European Review for Medical and Pharmacological Sciences 2020; 24: 4320-4327

Circ_0067934 correlates with poor prognosis and promotes laryngeal squamous cell cancer progression by sponging miR-1324

Y.-L. CHU

Department of Otorhinolaryngology, Air Force Hospital of the Northern Theater of the People's Liberation Army, Shenyang, China

Abstract. – OBJECTIVE: As a kind of non-coding RNA, circular RNA (circRNA) plays a regulatory role in many tumors. Our intention was to investigate the clinical significance, biological function, and molecular regulation mechanisms of circ_0067934 in laryngeal squamous cell cancer (LSCC) progression.

PATIENTS AND METHODS: The expression levels of circ_0067934 in LSCC tumor samples and cells were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The associations of circ_0067934 expression with clinicopathological features and overall survival in LSCC patients were statistically analyzed. The biological function of circ_0067934 in LSCC cells was analyzed by CCK-8, colony formation, EdU, and transwell assays. Dual-Luciferase reporter assay was conducted to verify that circ_0067934 may sponge miR-1324 to regulate proliferation and migration of LSCC.

RESULTS: Circ_0067934 expression was remarkably upregulated in LSCC tissues and cells. High level of circ_0067934 was significantly related to tumor size, lymph node status, and distant metastasis of LSCC. Furthermore, highly expressed circ_0067934 could result in notably worse survival. *In vitro* experiments showed that down-regulation of circ_0067934 inhibited proliferation and migration of LSCC cells. Mechanism analysis revealed that circ_0067934 regulated LSCC development by sponging miR-1324.

CONCLUSIONS: Circ_0067934 may function as an oncogene in LSCC, which provided a feasible prognostic biomarker and therapeutic target for LSCC.

Key Words:
Circ_0067934, Prognosis, Tumorigenesis, LSCC.

Introduction

Head and neck malignant tumors are one of the most frequent invasive tumors in the world[1]. Laryngeal cancer (LC) originates from the epithelial tissues of the laryngeal mucosa, accounting for about 25% of head and neck malignancies[2,3]. As the most common type of laryngeal cancer, the incidence of laryngeal squamous cell carcinoma (LSCC) is not high, but it leads to high mortality[4]. The development of LSCC is the result of a variety of factors, such as smoking, alcoholism, papillomavirus infection, and poor eating habits[5,6]. Diagnostic methods for early-stage laryngeal cancer are lacking, which significantly limit therapeutic efficacy. Patients with advanced laryngeal cancer have a wide range of surgical resections that affect swallowing, breathing, and pronunciation[7,8]. Although the treatments and diagnostic techniques have developed quickly, the 5-year overall survival rate of LSCC is still very low[7,9]. Therefore, the molecular mechanism of in-depth study of laryngeal cancer is imminent.

As a novel non-coding RNA, circular RNA (circRNA) has a large number of miRNA binding sites although it does not have 3' and 5' ends[10]. CircRNAs could be new diagnostic biomarkers and therapeutic targets for cancer due to its dysregulation in different diseases and tissues[11-15], such as lung cancer, breast cancer, colorectal cancer, hepatocellular carcinoma, and gastric cancer. Circ_0067934 is a circular RNA molecule of 170 nucleotides located on chromosome 3q26.2[16]. Dysregulation of circ_0067934 has been reported in various cancers[17-19]. However, the potential effects and molecular mechanisms of circ_0067934 in LSCC remain unclear.

In this study, we investigated the characterization of circ_0067934 in LSCC and adjacent normal tissues, and analyzed the clinical value of candidate circ_0067934 in LSCC. We also explored the biological function of circ_0067934 *in vitro*. Circ_0067934 may be a potential biomarker for diagnosing and treating LSCC.

Corresponding Author: Yanling Chu, Ph.D; e-mail: 283598876@qq.com

Patients and Methods

Tissues and Cell Lines

40 pairs of tumor tissue and adjacent non-tumor tissue samples were surgically removed from LSCC patients treated in the Air Force Hospital of the Northern Theater of the People's Liberation Army from July 2017 to July 2019. None of the enrolled patients had preoperative anti-tumor therapies. Sample collection was obtained with the consent of the patients and approved by Air Force Hospital of the Northern Theater of the People's Liberation Army Ethics Committee. These samples were stored at –80°C for further study. All human LSCC cells (TU212 and TU686) and 16HBE (human bronchial epithelial) cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). These cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, NY, USA) containing 10% fetal bovine serum (FBS, Gibco, NY, USA). They were cultured at 37°C, 5% CO_2.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total mRNA was isolated from LSCC tissues and cell lines by the TRIzol (Invitrogen, Carlsbad, CA, USA). Complementary deoxyribonucleic acid (cDNA) was synthesized by a reverse transcription system. The SYBR green kit (Ribobio, Guangzhou, China) was used for qRT-PCR. The expression levels of circ_0067934 and miR-1324 were detected using an ABI 7500 real-time PCR detection system (ABI, Applied Biosystems, Foster City, CA, USA). CircRNA levels were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). MiRNA levels were normalized by small nuclear U6. Relative expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: Circ_0067934, F: 5'-CCATAACCAGCCTTAACGC-3', R: 5'-GGCTAGGAGGTCCGAAGTGA-3'; miR-1324, F: 5'-GCGTGTCGACATCACGAATG-3', R: 5'-AGGTTGCCGTCAGGAACGA-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'- AACGCTTCACGAATTTGCGT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Cell Transfection

Si-hsa-circ_0067934, hsa-miR-1324 inhibitor, and negative control were obtained from Ribobio (Guangzhou, China). TU212 and TU686 cells were cultured in 6-well plates (Corning, Corning, NY, USA) and transfected with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's requirements.

Cell Counting Kit-8 Assay

The transfected LSCC cells were seeded into 96-well plates (Corning, Corning, NY, USA). After cell culture for 1, 2, 3, and 4 days, cell counting kit-8 reagent (Promega, Madison, WI, USA) was added to detect the cell proliferation according to the operation manual. The absorbance was detected at 450 nm.

Colony Formation Assay

The transfected LSCC cells were uniformly placed in 6-well plates (Corning, Corning, NY, USA) at 1500 cells/well. After 10 days, each dish was washed twice with phosphate-buffered saline (PBS; Beyotime, Shanghai, China) and stained with 0.1% crystal violet solution (Beyotime, Shanghai, China). Colonies containing over 50 cells were counted.

EdU Assay

The 5-ethynyl-2'-deoxyuridine (EdU) assay (Ribobio, Guangzhou, China) was conducted to detect cell proliferation viability. Transfected LSCC cells were seeded into 24-well plates (Corning, Corning, NY, USA) with 1×10^4 cells/well. EdU labeling, cell fixation, Apollo staining, and DNA staining were performed according to the instructions. Finally, images were obtained and analyzed by fluorescence microscopy.

Transwell Assay

About 2×10^5 transfected LSCC cells were seeded in the top chamber (Corning, Corning, NY, USA) of the insert mixed with serum-free medium, and medium supplemented with 10% FBS was added as a chemoattractant in the lower chamber (Corning, Corning, NY, USA). The cells were cultured for 48 h, and those migrating through the substrate to the other side of the insert were fixed with methanol at room temperature for 5 min, stained with crystal violet (Beyotime, Shanghai, China), and counted in 15 random regions per well.

Dual-luciferase Reporter Assay

Circ_0067934-wt or circ_0067934-mut was cloned into a Dual-Luciferase reporter vector (GP-mirGLO). 5×10^3 HEK293T cells were plat-

ed in a 24-well plate (Corning, Corning, NY, USA). Then, the cells were co-transfected with the Dual-Luciferase reporter plasmid and miR-1324 mimics or NC using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After 48 h, Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) was used to measure the relative Luciferase activity.

Statistical Analysis

All values were presented as means ± SD (standard deviation). Unpaired Student's *t*-test was used to compare the effects of different treatments. $p < 0.05$ indicated the statistically significant. All analyses of data were conducted with GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA).

Results

Circ_0067934 Was Overexpressed in LSCC Tissues and Cells

Circ_0067934 expressions in LSCC tissues and cell lines were detected by qRT-PCR. Circ_0067934 expression was significantly higher in LSCC tissues than in adjacent non-tumor tissues (Figure 1A). Similarly, the expression of circ_0067934 in LSCC cells was markedly higher than that of 16HBE (Figure 1B). To unveil the clinical role of circ_0067934 in LSCC, patients were divided into different subgroups according to the median value. As Table I suggested, high level of circ_0067934 was relevant to larger tumor size, stronger lymph node metastasis, and distant metastasis. In addition, LSCC patients with high level of circ_0067934 showed lower survival rates (Figure 1C). Overall, our data presented that circ_0067934 might participate in LSCC progression as a potential prognostic biomarker.

Circ_0067934 Knockdown Inhibited Proliferation and Migration In Vitro

To enquiry the effect of circ_0067934 *in vitro* on the biological function of LSCC cells, cell transfection was performed. Si-circ_0067934 was transfected into the TU212 and TU686 cell lines, respectively, and the efficiency of transfection was determined using qRT-PCR (Figure 2A). First, to verify the role of circ_0067934 on proliferation, CCK-8 assay, colony formation, and

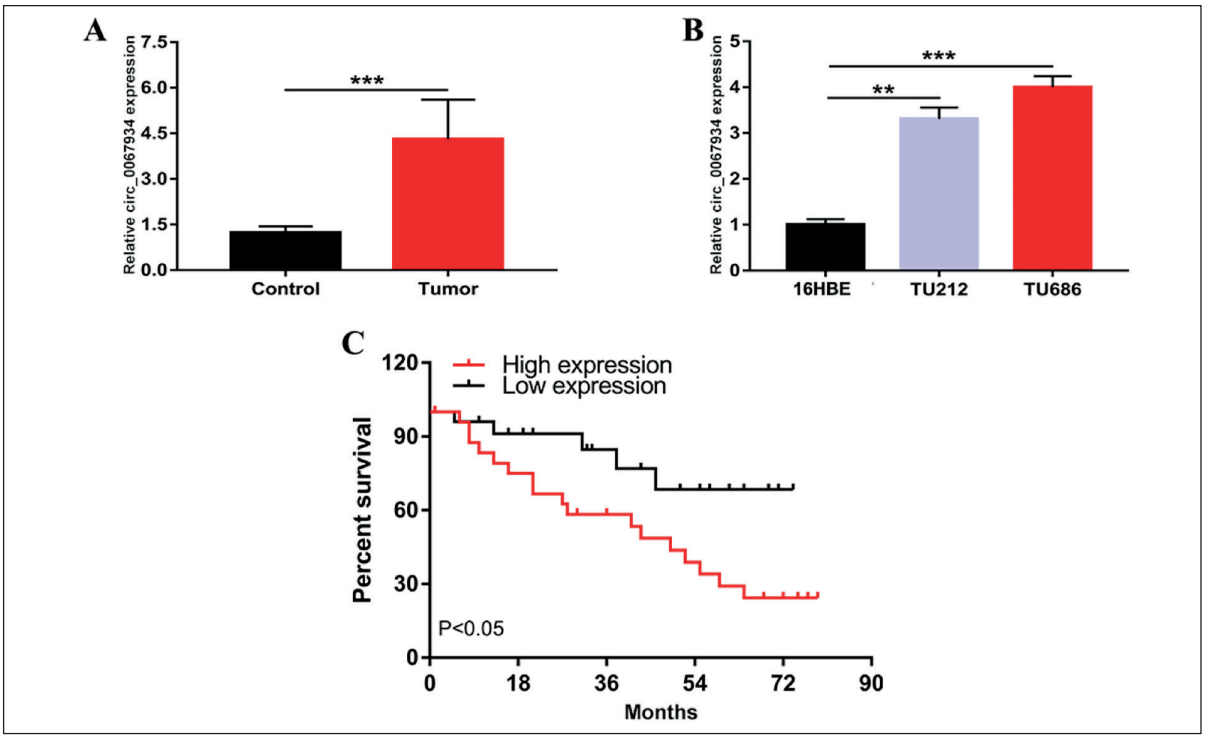

Figure 1. Circ_0067934 was upregulated in LSCC tissues and cells. **A,** Expression level of circ_0067934 in LSCC tissues and para-carcinoma tissues detected by qRT-PCR. **B,** Expression level of circ_0067934 in LSCC and normal cell lines detected by qRT-PCR. **C,** Survival curve analysis by Kaplan-Meier method. *** $p < 0.001$.

Table I. Expression of circ_0067934 according to patients' clinical features.

Factors	Circ_0067934 expression		*p*-value
	High	Low	
Gender			
Male	8	11	0.749
Female	12	9	
Age (years)			
≥ 50	13	8	0.113
< 50	7	12	
Tumour size (cm)			
> 5	15	7	0.011*
≤ 5	5	13	
Lymph node metastasis			
Yes	16	6	0.001*
No	4	14	
Distant metastasis			
Yes	14	5	0.004*
No	6	15	

The High or Low group was cut off by the median expression level of Circ_0067934.

EdU assays were performed. Viability of TU212 and TU686 cells was significantly reduced after circ_0067934 knockdown (Figure 2B). As shown in Figure 2C, the colony formation numbers of LSCC cells with knockdown of circ_0067934 were observably dropped. Similarly, the proportion of EdU-positive cells remarkably decreased after knockdown of circ_0067934 (Figure 2D). Besides, transwell assay was applied to validate the effect of circ_0067934 on cell migration. After inhibiting the expression of circ_0067934 in LSCC cells, the number of migratory cells was significantly reduced (Figure 2E). These results suggested that circ_0067934 contributed to cell proliferation and migration *in vitro*.

Circ_0067934 May Be a Sponge for MiR-1324

Circ_0067934 could promote cell proliferation and migration in LSCC. However, the molecular mechanism of this phenomenon remains unknown. The binding sites between circ_0067934 and miR-1324 were predicted by TargetScan (Figure 3A). To further verify this regulatory relationship, the Dual-Luciferase reporter assay was performed. Overexpression of miR-1324 suppressed Luciferase activity of circ_0067934 wt-3'UTR, rather than the mut-3'UTR (Figure 3B). Further, miR-1324 expression in LSCC tissues was significantly lower than in adjacent normal tissues (Figure 3C). The expression level of miR-1324 in LSCC tissues was negatively correlated with that of circ_0067934 (Figure 3D). Similarly, the expression level of miR-1324 in the LSCC cell lines was also significantly lower than 16HBE (Figure 3E). The above findings proved that circ_0067934 could sponge miR-1324.

MiR-1324 Mediated the Functional Effects of Circ_0067934

To investigate whether circ_0067934 could regulate cell proliferation and migration by modulating miR-1324, we reversed the expression of miR-1324 in si-circ_0067934 group (Figure 4A). Knockdown of miR-1324 repaired the effects of si-circ_0067934 on cell proliferation and migration of TU212 and TU686 cells (Figure 4B, 4C, 4D, and 4E). Considering these results, it is suggested that circ_0067934 could promote proliferation and migration of LSCC cells by sponging miR-1324.

Discussion

LSCC remains the leading cause of tumor-related death worldwide. Although new detective and therapeutic methods are constantly emerging, the prognosis of LSCC is unsatisfactory[20]. Consequently, it is worthwhile and significant to find out new molecular markers for early diagnosis and treatment of LSCC.

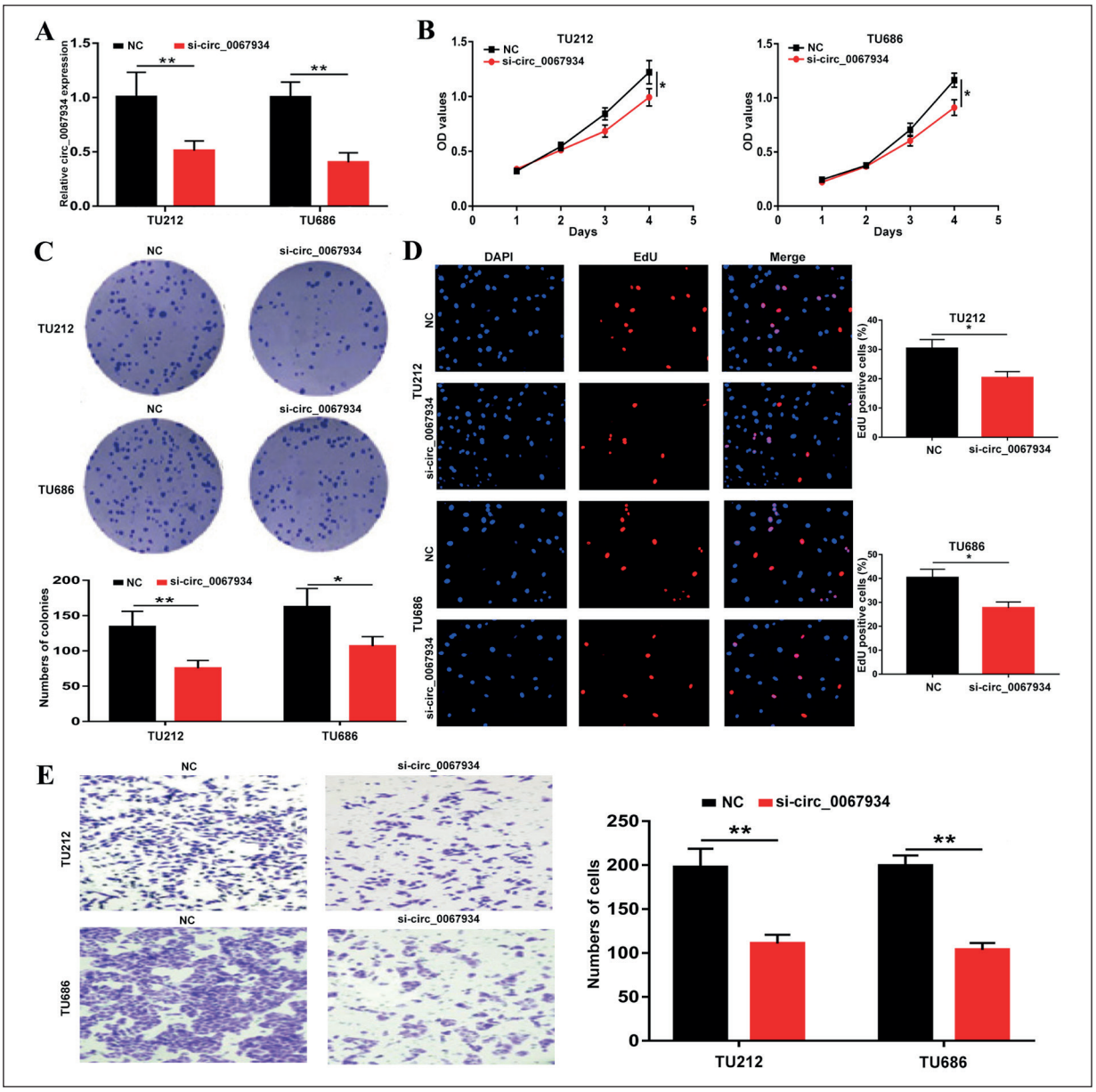

Figure 2. Circ_0067934 knockdown inhibited proliferation and promotes apoptosis *in vitro*. **A,** Analysis of circ_0067934 expression level in TU212 and TU686 cells after transfection by qRT-PCR. **B,** Cell counting kit-8 assay indicated that knockdown of circ_0067934 inhibited proliferation. **C,** Effects of circ_0067938 on cell proliferation were detected by colony formation assay (original magnification, ×40). **D,** Effects of circ_0067938 on cell proliferation were detected by EdU assay. (original magnification, x100) **E,** Transwell assay indicated that knockdown of circ_0067934 inhibited migration (original magnification, ×100). *$p < 0.05$, **$p < 0.01$.

Important roles of circular RNAs in tumor development have been identified[21,22]. Nevertheless, the function of circ_0067934 in LSCC is still unclear. Circ_0067934 is a circular RNA molecule of 170 nucleotides, which is generated from chromosomal region 3q26.2[16]. In this study, it was first ascertained that circ_0067934 expression was markedly overexpressed in LSCC tissues and cells. High level of circ_0067934 was related to tumor size, lymph node metastasis, distant metastasis, and poor prognosis, suggesting that circ_0067934 may contribute to LSCC development and progression. To find out the biological function of circ_0067934 in LSCC, circ_0067934

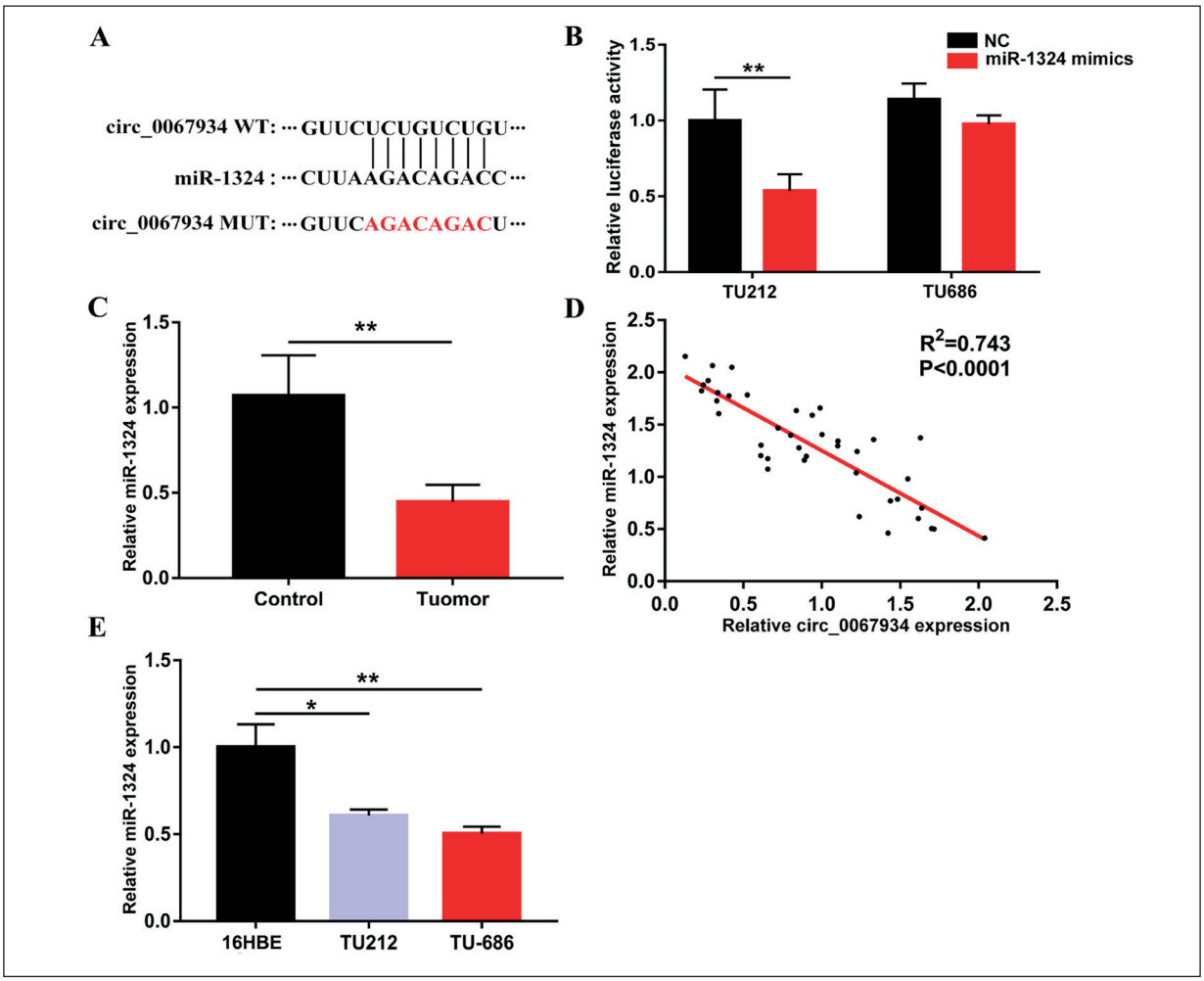

Figure 3. MiR-1324 may be a potential target of circ_0067934. **A,** Binding sites of circ0067934 and miR-1324. **B,** Overexpression of miR-1324 decreased the luciferase activity of wt 3'-UTR of circ_0067934, while alteration of miR-1324 had no effect on the luciferase activity of mut 3'-UTR of circ_0067934 in HEK293T. **C,** Expression level of miR-1324 in LSCC tissues and para-carcinoma tissues detected by qRT-PCR. **D,** Pearson correlation between circ_0067934 and miR-1324 levels. **E,** Expression level of miR-1324 in LSCC and normal cell lines detected by qRT-PCR. $^{**}p < 0.01$.

expression was knocked down in the LSCC cell lines. The experimental data revealed that knockdown of circ_0067934 inhibited cell proliferation and migration. Circ_0067934 may be an oncogene in LSCC.

Later, the downstream mechanism of circ_0067934 was analyzed and explored. MicroRNAs (miRNAs) are small noncoding RNAs, with about 22 nucleotides[23]. It has been showed that circRNAs can participate in tumor progression by regulating miRNAs. Of note, circTP63 could promote lung squamous cell carcinoma progression by regulating miR-873-3p[26]. Also, circ_0058124 could promote papillary thyroid cancer progression and metastasis by modulating miR-218-5p[27]. With bioinformatic analysis and Dual-Luciferase reporter assay, circ-0067934 was detected to act as a molecular sponge for miR-1324. MiR-1324 is reported to inhibit cell proliferation and metastasis in hepatocellular carcinoma[28]. We demonstrated for the first time that miR-1324 was down-regulated in LSCC tissues and cells, and knockdown of miR-1324 promoted cell proliferation and migration. These results revealed that circ_0067934 could promote LSCC cell growth and migration by modulating the expression of miR-1324. In summary, our findings suggested that circ_0067934 may be an effective marker for LSCC.

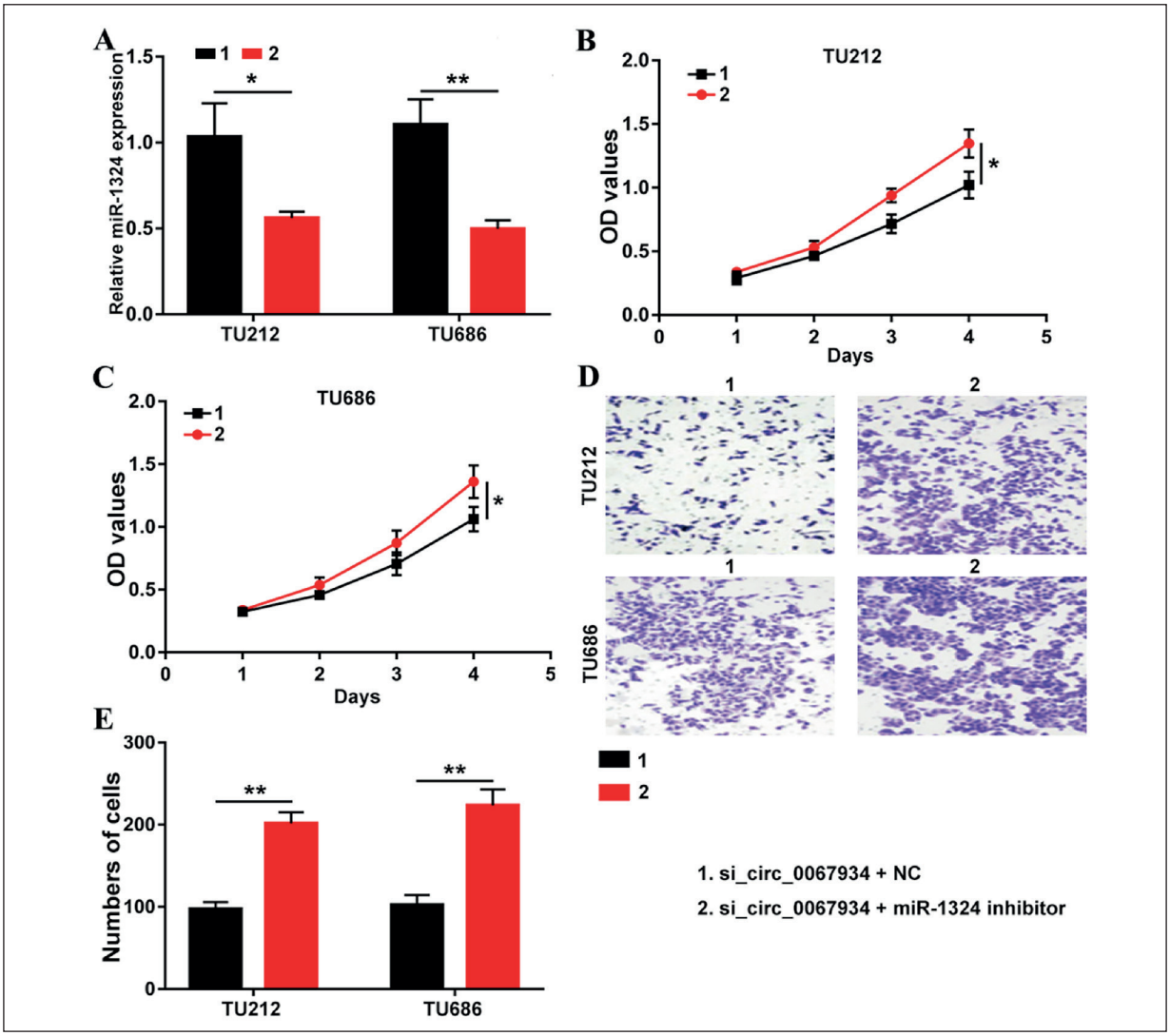

Figure 4. Circ_0067934 promoted proliferation and migration by modulating miR-1324. **A,** Analysis of miR-1324 expression level in TU212 and TU686 cells after transfection by qRT-PCR. **B,** and **C,** Effects of miR-1324 on cell proliferation were detected by CCK-8 assay. **D,** and **E,** Effects of miR-1324 on cell migration were detected by transwell assay (original magnification, ×100). *$p < 0.05$, **$p < 0.01$.

Conclusions

We first demonstrated that circ_0067934 expression was upregulated in LSCC tissues and may be a negative prognostic factor. Moreover, circ_0067934 was implicated in LSCC cell proliferation and migration, suggesting that circ_0067934 could be an effective therapeutic target for LSCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Zhang X, Cavus O, Zhou Y, Dusitkasem S. Airway management during anesthetic induction of secondary laryngectomy for recurrent laryngeal cancer: three cases of report and analysis. Front Med (Lausanne) 2018; 5: 264.
2) Pajic MI, Jelic D, Matic I, Maslovara S, Mendes T. Presence of Helicobacter pylori in the stomach and laryngeal mucosal linings in patients with laryngeal cancer. Acta Clin Croat 2018; 57: 91-95.
3) Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.

4) Xu Z, Xi K. LncRNA RGMB-AS1 promotes laryngeal squamous cell carcinoma cells progression via sponging miR-22/NLRP3 axis. Biomed Pharmacother 2019; 118: 109222.

5) Chen X, Gao L, Sturgis EM, Liang Z, Zhu Y, Xia X, Zhu X, Chen X, Li G, Gao Z. HPV16 DNA and integration in normal and malignant epithelium: implications for the etiology of laryngeal squamous cell carcinoma. Ann Oncol 2017; 28: 1105-1110.

6) Khan JA, Siddque MA, Haque MN, Kundu SC, Ahmed MU, Bhuiyan AS. Etiology of laryngeal squamous cell carcinoma: study of 50 cases in Mymensingh medical College Hospital. Mymensingh Med J 2015; 24: 492-496.

7) Thompson LD. Laryngeal dysplasia, squamous cell carcinoma, and cariants. Surg Pathol Clin 2017; 10: 15-33.

8) Chan JYK, Zhen G, Agrawal N. The role of tumor DNA as a diagnostic tool for head and neck squamous cell carcinoma. Semin Cancer Biol 2019; 55: 1-7.

9) Marur S, Forastiere AA. Head and neck squamous cell carcinoma: update on epidemiology, diagnosis, and treatment. Mayo Clin Proc 2016; 91: 386-396.

10) Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, Maier L, Mackowiak SD, Gregersen LH, Munschauer M, Loewer A, Ziebold U, Landthaler M, Kocks C, le Noble F, Rajewsky N. Circular RNAs are a large class of animal RNAs with regulatory potency. Nature 2013; 495: 333-338.

11) Gao S, Yu Y, Liu L, Meng J, Li G. Circular RNA hsa_circ_0007059 restrains proliferation and epithelial-mesenchymal transition in lung cancer cells via inhibiting microRNA-378. Life Sci 2019; 233: 116692.

12) Chen LH, Wang LP, Ma XQ. Circ_SPECC1 enhances the inhibition of miR-526b on downstream KDM4A/YAP1 pathway to regulate the growth and invasion of gastric cancer cells. Biochem Biophys Res Commun 2019; 517: 253-259.

13) Zhu CL, Sha X, Wang Y, Li J, Zhang MY, Guo ZY, Sun SA, He JD. Circular RNA hsa_circ_0007142 is upregulated and targets miR-103a-2-5p in colorectal cancer. J Oncol 2019; 2019: 9836819.

14) Kong Y, Yang L, Wei W, Lyu N, Zou Y, Gao G, Ou X, Xie X, Tang H. CircPLK1 sponges miR-296-5p to facilitate triple-negative breast cancer progression. Epigenomics 2019; 11: 1163-1176.

15) Wang L, Long H, Zheng Q, Bo X, Xiao X, Li B. Circular RNA circRHOT1 promotes hepatocellular carcinoma progression by initiation of NR2F6 expression. Mol Cancer 2019; 18: 119.

16) Wang J, Li H. CircRNA circ_0067934 silencing inhibits the proliferation, migration and invasion of NSCLC cells and correlates with unfavorable prognosis in NSCLC. Eur Rev Med Pharmacol Sci 2018; 22: 3053-3060.

17) Xin J, Zhang XY, Sun DK, Tian LQ, Xu P. Up-regulated circular RNA hsa_circ_0067934 contributes to glioblastoma progression through activating PI3K-AKT pathway. Eur Rev Med Pharmacol Sci 2019; 23: 3447-3454.

18) Hu C, Wang Y, Li A, Zhang J, Xue F, Zhu L. Overexpressed circ_0067934 acts as an oncogene to facilitate cervical cancer progression via the miR-545/EIF3C axis. J Cell Physiol 2019; 234: 9225-9232.

19) Xia W, Qiu M, Chen R, Wang S, Leng X, Wang J, Xu Y, Hu J, Dong G, Xu PL, Yin R. Circular RNA has_circ_0067934 is upregulated in esophageal squamous cell carcinoma and promoted proliferation. Sci Rep 2016; 6: 35576.

20) Cossu AM, Mosca L, Zappavigna S, Misso G, Bocchetti M, De Micco F, Quagliuolo L, Porcelli M, Caraglia M, Boccellino M. Long non-coding RNAs as important biomarkers in laryngeal cancer and other head and neck tumours. Int J Mol Sci 2019; 20. pii: E3444.

21) Fu B, Zhang A, Li M, Pan L, Tang W, An M, Liu W, Zhang J. Circular RNA profile of breast cancer brain metastasis: identification of potential biomarkers and therapeutic targets. Epigenomics 2018; 10: 1619-1630.

22) Okcanoglu TB, Gunduz C. Circular RNAs in leukemia. Biomed Rep 2019; 0: 1-5.

23) He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004; 5: 522-531.

24) Caiment F, Gaj S, Claessen S, Kleinjans J. High-throughput data integration of RNA-miRNA-circRNA reveals novel insights into mechanisms of benzo[a]pyrene-induced carcinogenicity. Nucleic Acids Res 2015; 43: 2525-2534.

25) Sulaiman SA, Abdul MN, Mohamad HE, Abu N, Jamal R. Prospective advances in circular RNA investigation. Adv Exp Med Biol 2018; 1087: 357-370.

26) Cheng Z, Yu C, Cui S, Wang H, Jin H, Wang C, Li B, Qin M, Yang C, He J, Zuo Q, Wang S, Liu J, Ye W, Lv Y, Zhao F, Yao M, Jiang L, Qin W. CircTP63 functions as a ceRNA to promote lung squamous cell carcinoma progression by upregulating FOXM1. Nat Commun 2019; 10: 3200.

27) Yao Y, Chen X, Yang H, Chen W, Qian Y, Yan Z, Liao T, Yao W, Wu W, Yu T, Chen Y, Zhang Y. Hsa_circ_0058124 promotes papillary thyroid cancer tumorigenesis and invasiveness through the NOTCH3/GATAD2A axis. J Exp Clin Cancer Res 2019; 38: 318.

28) Zhu Q, Lu G, Luo Z, Gui F, Wu J, Zhang D, Ni Y. CircRNA circ_0067934 promotes tumor growth and metastasis in hepatocellular carcinoma through regulation of miR-1324/FZD5/Wnt/beta-catenin