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 RNAs (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 Two OSCC cells were transfected with siRNAs targeting linc01234, and RT-qPCR, CCK-8, EDU, wound healing and Transwell and western blot assays were performed to analyze the effect of linc01234 on cell proliferation, migration and invasion. Bioinformatic analysis, luciferase assays and RT-qPCR identified a competitive endogenous RNAs (ceRNAs) among linc01234, miR-433-3p and PAK4. Results We found that linc01234 was significantly upregulated in OSCC tissues and cell lines and positively associated with T stage, lymphnode metastasis, differentiation. Kaplan-Meier analysis of OSCC reveals a positive correlation between linc01234 and the overall survival. Following the linc01234 deletion, the cell proliferation and metastasis abilities in CAL27 and SCC25 cells were found to be extremely reduced. Mechanism studies indicated that linc01234 located in cytoplasm and shared microRNA (miRNA) response elements with miR-433-3p. Luciferase assays indicated that miR-433-3p bind to the 3'-UTR of PAK4. Conclusions Our results indicated that linc01234 functioned as an oncogene in OSCC 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 Xiang-ya 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 Xiang-ya 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 purchased from the Cell Bank of Chinese Academy of Sciences (Beijing, China). 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 PCR assays

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 assay

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 assay

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 SCC25cells 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) at 4°C overnight, 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. The correlations between linc01234 expression and clinicopathological parameters were analyzed using the χ^2 test. Differences were analyzed using the Student's t-test or one-way ANOVA followed by the Student-Newman-Keuls-q test. P<0.05was considered to indicate a statistically significant difference.

Results

Linc01234 is increased in OSCC tissues and cell lines.

To explore the expression levels and clinical significances of linc01234 in OSCC, we first examined the expression 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 Xiang-ya 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. 1B). Following culture of four OSCC cell lines and the negative control NOK cells, it was identified that linc01234 expression levels were significantly increased in all OSCC cell lines (P<0.05; Fig. 1C), compared with that in NOK cell line.

Linc01234 is closely associated with OSCC patients' prognosis.

In order to investigate the clinical significance of linc01234 in OSCC progression, the correlativity between linc01234 expression and clinicopathological features was analyzed by χ2 test. It was identified that linc01234 expression was closely correlated with T stage, N stage and pathological stage (P<0.05), however there was no statistical correlation between linc01234 and age, gender (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 HNSCC 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, the results of the EDU assays demonstrated that most EDU+ cells showed a glowing green fluorescence in the OSCC cells transfected with linc01234 siRNAs (Figure 2C), indicating that the ability of DNA synthesis was remarkedly decreased after linc01234 siRNAs transfection. These 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 role linc01234 on cell migration and invasion in CAL27 and SCC25 cells was further explored. As showed in Figure 3A and B, a great number of CAL27 and SCC25 cells in siNC group migrated to the lower surface of the upper chamber. However, in the linc01234-deleted cells, only an average of OSCC cells migrated to the lower surface, which was the opposite to the control OSCC cells. As shown in Figure 3C and D, the wound healing area of siNCs group was significantly greater than in the linc01234 siRNAs group. 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 base, 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

assays to further validate this hypothesis. Our results indicated that the miR-433–3p mimics + linc01234 wild-type (linc01234 WT) group but not miR-433–3p mimics + linc01234 mutant type (linc01234 MUT) group showed an obviously reduced luciferase activity in OSCC cells (Figure 4D). Additionally, linc01234 inhibition significantly elevated miR-433–3p expression levels in CAL27 and SCC25 cells (Figure 4E). In summary, we comfirmed that linc01234 could sponge miR-433–3p in OSCC cells.

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

We have proved 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 screen the potential targets of the linc01234/miR–433–3p axis. Using 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 significantly downregulated in HNSCC samples in Starbase database (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 cotransfected miR–433–3p mimics and these luciferase reporter vectors into OSCC cells. We indicated that the luciferase activities dramatically declined in OSCC cells cotransfected 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 proved that PAK4 was a direct target of miR–433–3p in OSCC cells.

Discussion

Recently, increasing evidences have proved that the aberrant expression of lncRNAs might facilitate to the development and progression of solid tumors and particular lncRNAs were 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 numerous OSCC 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 OS than patients with low linc01234 expression based on Starbase 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-PCR results showed that linc01234 deletion increased the expression levels of miR-433–3p, which was dramatically downregulated in HNSCC tissues. Our data suggested that linc01234 exerted its function through competing with miR-433–3p in OSCC. However, the underlying mechanism of linc01234/miR–433–3p axis regulating OSCC progression is still 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 an SE-associated candidate oncogene and promoted the proliferation of OSCC cells [33]. In our study, we screened PAK4 as a potential target gene of miR-433–3p based on 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 founding suggested that linc01234 modulated OSCC carcinogenesis through miR-433–3p-mediated PAK4.

Conclusions

In summary, our study has confirmed that linc01234 was increased in OSCC tissues and cell lines, and could be an independent prognostic predictor for OSCC. In mechanism, linc01234 deletion suppressed the proliferation and metastasis of OSCC cells via regulating miR-433–3p/PAK4 axis. Thus, our results provide a novel sight into diagnosis and targeted therapy for OSCC.

Declarations

Conflicts of Interest

The Authors declare no conflicts of interest.

Acknowledgements

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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

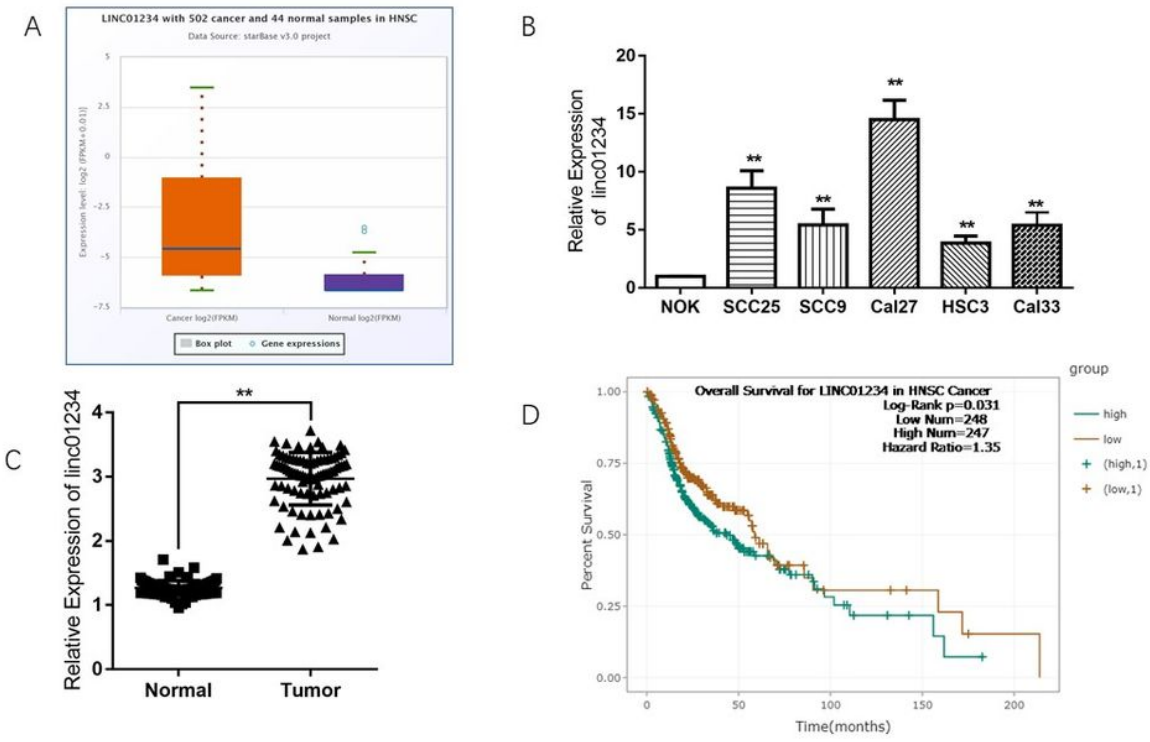

Figure 1

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 significantly increased in OSCC cell lines, compared with that in the NOK cell line. (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.

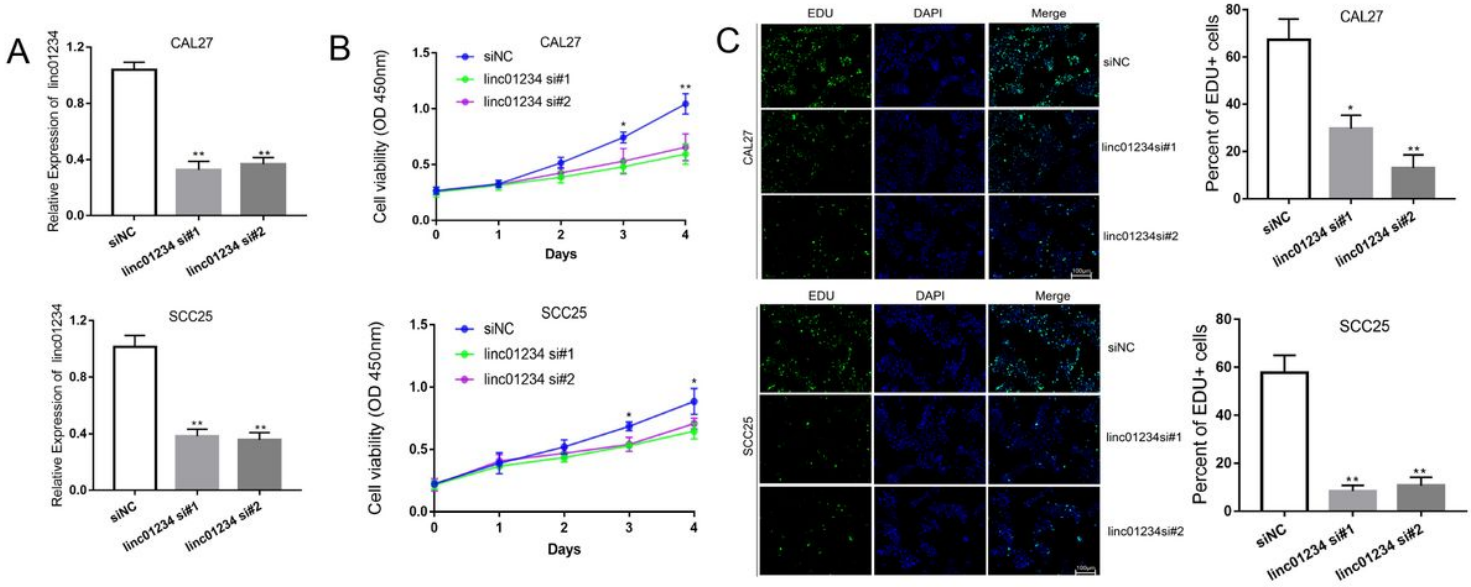

Figure 2

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.

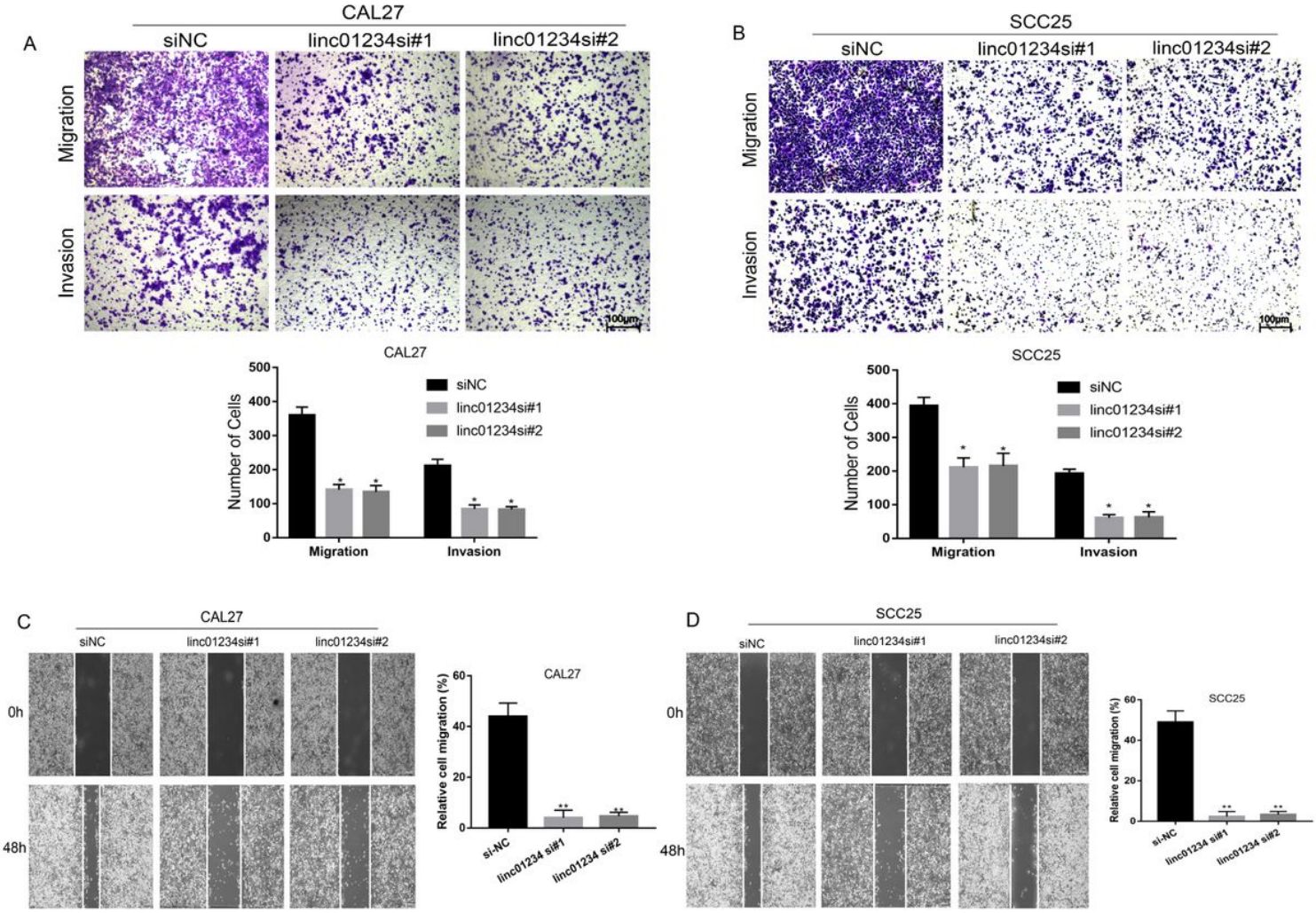

Figure 3

Figure 3. Decreased LINC01234 inhibits migration and invasion in vitro. (A) The ability of cell migration and invasion in CAL27 cells with linc01234 knockdown were detected by Transwell assays. (B) The ability of cell migration and invasion in SCC25 cells with linc01234 knockdown were detected by wound healing assays. * P<0.05; **,P<0.01 vs control.

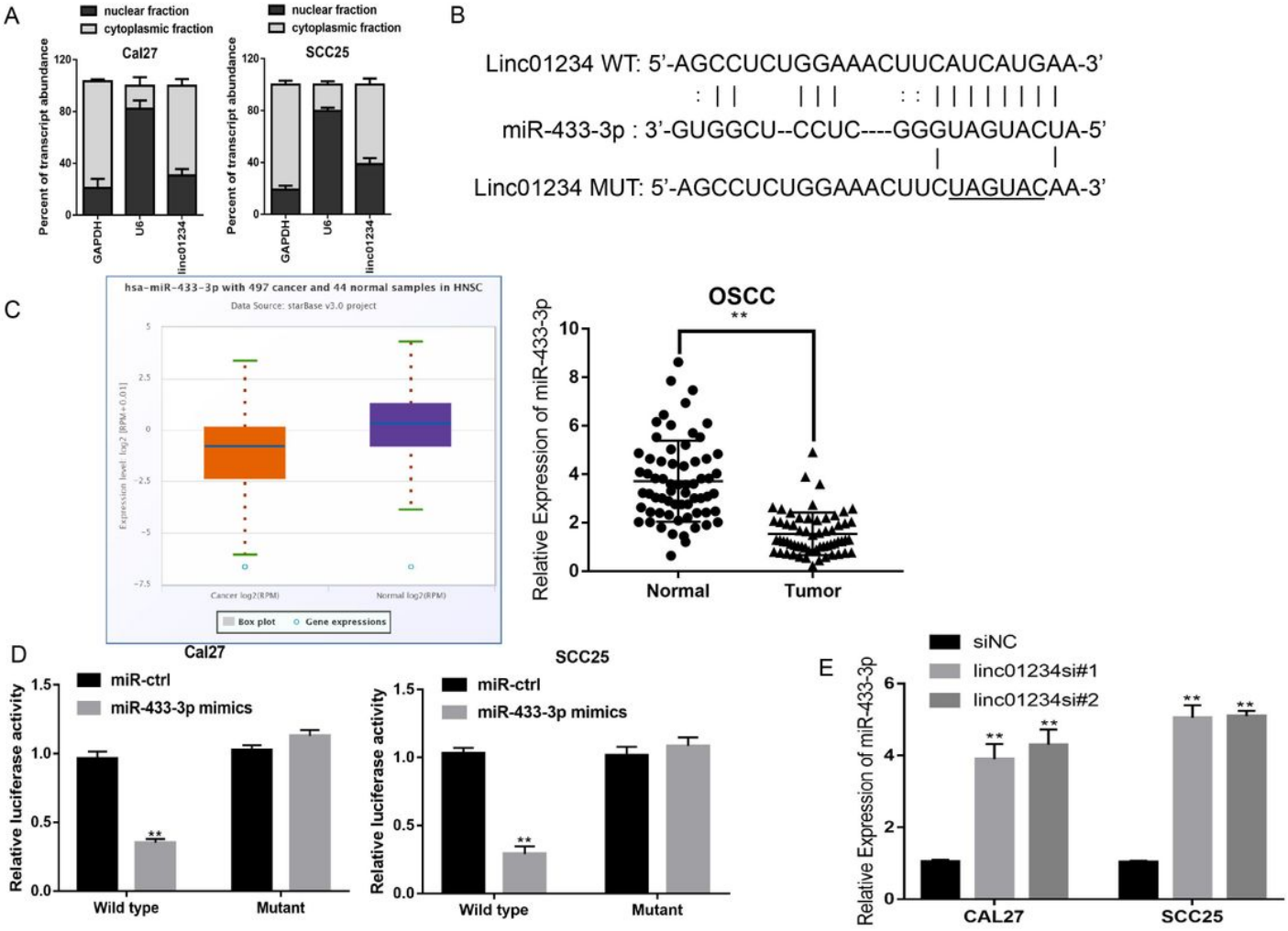

Figure 4

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 qRT-PCR assay. *P < 0.05, **P < 0.01

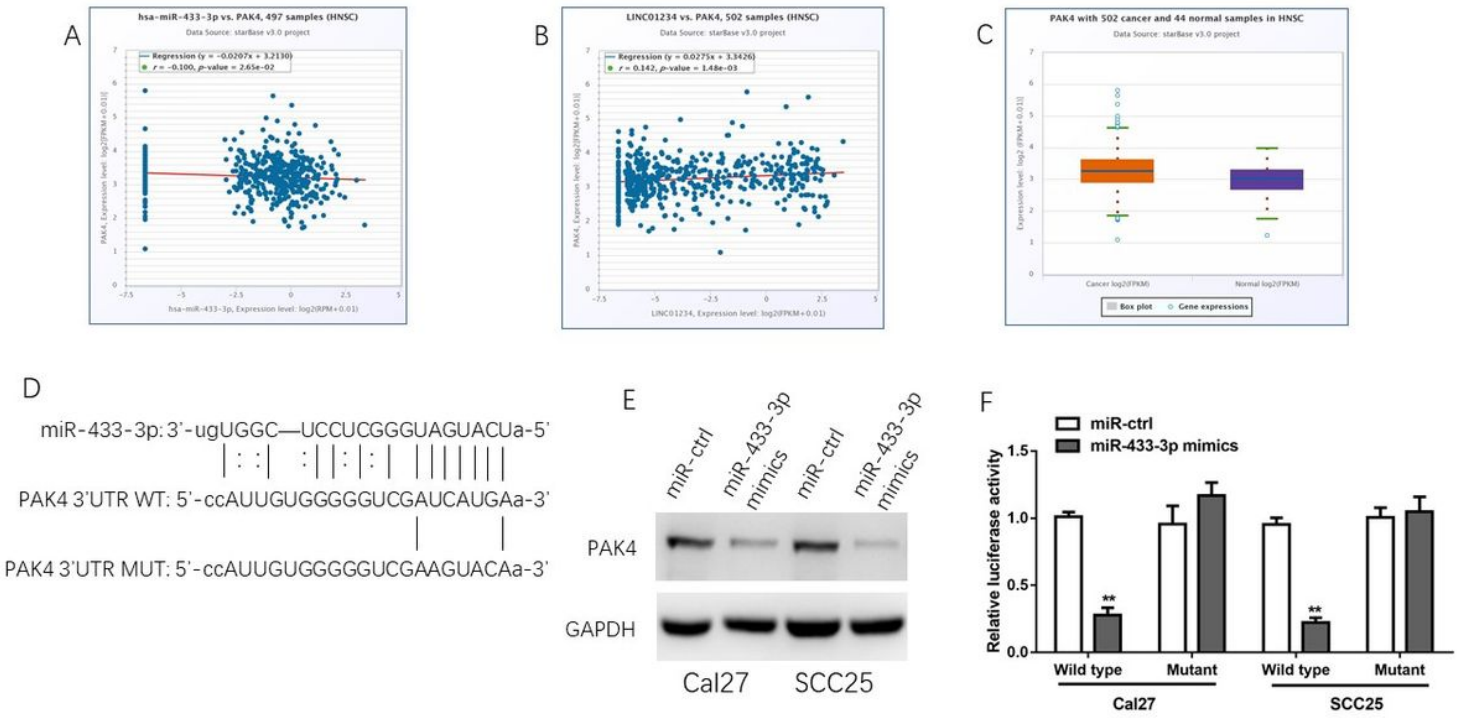

Figure 5

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 and PAK4 was showed. (E) PAK4 expression was inhibited in response to linc01234 deletion. (F) The interaction between miR-433-3p and PAK4 in OSCC cells was examined by luciferase reporter assay. *P< 0.05, **P < 0.01.