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Linc01234 promotes cell proliferation and metastasis in oral squamous cell carcinoma via miR-433/PAK4 axis

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Abstract

Background: Mounting studies demonstrate long non-coding RNA s (lncRNAs) play an important role in tumor progression. However, the potential biological functions and clinical importance of Linc01234 in oral squamous cell carcinoma (OSCC) still remain unclear. Methods: We evaluated the expression profile and prognostic values of Linc01234 in OSCC tissues via RT-qPCR. Then, functional experiments in vitro were performed to investigate the effects of Linc01234 on tumor growth, migration and invasion in OSCC. Mechanistically, RT-qPCR, bioinformatic analysis and dual luciferase reporter assays were performed to identify a competitive endogenous RNA s (ceRNA s) among Linc01234, miR-433-3p and PAK4. Results: We found that Linc01234 was evidently upregulated in OSCC tissues and cell lines, and its level was positively associated with T stage, lymphnode metastasis, differentiation and poor prognosis of patients with OSCC. Our results found that Linc01234 inhibited cell proliferation and metastasis abilities in CAL27 and SCC25 cells following its deletion. Mechanistic analysis indicated that Linc01234 may act as a ceRNA (competing endogenous RNA) of miR-433-3p to relieve the repressive effect of miR-433-3p on its target PAK4. Conclusions: Our results indicated that Linc01234 promotes OSCC progression through Linc01234/miR-433/PAK4 axis and might be a potential therapeutic target for OSCC.

Background

Oral squamous cell carcinoma (OSCC) is one of the most prevalent subsets of head and neck cancers, ranking as the eighth caner in all malignant tumors worldwide [1]. A new global cancer statistic in 2018 showed that 447,751 newly diagnosed cases of oral cancer and oropharyngeal tumors in the whole world [2]. Although new technologies in diagnosis and treatment have greatly improved the overall survival rates, the prognosis of OSCC still remains dismal [3]. Therefore, this severe form makes it necessary to explore the underlying mechanism and provide novel therapeutic targets for OSCC.

Recently, it has been verified that long non-coding RNAs (lncRNAs) function as an oncogene or a tumor suppressor regulating the biological behaviors of diverse neoplasms [4,5]. At present, numerous evidences have confirmed that lncRNAs involved in cell proliferation, apoptosis, migration and metabolism in OSCC [6]. For example, lncRNA LEF1-AS1 was remarkedly upregulated in OSCC tissues and served as an oncogene in OSCC through suppressing Hippo signaling pathway [7]. LncRNA MEG3 executed an antitumor effect on cell growth and metastasis in OSCC through suppressing the activity of WNT/β-catenin pathway [8]. In addition, aberrant lncRNAs also served as prognostic indicators for tumor recurrence and metastasis. For example, SNHG15 was reported to be significantly upregulated in tumors and enhanced SNHG15 expression could be considered as a promising biomarker for cancer diagnosis, prognosis or treatment [9]. However, the detailed biological importance of most lncRNAs in OSCC development remains blurry.

Linc01234 (ENSG00000249550) is a conserved long noncoding RNA located at 12q24.13 and aberrantly expressed in several cancers [10-12]. Linc01234 was closely correlated with a poor survival in OSCC and breast cancer through screening the TCGA database [13,14]. Although Linc01234 has been reported as an

oncogene promoting OSCC growth and inhibiting apoptosis [11], the clinical importance and underlying mechanism of Linc01234 in OSCC progression is still unclear.

Here, our study is conducted to examine the expression levels of Linc01234 in clinical OSCC samples and a series of OSCC cell lines. Then, we investigate the effects of Linc01234 on cell proliferation and migration in OSCC through gain-of-function and loss-of-function experiments. The present study also analyzed the association between Linc01234 expression and clinical features and prognosis of OSCC patients and might provide new light on targeted therapy and diagnosis of oral cancer.

Methods

Clinical specimens of OSCC

OSCC specimens were randomly selected from 88 patients who had undergone surgery at Affiliated Haikou Hospital and Xiangya Hospital and surgically proven primary OSCC. None of the OSCC patients had received chemoradiotherapy prior to surgery. We collected tumor tissues and their adjacent non-cancerous tissues from each case. All specimens were immediately frozen in liquid nitrogen until used for the subsequent RNA extraction. This study was approved by the Ethics Committee of Affiliated Haikou Hospital and Xiangya Hospital. All patients have been informed and written the consents.

Cell culture and transfection

The CAL27, SCC9 and SCC25 cell lines were purchased from American Type Culture Collection (Manassas, VA, USA), The HSC3, NOK and CAL33 cell lines were kindly gifted from Guanghua School of Stomatology of Sun yet-san University. CAL27, CAL33 and HSC3 cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA). SCC25 and SCC9 cells were cultured in DMEM/F-12 (Gibco) supplemented with 10% FBS. Normal oral keratinocytes (NOKs) were cultured in KSFM medium (Gibco) supplemented with EGF. Linc01234 siRNAs and control siRNAs were purchased from RiboBio (Guangzhou, China). CAL27 and SCC25 cells were transfected with Linc01234 siRNAs or negative controls (50nM) using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturers' indications.

Real-time quantitative PCR(RT-qPCR)

Total RNA was extracted from OSCC tissues and cells by TRIzol Reagent (Invitrogen Life Technologies). 1000ng total RNAs were reverse transcribed using the PrimeSxript RT reagent Kit (Takara, Tokyo, Japan). RT-qPCR was performed using SYBR Green qPCR Mix (Takara, Tokyo, Japan) with a Roche LightCycler 480 system. The ΔCt values of Linc01234 were normalized to GAPDH or U6.

Cell proliferation assays

For EDU assays, CAL27 and SCC25 cells ($2x10^4$/well) were cultured in 6-well plates prior to the transfection. 48h later, the ability of cell proliferation was detected by 5-ethynyl-2'-deoxyuridine (EDU) assay (Life Technologies Corporation, USA) as previously reported [16]. Then, CAL27 and SCC25 cells were stained with DAPI and visualized by a fluorescence microscope (Olympus, Tokyo, Japan). The EDU+ rate was measured with the ratio of the number of EDU-positive cells (green cells) to the total number of DAPI-positive cells (blue cells). For the CCK8 assays, CAL27 and SCC25 cells were cultured in the 96-well plate for 4 consecutive days. Then, the new medium containing 10μl CCK8 solution was replaced in each well, followed by a 1h incubation at 37 °C, and the absorbance was measured at 450nm.

Transwell assays

$1×10^5$ OSCC cells were suspended in serum-free DMEM medium and seeded in the upper chambers (BD Biosciences, San Jose, CA, USA) pre-coated with Matrigel (Corning, New York, NY, USA) (for invasion assays) or boyden chamber without Matrigel (for migration assays) and incubated for 24h. The migrated and invaded OSCC cells on the lower compartment were stained and counted with a microscope (Leica, Wetzlar, Germany).

Wound-healing assays

Two OSCC cells were cultured in 6-well plates until 90% confluent. After scratch on the bottom, CAL27 and SCC25 cells were washed with PBS and taken photographs using a microscope (Leica, Wetzlar, Germany) at 0 h and 48 h, respectively.

Subcellular fraction

The subcellular fractionation was performed with a PARIS Kit purchased from Invitrogen (Carlsbad, CA, USA). $1 × 10^7$ CAL27 and SCC25 cells were collected and isolated using a previously established protocol [15].

Dual-luciferase reporter assays

The Linc01234 fragments or 3'-UTR sequence of PAK4 with potential miR-433-3p binding site or mutants were cloned into the pMIR-REPORT plasmids. The wild type or mutant plasmids were co-transfected into OSCC cells as well as miR-433-3p mimics or miR-NC. 48h later, luciferase activity was examined with a dual luciferase assay kit (Promega, Madison, WI, USA) and these results were normalized to Renilla activity.

Western blot assays

CAL27 and SCC25 cells were collected and lysed by RIPA buffer (Beyotime, China). The protein extracts were separated with SDS-PAGE gel and transferred into PVDF membranes (Millipore Corporation, USA). Then, the PVDF membranes were blocked in 5% nonfat milk solution at RT and cultured with were incubated with anti-PAK4 (ab62509, Abcam, Cambridge, UK) and anti-GAPDH (AC003, ABclonal, China) overnight at 4℃, followed by incubating with the suitable secondary antibodies. The reaction was detected by an enhanced chemiluminescence (ECL) detection system (Millipore, MA, USA)

Statistical analysis

All data were calculated with SPSS 22.0 (IBM Corp., Armonk, NY, USA) and expressed as the mean ± standard deviation of at least three independent experiments. For CCK8 experiments, colony formation assay, Transwell, wound-healing assays, dual-luciferase reporter detection, RT-qPCR and western blotting, each of them was independently repeated 3 times. Comparisons were performed using two-tailed Student's *t*-test or two-way ANOVA. The correlation between Linc01234 expression and clinicopathological parameters was analyzed using the $\chi 2$ test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Linc01234 is increased in OSCC tissues and cell lines

To explore the expression value and clinical significance of Linc01234 in OSCC, we first examined the levels of Linc01234 in Starbase database and specimens collected in our hospitals. As showed in Figure 1A, Linc01234 was dramatically upregulated in HNSCC samples (Cancer) compared with that in normal tissues (Normal) ($P<0.0001$). We further detected the expression levels of Linc01234 in 88 OSCC specimens and adjacent oral normal tissues, which were collected from surgical resection in Affiliated Haikou Hospital and Xiangya Hospital. The results of RT-qPCR indicated that the mRNA levels of Linc01234 in OSCC tissues were significantly higher than that in adjacent non-tumor tissues ($P<0.01$; Fig. 1C). Following culture of four OSCC cell lines and the negative control NOK cells, it was identified that Linc01234 expression level was significantly increased in all OSCC cell lines ($P<0.05$; Fig. 1B), compared with that in the NOK cell line.

Linc01234 is closely associated with OSCC patients' prognosis

In order to investigate the clinical significance of Linc01234 in OSCC progression, the association between Linc01234 expression and clinicopathological features was analyzed by $\chi 2$ test. We found that Linc01234 expression was closely correlated with T stage, N stage and pathological stage (P<0.05), however there was no statistical significance between Linc01234 and age, gender, tumor site and distant metastasis (Table 1). In addition, Cox analysis showed that Linc01234 expression could be an independent predictor for OSCC patients' prognosis as well as T stage, N stage and advanced pathological stage (Table 2), indicating that Linc01234 could be an independent prognosis factor for OSCC with the aggressive phenotype. In addition, Starbase database showed that high expressed Linc01234 in head and neck cancer patients was closely correlated with short overall survival (OS) (Figure 1D).

Silence of Linc01234 represses OSCC cell growth

To explore the biological role of Linc01234 in OSCC, we transfected siRNAs targeting Linc01234 in CAL27 and SCC25 cells and examined Linc01234 levels by RT-qPCR. Following transfection, these siRNAs apparently decreased the transcription of Linc01234 in CAL27 and SCC25 cells (Figure 2A), suggesting a high knockdown efficiency of these siRNAs. Using CCK8 assays, the proliferative rates were significantly decreased by Linc01234 inhibition in CAL27 cells and SCC25 cells (Figure 2B), respectively. Furthermore, EDU-positive cells in Linc01234 deleted OSCC cells was remarkedly lower than that in the control group and siNC group (Figure 2C), indicating a decreased DNA synthesis ability in OSCC cells with Linc01234 siRNAs. Our data suggested that Linc01234 promotes OSCC cell proliferation by enhanced DNA synthesis.

Knockdown of Linc01234 inhibited migration and invasion in OSCC cells

In view of a strong association of Linc01234 expression with lymph node metastasis (N stage), the effect of Linc01234 on cell migration and invasion in OSCC cells was further explored. As showed in Figure 3A and B, a great number of CAL27 and SCC25 cells in the control and siNC group migrated to the lower surface of the upper chamber. But only an average of cells migrated to the lower surface in Linc01234-deleted groups. Figure 3C and D indicated the wound healing area of control and siNCs group was significantly greater than that in Linc01234-deleted groups. These observations indicated that Linc01234 may function as a positive regulator of cell metastasis in OSCC.

Linc01234 sponges miR-433-3p in OSCC cells

Numerous studies have reported that lncRNAs could act as microRNA (miRNA) sponges, to regulate endogenous miRNAs for their binding to the target mRNAs, and inhibit the expression of these target mRNAs [16]. First, we found that Linc01234 was mainly expressed in cytoplasm of CAL27 and SCC25 cells analyzing by RT-qPCR (Figure 4A). Then, we screened the potential target miRNAs using LncBase Predicted version (v.)2 of DIANA tools and obtained 35 potential miRNAs. Screening Pubmed database, we identified that miR-433-3p, a famous tumor suppresser, was a possible downstream target of Linc01234 (Figure 4B). Furthermore, miR-433-3p decreased in OSCC samples compared with the normal tissues analyzed by RT-qPCR and Starbase database (Figure 4C). Then, we next performed a dual-luciferase assay to validate this hypothesis. Our results indicated that the miR-433-3p mimics + Linc01234 wild-type (Linc01234 wild) group but not miR-433-3p mimics + Linc01234 mutant type (Linc01234 mutant) group showed an obviously reduced luciferase activity in OSCC cells (Figure 4D). Additionally, Linc01234 inhibition significantly elevated miR-433-3p expression level in CAL27 and SCC25 cells (Figure 4E). In summary, we confirmed that Linc01234 could sponge miR-433-3p in OSCC cells.

miR-433-3p directly binds to the 3'UTR of PAK4

We have confirmed that Linc01234 served as an oncogene in OSCC progression and confirmed that Linc01234 functioned as a ceRNA regulating miR-433-3p expression. Thus, we further investigate the potential target of the Linc01234/miR-433-3p axis. Using Targetscan and Starbase tools, we found the expression of PAK4 was positively correlated with Linc01234, but negatively correlated with miR-433-3p, and miR-433-3p have binding sites in the 3'UTR of PAK4. In addition, PAK4 was remarkedly downregulated in head and neck cancer analyzed by Starbase (Figure 5A-D). To confirm whether PAK4 was a direct target of miR-433-3p, we purchased wild-type and mutant luciferase reporter plasmids containing the complementary sequence of miR-433-3p with PAK4, and co-transfected miR-433-3p mimics and these luciferase reporter vectors into OSCC cells. We indicated that the luciferase activities dramatically declined in OSCC cells co-transfected with miR-433-3p mimics and wild-type PAK4 vectors, but no change in luciferase activity of OSCC cells transfected with mutant PAK4 plasmids (Figure 5F). Finally, miR-433-3p overexpression significantly suppresses PAK4 expression in OSCC cells (Figure 5E). Thus, we suggest that PAK4 was a direct target of miR-433-3p in OSCC cells.

Discussion

Recently, increasing evidences have proved that an aberrant lncRNA might facilitate to the development and progression of solid tumors and particular lncRNA was identified as independent biomarker [17-19]. Due to the unknown biological functions of lncRNAs and limited understand of the molecular mechanisms, more studies are needed. Our present study investigated an OSCC prognosis-related lncRNA—Linc01234, and elucidated its functional roles in OSCC progression.

In the present research, we found that Linc01234 was significantly upregulated in clinical OSCC samples and cell lines. High expressed Linc01234 was positively related to advanced T stage, lymph node metastasis and poor pathological differentiation. In addition, OSCC patients with high Linc01234 expression had a worse overall survival (OS) than patients with low Linc01234 expression based via database analysis. Cox analysis also indicated that Linc01234 expression could be an independent predictor for OSCC patients' prognosis as well as T stage, N stage and advanced pathological stage. In biological functional experiments, Linc01234 inhibition prominently contributed to the decreased proliferative activity and metastasis in CAL27 and SCC25 cells. In summary, our results suggested that Linc01234 plays a cancer-promoting role in cell growth and metastasis in OSCC.

Growing evidences indicated that lncRNAs and mRNAs could cross regulate each other via competing for shared miRNA response elements (MREs) [16,20]. Specifically, many lncRNAs act as a sponge in the regulation of miRNA target gene involving OSCC carcinogenesis [21,22]. miR-433, a well-characterized miRNA, was found as a tumor suppresser in different neoplasms [23,24]. Furthermore, Wang et al reported that miR-433 was downregulated in OSCC tissues and evaluated miR-433 expression markedly suppressed cells proliferation, invasion and migration through targeting HDAC6 [25]. In our study, we found that Linc01234 contained miRNA response elements for miR-433-3p with a 13nt complementarity. Dual-luciferase assays confirmed a direct correlation between miR-433-3p and Linc01234. RT-qPCR results showed that Linc01234 deletion increased the expression levels of miR-433-3p, which was dramatically downregulated in head and neck cancer tissues. Our data suggested that Linc01234 may act as a ceRNA (competing endogenous RNA) of miR-433-3p to relieve the repressive effect of miR-433-3p on its target. However, the underlying mechanism of Linc01234/miR-433-3p axis in OSCC remains unclear.

p21-Activated kinase 4 (PAK4), a member of the PAK family, regulates a wide range of cellular functions, including cell adhesion, migration, proliferation, and survival [26,27]. Previous studies have reported that dysregulation of PAK4 expression contributes to development and progression of various tumors [28,29]. Several studies have reported that PAK4 could be regulated by many miRNAs in various cancers, including miR-485, miR-199a-3p [30-32]. Especially, in OSCC, PAK4 served as a super enhancer-associated candidate oncogene and promoted the proliferation of OSCC cells [33]. In our study, we identified PAK4 as the potential target of miR-433-3p based on Targetscan and Starbase prediction. Dual-luciferase assay have confirmed that miR-433-3p bind to PAK4 directly. Furthermore, PAK4 protein levels in CAL27 and SCC25 cells with miR-433-3p overexpression were significantly inhibited. Overall, our findings suggested that Linc01234 modulated OSCC carcinogenesis through miR-433-3p-regulated PAK4.

Conclusions

In summary, our study has confirmed that Linc01234 promotes OSCC progression through Linc01234/miR-433-3p/PAK4 axis and it may serve as a new diagnostic marker or target for treatment of OSCC patients.

Abbreviations

LncRNA: long non-coding RNA; OSCC: oral squamous cell carcinoma; ceRNA: competitive endogenous RNA; PAK4: p21-Activated kinase 4; MRE: miRNA response element; HDAC6: Histone Deacetylase 6; OS: overall survival; NOK: normal oral keratinocytes.

Declarations

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The manuscript does not contain any individual person's data.

Authors' contributions

DL and RZ carried out the functional experiments in vitro; YW collected the OSCC tissues and examined the mRNA and protein expression. DL, PX, RZ and XJ wrote the manuscript. All the authors contributed to the design of the project. All the authors have read and approved the manuscript.

Conflicts of Interest

The Authors declare no conflicts of interest.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Affiliated Haikou Hospital and Xiang-ya Hospital. The informed consent forms were obtained from the OSCC patients.

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Availability of data and materials

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

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Tables

Table 1. The association between linc01234 expression and clinicopathological parameters in patients with OSCC.

Clinicopathological parameters	Linc01234 expression		$\chi 2$	P value
	Low(N=44)	High(N=44)		
Age			0.16	0.665
<60	27	25		
≥60	17	19		
Gender			0.05	0.829
Male	25	26		
Female	19	18		
Tumor site			0.18	0.669
Tongue	22	24		
Non-tongue	22	20		
T stage			9.08	0.002
T_{1-2}	32	18		
T_{3-4}	12	26		
Lymphnode metastasis			3.93	0.048
No	32	23		
Yes	12	21		
Distance metastasis			0.72	0.395
Yes	6	9		
No	38	35		
Differentiation				
Well and moderately	34	26	4.87	0.027
Poorly	8	18		

Table II. Univariate and multivariate Cox proportional hazards analysis of linc01234 expression and OS in patients with OSCC.

Clinicopathological parameters	Univariate Cox analysis		Multivariate Cox analysis	
	RR (95%CI)	P value	RR (95%CI)	P value
Age	0.91 (0.43-1.77)	0.588		
Gender	1.04 (0.65–1.51)	0.903		
Tumor site	1.15 (0.98–1.35)	0.094		
T stage	1.72 (1.18–2.26)	0.020	1.82 (1.42-2.94)	0.003
Lymphnode metastasis	1.41 (1.11–1.92)	0.036	1.52 (1.05-1.86)	0.031
Distance metastasis	0.95 (0.61–1.47)	0.834		
Differentiation	1.44 (1.18–1.82)	0.010	1.15 (1.02–1.30)	0.028
Linc01234 expression	1.64 (1.38–2.55)	0.008	1.99 (1.33-3.05)	0.001

Figures

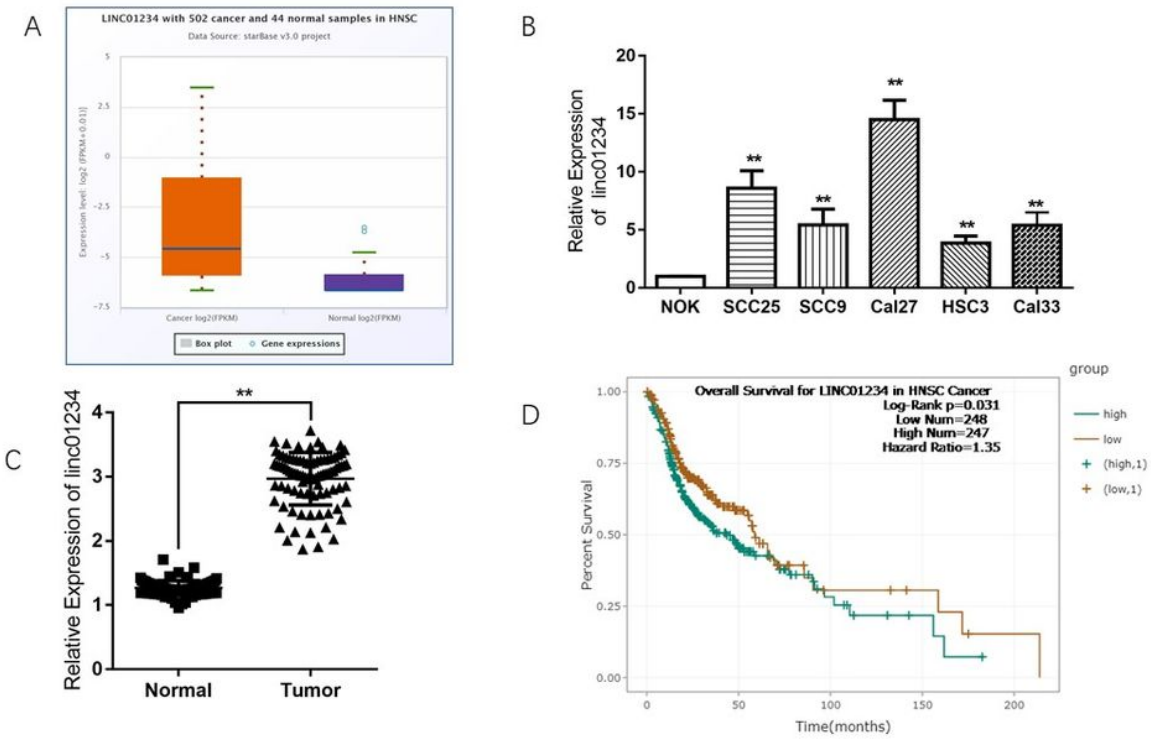


Figure 1

Linc01234 was upregulated in OSCC tissues and cell lines. (A) Linc01234 expression was significantly increased in OSCC tissues, compared with in adjacent non-tumor tissues via Starbase analysis. (B) Linc01234 expression was examined in OSCC and NOK cells via RT-qPCR assays. (C) Linc01234 expression was significantly increased in OSCC tissues, compared with in adjacent non-tumor tissues analyzed by RT-qPCR. (D) Kaplan-Meier analysis for the effects of Linc01234 expression on the OS of HNSCC patients based on Starbase database. **,P<0.01;***,P<0.001 vs control.

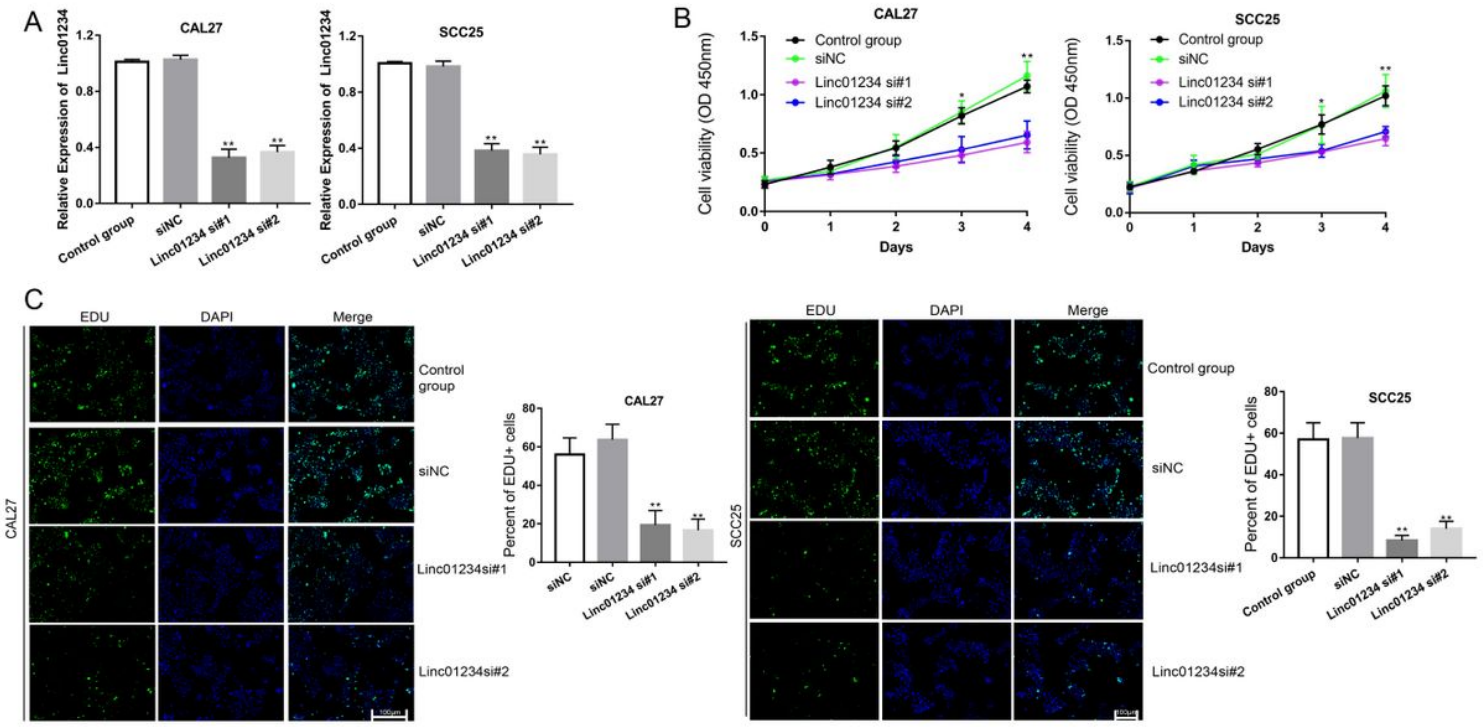


Figure 2

Decreased Linc01234 inhibits cell proliferation in vitro. (A) The relative expression of Linc01234 was detected with RT-qPCR when CAL27 and SCC25 cells were transfected with siNC, Linc01234 si#1 or Linc01234 si#2. (B) CCK8 assays were performed to detect the cell proliferation ability after CAL27 and SCC25 cells transfected with siNC, Linc01234 si#1 or Linc01234 si#2. (C) EDU assays were performed to detect the cell proliferation ability in CAL27 and SCC25 cells transfected with siNC, Linc01234 si#1 or Linc01234 si#2. *P<0.05; **,P<0.01 vs control.

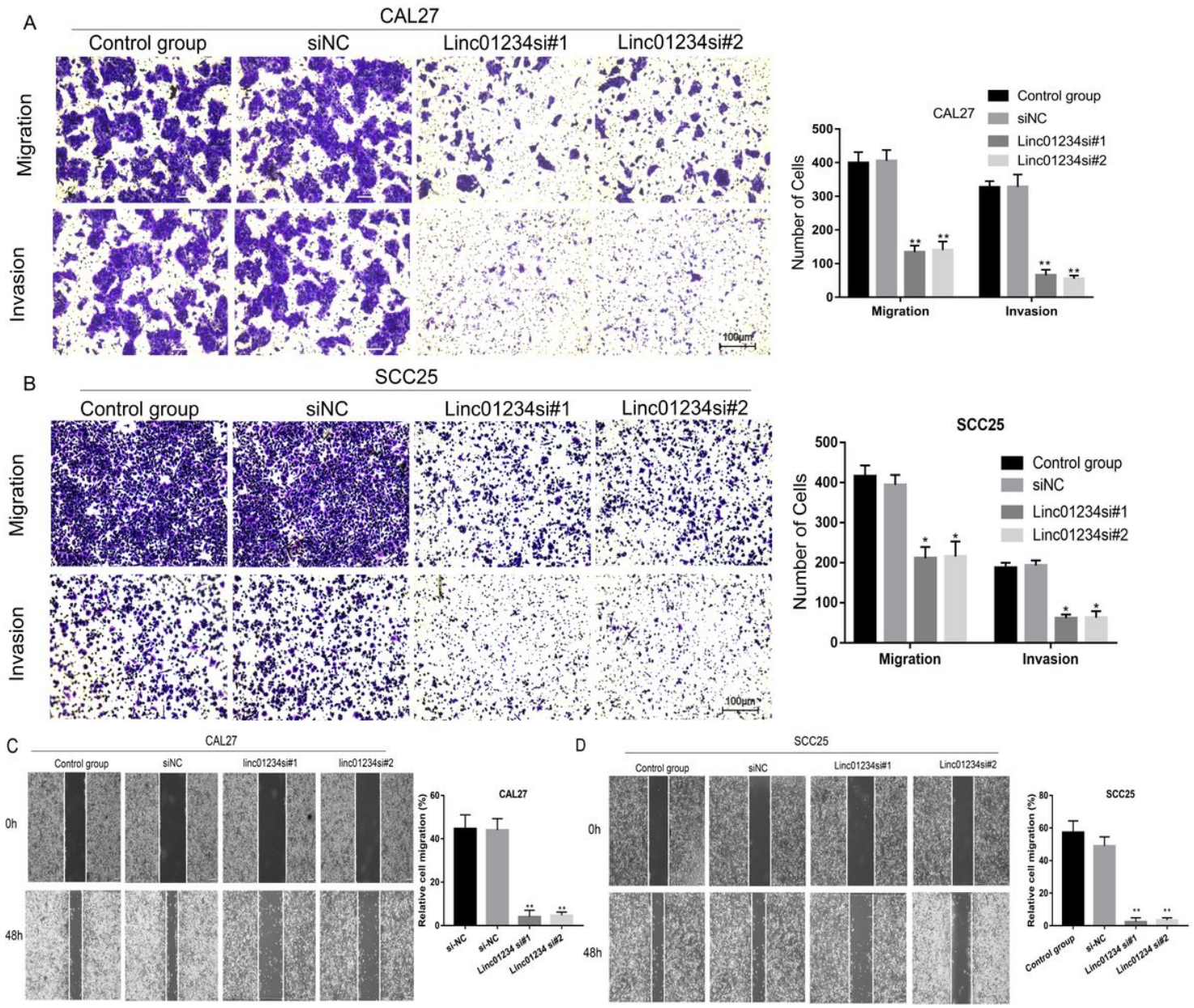


Figure 3

Decreased Linc01234 inhibits migration and invasion in vitro. (A,B) The ability of cell migration and invasion in CAL27 and SCC25 cells with Linc01234 knockdown were detected by Transwell assays. (C,D) The migration ability of CAL27 and SCC25 cells with Linc01234 knockdown were detected by wound healing assays. * P<0.05; **,P<0.01 vs control.

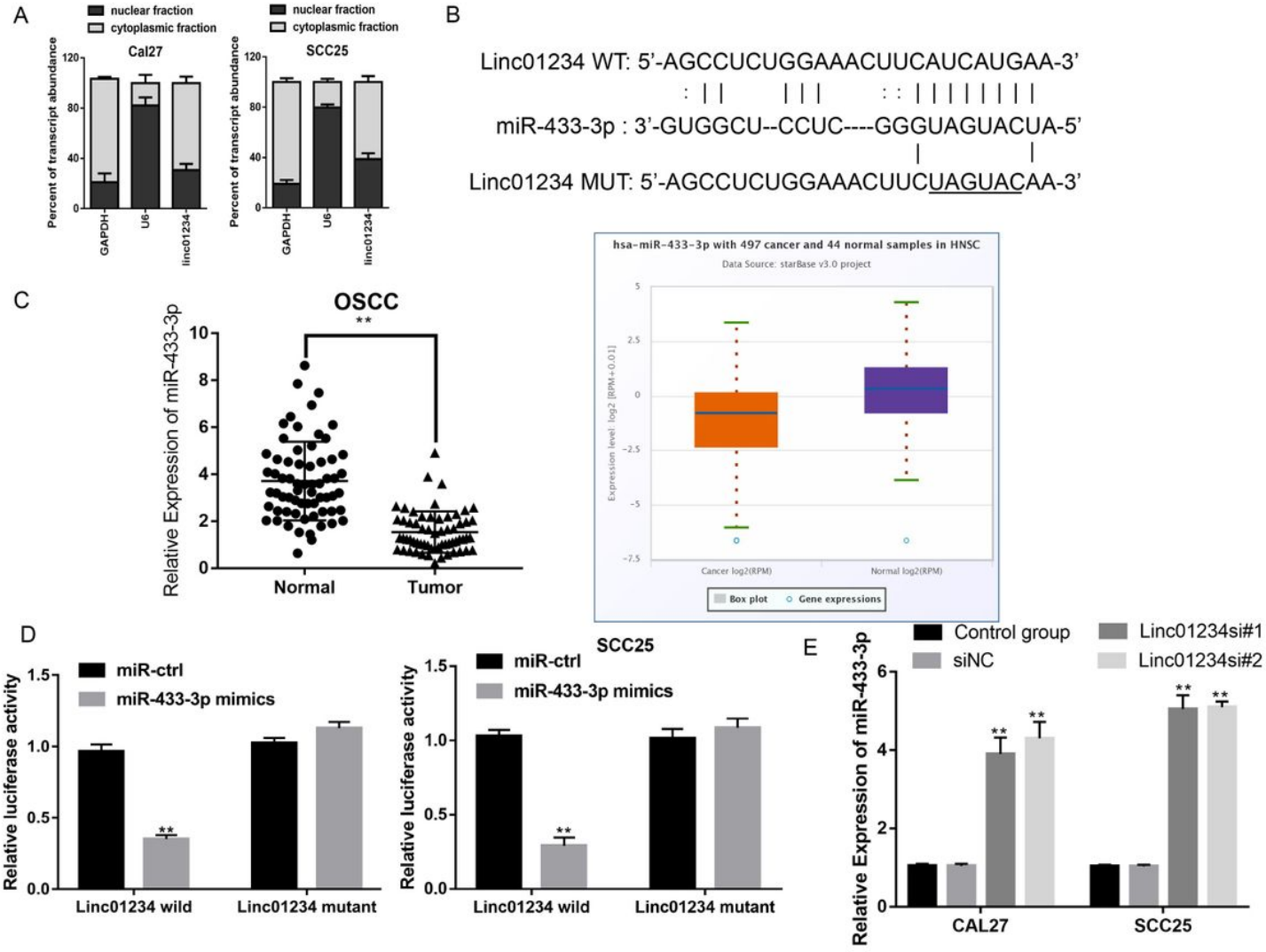


Figure 4

Linc01234 could sponge miR-433-3p in OSCC. (A) The cellular localization of Linc01234 was determined by Subcellular fractionation and RT-qPCR assay. GAPDH: cytoplasmic control, U6: nuclear control. (B) Schematic illustration of the predicted binding sites between Linc01234 and miR-433-3p and mutation of potential miR-433-3p binding sequence in Linc01234. (C) miR-433-3p expression was examined in OSCC tissues using RT-qPCR and Starbase analysis. (D) Luciferase reporter assay indicated the direct bind between Linc01234 and miR-433-3p. (E) The mRNA level of miR-433-3p after Linc01234 knockdown was observed by RT-qPCR assay. *P < 0.05, **P < 0.01

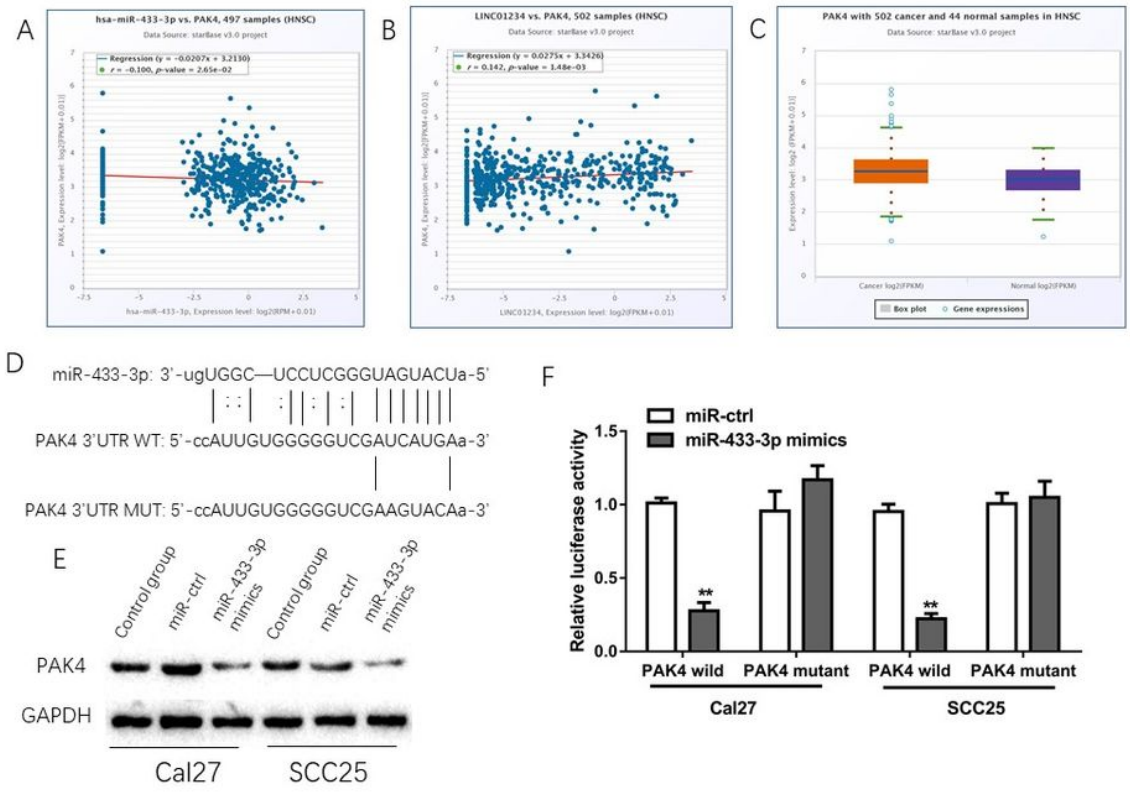


Figure 5

PAK4 is a target gene of miR-433-3p in OSCC. (A and B) revealed the correlation between PAK4 expression and miR-433-3p expression or Linc01234 expression in HSCC tissues. (C) PAK4 expression in HSCC tissues and normal tissues was veritied by Starbase analysis. (D) The putative binding sites of miR-433-3p and PAK4 was showed. (E) The expression of PAK4 was detected by RT-qPCR in OSCC cells with miR-433-3p overexpression. (F) The direct interaction between miR-433-3p and PAK4 in OSCC cells was examined by luciferase reporter assay. *P< 0.05, **P < 0.01.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfigure2.JPG
- Supplementaryfigure3.JPG
- Supplementaryfigure1.JPG