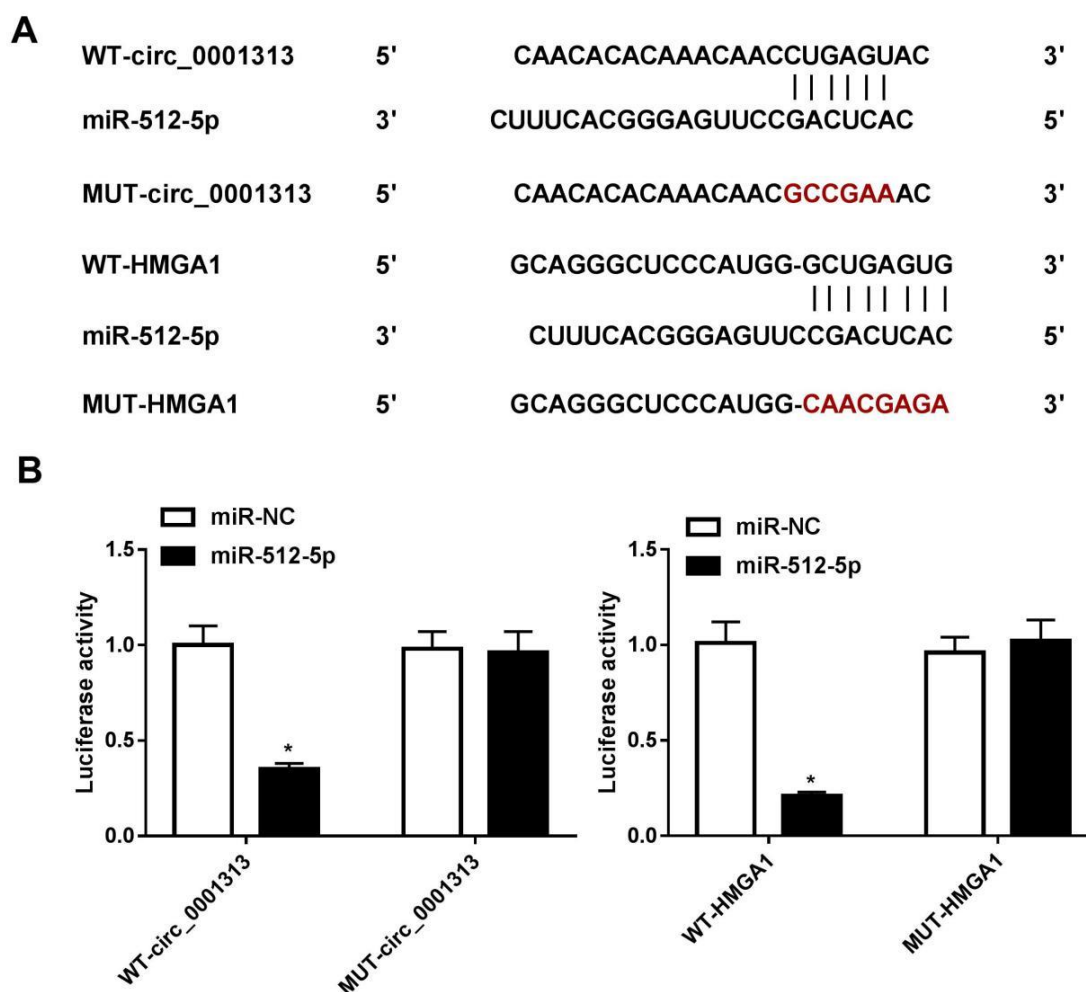


Figure 4: Verification of target relationship between miR-512-5p and circ_0001313 or HMGA1. A: Complementary sequence between miR-512-5p and circ_0001313 or HMGA1. B: Dual-luciferase reporter assay for the luciferase activity of WT-circ_0001313, MUT-circ_0001313, WT-HMGA1 and MUT-HMGA1 in TU686 cells co-transfected with miR-NC or miR-512-5p. * $P < 0.05$.

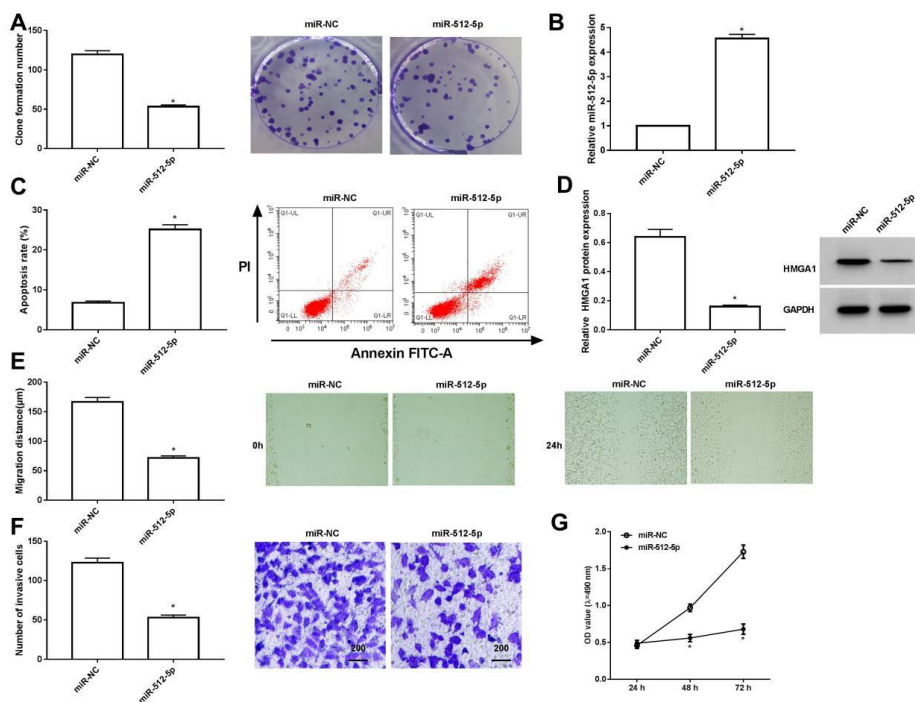


Figure 5: Effect of miR-512-5p on proliferation, apoptosis, migration and invasion of TU686 cells. TU686 cells were transfected with miR-NC or miR-512-5p. A: Colony formation assay for the clonogenicity in transfected cells. B: QRT-PCR assay for the miR-512-5p expression in transfected cells. C: Flow cytometry for the apoptosis of transfected cells. D: Western blot assay for the HMGA1 protein expression in transfected cells. E: Wound healing assay for the migration in transfected cells. F: Transwell assay for the invasion in transfected cells. G: MTT assay for the cell viability of transfected cells. * $P < 0.05$.

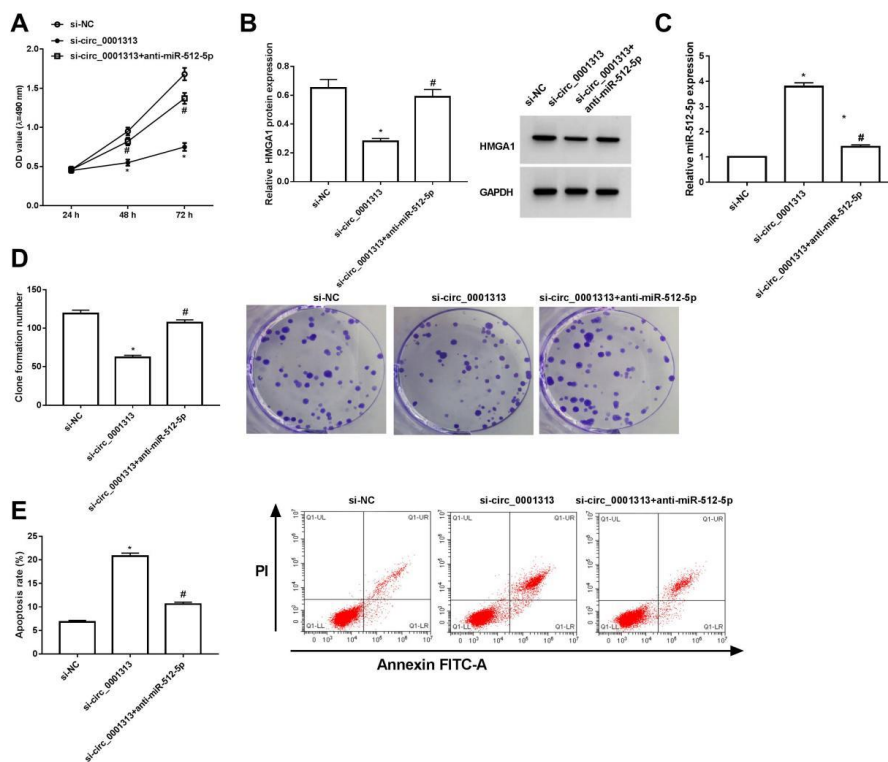


Figure 6: Co-effect of circ_0001313 interference and miR-512-5p inhibition on proliferation and apoptosis of TU686 cells. TU686 cells were transfected with si-NC, si-circ_0001313 or si-circ_0001313+anti-miR-512-5p. A: MTT assay for the cell viability of transfected cells. B: Western blot assay for the HMGA1 protein expression in transfected cells. C: QRT-PCR assay for the miR-512-5p expression in transfected cells. D: Colony formation assay for the clonogenicity in transfected cells. E: Flow cytometry for the apoptosis of transfected cells. * $P < 0.05$.

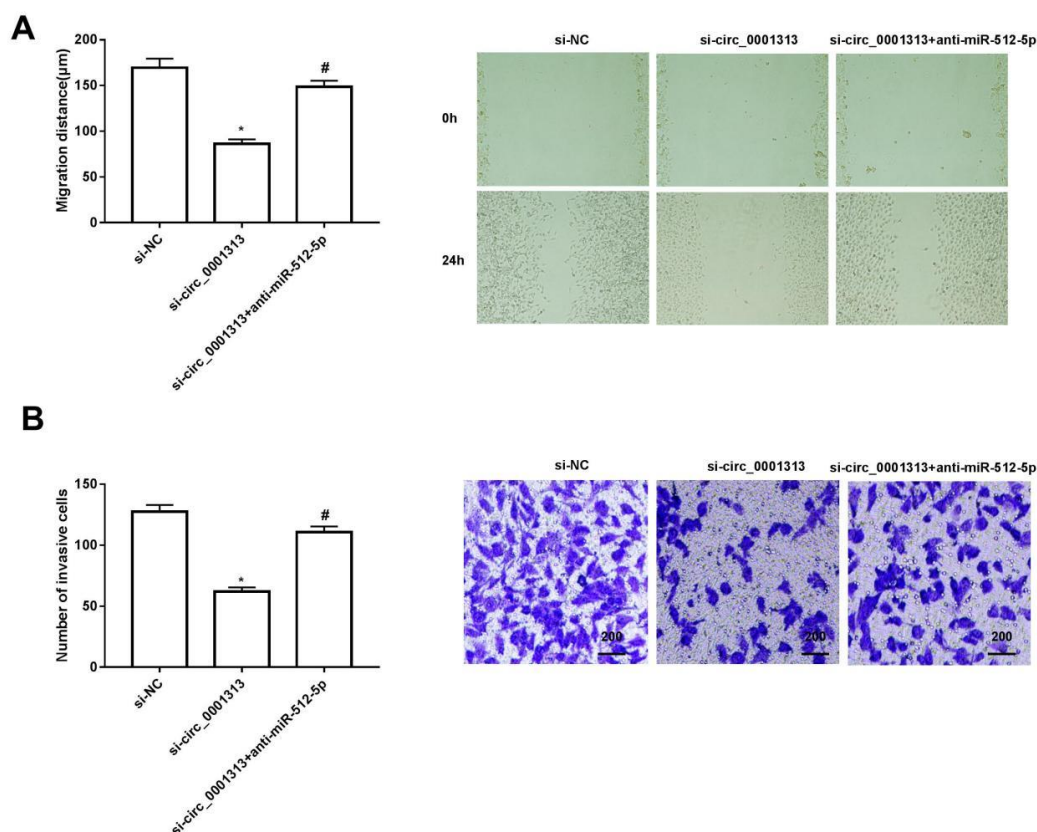


Figure 7: Co-effect of circ_0001313 interference and miR-512-5p inhibition on migration and invasion of TU686 cells. TU686 cells were transfected with si-NC, si-circ_0001313 or si-circ_0001313+anti-miR-512-5p. A: Wound healing assay for the migration in transfected cells. B: Transwell assay for the invasion in transfected cells. * $P < 0.05$.

DISCUSSION

Studies proved that the deregulated circRNAs was connected with multiple human cancers. CircRNAs are often considered as promising biomarkers for cancer diagnosis and treatment [15,16]. It was reported that circCCDC66 aggravated the malignant phenotypes of osteosarcoma cells via miR-338-3p and PTP1B pathway [17]. The abundance of circCCDC66 is increased in cervical cancer. CircCCDC66 deficiency in cervical cancer cells impede cell survival and mobility through miR-452-5p/REXO1 axis [18]. Depletion of circCCDC66 in gastric cancer cells significantly curbed cell proliferative ability and mobility [19]. Nevertheless, the function of circ_0001313 in laryngeal carcinoma has not been uncovered. In our data, laryngeal carcinoma tissues manifested increased circ_0001313 expression levels relative to normal tissues. Interference of circ_0001313 hindered the growth and mobility of TU686 cells, indicating that circ_0001313 was an oncogenic factor in laryngeal carcinoma.

MiR-512-5p hamper NSCLC tumorigenesis by targeting β -catenin [20]. The growth of colorectal

cancer cells is boosted by circ-0004277 via increasing PTMA through sequestering miR-512-5p [21]. A declined miR-512-5p content in laryngeal carcinoma was observed in this study. Enforced miR-512-5p expression suppressed the mobility and survival of TU686 cells suggesting the tumor-suppressor role. In addition, miR-512-5p was verified to interact with HMGA1. Furthermore, previous studies have shown that HMGA1 contributed to liver metastasis in colon cancer via stimulating GLUT3 accumulation [22]. Additionally, PD-L1 was able to crucially facilitate the expansion of colorectal cancer self-renewal via mediating the activation of HMGA1-dependent pathways [23]. MiR-125b induced cervical cancer growth by altering HMGA1 [24]. MiR-195 targetedly declined HMGA1 in renal cell carcinoma, thereby hindering the survival and metastasis of tumor cells [25]. Therefore, HMGA1 might exert key roles in tumorigenesis of many cancers. Moreover, miR-512-5p affected laryngeal carcinoma development by targeting HMGA1.

Furthermore, circ_0001313 sponged miR-512-5p and interference of miR-512-5p weakened the impact of circ_0001313 depletion on growth and

mobility of TU686 cells, which suggested that circ_0001313 might influence laryngeal carcinoma progression by miR-512-5p. Former research uncovered that circ_0067835 increased HMGA1 expression in endometrial cancer cells to facilitate cell metastasis and growth via segregating miR-324-5p [26]. Therefore, circRNAs could affect the expression of HMGA1 by sponging miRNAs. Here, interference of circ_0001313 decreased the content of HMGA1, which was weakened through the deficiency of miR-512-5p.

CONCLUSION

Depletion of circ_0001313 partially inhibited migration, growth, and invasiveness of laryngeal carcinoma TU686 cells through regulation in miR-512-5p/HMGA1 axis.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xiaofeng Wang¹ and Qiaoling Guo¹ contributed to the study design, data analysis, writing and revision equally. Xianyang Luo, Junhua Wu and Bangyang Zhang contributed to the experiments, data formation and revision. Yi Zhou contributed to the study design and revision.

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