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Original Research Article

Long non-coding RNA LINC00470 regulates the growth and epithelial-to-mesenchymal transition of human oral cancer cells

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Abstract

Purpose: *To investigate the role of long non-coding RNA LINC00470 in human oral cancer tissues and cell lines (OC3, SCC9 and CAL27).*
Methods: *Expression of mRNA was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Cell viability and proliferation were measured using MTT and EdU assays, respectively, while cell migration and invasion were determined with transwell assay. Protein expression was assayed with western blotting.*
Results: *There was significant up-regulation of LINC00470 in the cancer tissues and cell lines (OC3, SCC9 and CAL27) ($p < 0.05$). Silencing of LINC00470 led to significant inhibition of the proliferation and colony-formation potential of the oral cancer cells ($p < 0.05$). Furthermore, silencing of LINC00470 in oral cancer cell lines caused significant induction of apoptosis, suppression of migration and invasion, and restoration of epithelial-to-mesenchymal transition (EMT).*
Conclusion: *These results strongly demonstrate the oncogenic role of LINC00470 in oral cancer, thereby suggesting its possible prognostic and therapeutic potential in this lethal malignancy.*

Keywords: *Oral cancer, Long non-coding RNA, LINC00470, Apoptosis, Metastasis, EMT*

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INTRODUCTION

Oral cancer is one of the frequently-diagnosed human cancers, and in terms of over-all disease prevalence, it is considered the sixth most common cancer at the global level [1]. Oral squamous cell carcinoma (OSCC) is the most common type of oral carcinoma, with annual cases of more than half a million, and 5-year survival rate of about 50 % [2]. Moreover, OSCC is associated with post-surgical local recurrence and distant metastasis which account for its poor prognosis and lethality [3]. A number of recent studies on oral cancer have reported the possible applicability of regulatory RNAs such as micro-RNAs and long non-coding RNAs for prognostic and therapeutic purposes [4]. Long non-coding RNAs (lncRNAs) are a heterogeneous class of non-protein coding RNA transcripts comprising more than 200 nucleotides which are transcribed by RNA

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polymerase II. The lncRNAs often exhibit tissue-specific expression patterns, and they are crucial in cellular signalling pathways involved in regulation of the hallmarks of animal growth, differentiation and development [5]. Dysregulation of lncRNAs has been shown to be closely linked with various human pathological conditions such as different types of human cancers. Deregulation of LncRNAs has been reported in human oral cancer [6]. One of the LncRNAs i.e., LINC00470 is known for its oncogenic role in human cancers such as glioblastoma and gastric cancer [7]. Moreover, oral cancer cells have been reported to accelerate proliferation and metastasis in melanoma [8]. However, the function of LINC00470 in human oral cancer has not yet been elucidated. Therefore, the current study was aimed at elucidation of the role of LINC00470 in human oral cancer cells and tissues.

METHODS

Human tissues and cell lines

After obtaining signed informed consent, oral cancer tissue samples were taken from oral cancer patients immediately after surgery, prior to application of chemotherapy/radiotherapy, at Huzhou Central Hospital (Zhejiang,China). Normal tissues were obtained from patients who underwent surgery for non-neoplastic diseases of the head and neck at the same hospital. The study on human tissues was approved by the institutional ethics committee of Huzhou Central Hospital as per approval no. SXH/23/2020. The study was carried out in line with the guidelines in the Helsinki Declaration [9]. Clinical specimens were preserved by refrigeration at -80 ^{o}C, prior to use. Three oral cancer cell lines i.e. OC3, SCC9 and CAL27, along with NOK-16B normal oral epithelial cells were procured from American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines were cultured in 10 % Dulbecco's Modified Eagle medium (high glucose; Invitrogen, CA) supplemented with 10 % FBS (Gibco) at 37 ^{o}C in a 5 % CO_2 humidified incubator. Si-LINC00470 and si-NC (GenePharma, Shanghai, China) were stably transfected into CAL27 cancer cells using Lipofectamine 2000 (Invitrogen, USA) as per the manufacturer's instructions. The efficacy of transfection was determined by qRT-PCR.

RNA isolation and gene expression analysis

Total proteins were extracted from tissue samples and cell lines using TRIzol reagent (Thermo Fisher Scientific). The total RNA from each sample was subjected to reverse transcription to synthesize cDNA using iScript cDNA Kit (Bio-Rad Laboratories, Inc.). With cDNA as template, specific primers were used to amplify target genes through qRT-PCR. The PCR reactions were carried out using SYBR Green PCR Master Mix (Thermo Fisher Scientific). The expression levels of LINC00470, NCadherin, α-catenin, fibronectin and vimentin were determined using specific primers as indicated in Table 1.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. The qRT-PCR reactions were carried out in triplicate, and Ct values were used for quantitative gene expression analysis with the $2^{-\Delta\Delta Ct}$ method.

MTT proliferation assay

Stably-transfected CAL27 cancer cells were plated at a density of approximately 2×10^4 cells/well in a 96-well plate, and allowed to grow for 12, 24, 36, 48, 60 or 72 h at 37 ^{o}C. After the incubation, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Millipore Sigma) solution was added to each well, followed by incubation at 37 ^{o}C for 2 h. Thereafter, 200 µL of dimethyl sulfoxide (DMSO) was added to each well so as to solubilize the formazan crystals formed. The absorbance of each well was read at a wavelength of 570 nm in a microplate reader.

EdU incorporation and clonogenic assays

For EdU incorporation, stably-transfected CAL27 cells were incubated with 10 µM 5-ethynyl-2′-deoxyuridine (EdU) for 2 h in a 12-well plate.

Table 1: Primer sequence used in PCR

Gene	Sense (5′-3′)	Antisense (5′-3′)
LINC00470	CGTAAGGTGACGAGGAGCTG	GGGGAATGGCTTTTGGGTCA
N-cadherin	CGAATGGATGAAAGACCCATCC	TAGCAGCTTCAACGGCAAAGTTC
α-Catenin	CTCTACTGCCACCAGCTGAACATC	ATGCCTTCACTGTCTGCACCAC
Fibronectin	CCCACCGTCTCAACATGCTTAG	CTCGGCTTCCTCCATAACAAGTAC
Vimentin	TGAGTACCGGAGACAGGTGCAG	GGAGCCACTGCCTTCATAGTCAA
GAPDH	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT

The EdU incorporation assay was performed using Click-iT® EdU Alexa Fluor® Imaging Kit (cat#C10337, Molecular Probes, USA) as per the manufacturer's guidelines. Counterstaining was done using 4',6-diamidino-2-phenylindole (DAPI). Finally, the EdU-positive cells were analysed and counted using a fluorescence microscope (Olympus).

Oral cancer cell lines CAL27 transfected with si-LINC00470 or si-NC were seeded in 6-well plates at a density of 4×10^3 cells/well. The cells were incubated at 37 ^oC for a period of 2 weeks during which the culture medium was changed every 4 days. Thereafter, the cell colonies were washed with PBS, fixed using 70 % ethanol, stained with 0.5 % crystal violet, photographed and counted under a stereomicroscope.

Annexin V-FITC/PI staining

Each oral cancer cell line was seeded in a 12-well plate at a density of approximately 2×10^4 cells/well, and cultured for 24 h at 37 ^oC, after which the cells were harvested through centrifugation. The cells were treated with 2 % BSA and resuspended in 150 µL of PBS. This was followed by sequential staining with Muse Annexin V and Dead Cell Reagent (100 µL) for 30 min in the dark at room temperature. Finally, the signals were analyzed using the Muse Cell Soft V1.4.0.0 Analyzer Assay system (Millipore).

Western blotting

Radio-immunoprecipitation assay (RIPA) lysis buffer was used to extract total proteins from the transfected cells. The proteins were resolved on 10 % SDS-PAGE, followed by blotting onto nitrocellulose membranes. Following incubation with specific primary antibodies at 4 °C overnight, the membranes were incubated with a horse radish peroxidase-conjugated secondary antibody for 2 h at room temperature. Finally, the protein bands were analyzed using an enhanced chemiluminescence kit (ECL kit, Santa Cruz Biotechnology), and their intensities were quantified using Quantity One system (Bio-Rad, Hercules, CA, USA). β-Actin was used as loading control.

Transwell assays

The invasion and migration of CAL27 cancer cells transfected with si-LINC00470 or si-NC were studied using transwell chambers (Corning) with or without Matigel (BD Bioscience) coating, respectively. Briefly, 10^5 cancer cells were loaded in the upper chamber of transwell plate, while the lower chamber contained only culture medium supplemented 10 % FBS. The transwell chambers were incubated for 24 h at 37 ^oC. Cells which did not migrate were cleared with a cotton swab, followed by staining with crystal violet, and incubation for 5 min. Then, different sections of the cells were examined and photographed under a light microscope (TS100; Nikon Corporation, Tokyo, Japan) at x200 magnification. The cell invasion potency of the CAL27 cancer cells was determined using a similar procedure as described above, except that the transwell chambers used were coated with Matrigel.

Statistical analysis

Each experiment was performed in three replicates. The results are presented as mean ± standard deviation (SD). Statistical analysis was done using GraphPad prism 7.0 offline software. Two-tailed *t*-test was used to determine statistical difference between two treatment groups. Statistical differences were deemed significant at $p < 0.05$.

RESULTS

Up-regulation of LINC00470 in oral cancer

Results from qRT-PCR showed that the expression of LIC00470 was significantly up-regulated in oral cancer tissues ($p < 0.05$, Figure 1 A). Moreover, LINC00470 was significantly expressed in the oral cancer cell lines (OC3, SCC9 and CAL27), when compared to NOK-16B normal epithelial cells ($p < 0.05$, Figure 1 B). The highest expression level of LINC00470 occurred in CAL27 cell line. Therefore, the CAL27 cell line was used in subsequent studies.

LINC00470 silencing inhibit oral cancer cell proliferation

The RNA interference-based approach was used to carry out transcriptional knock-down of LINC00470 in CAL27 cancer cells, and the knock-down was confirmed using qRT-PCR (Figure 1 C). The si-LINC00470 cancer cells exhibited more than 5-fold down-regulation of LINC00470. Next, MTT assay was used to determine the effect of LINC00470 silencing on proliferation of oral cancer cells. The results revealed that CAL27 cancer cells transfected with si-LINC00470 exhibited significant inhibition of proliferation when cultured for different durations (Figure 1 D). The results indicate that LINC00470 negatively regulated the proliferation of oral cancer cells.

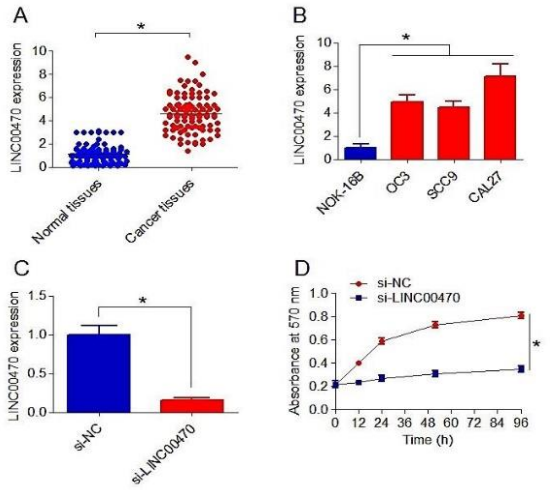

Figure 1: LINC00470 was upregulated in oral cancer tissues and cells. Expressions of LINC00470 in (A) normal and oral cancer tissues, (B) normal NOK-16B cells and oral cancer cells (OC3, SCC0 and CAL27), and in (C) CAL27 cells transfected with si-NC and si-LINC00470, (D) Cell viability of CAL27 cells transfected with si-NC and si-LINC00470. The experiments were performed in three replicates, and the data are presented as mean ± SD (*p < 0.05)

LINC00470 decreased cell viability via induction of apoptosis

The effect of LINC00470 knock-down on viability of oral cancer cells was determined using EdU incorporation assay. Oral cancer cells (CAL27) transfected with si-LINC00470 showed significantly lower EdU incorporation than the negative control (Figure 2 A). The relative percentage of EdU-positive cancer cells was less than 30, which was lower than that of control cells in which LINC00470 was silenced. Moreover, the colony-forming potential was significantly decreased by LINC00470 knock-down (p < 0.05). Furthermore, LINC00470-transfected CAL27 oral cancer cells showed only 30 % percent relative colony number, relative to negative control cells (Figure 2 B). Annexin V-FITC/PI staining procedure was used to identify the possible mechanism that underlying the anti-growth effects of LINC00470 silencing on CAL27 oral cancer cells, and levels of cell apoptosis were deduced using flow cytometry. The percentage of apoptotic cells were 35 % for siLIC00470-transfected CAL27 cancer cells, as against only 9 % for negative control cells (Figure 2 C). Western blotting showed that Bax protein expression was significantly increased, while Bcl-2 protein expression was significantly down-regulated in CAL27 oral cancer cells with LINC00470 silencing (p < 0.05; Figure 2 D). The results suggest that silencing of LIC00470 induced apoptosis in CAL27 oral cancer cells.

LINC00470 suppressed CAL27 oral cancer cell migration, invasion and EMT

Transwell assay was performed to determine the effect of LIC00470 silencing on migration and invasion of CAL27 oral cancer cells. It was found that migration of CAL27 oral cancer cells was significantly decreased by repression of LINC00470 (Figure 3 A). Cancer cell migration was reduced by more than 70 % by LINC00470 silencing. Similarly, invasion of CAL27 oral cancer cells was significantly decreased by LINC0040 knock-down, while LINC00470 silencing reduced cell invasiveness to only 30 % (Figure 3 B). In addition, qRT-PCR assay showed that LINC00470 silencing in CAL27 oral cancer cells significantly enhanced the expressions of epithelial markers (E-cadherin and α-catenin), while it significantly down-regulated the expressions of mesenchymal markers i.e. fibronectin and vimentin (Figure 3 C). These results are indicative of pro-metastatic regulatory role of LINC00470 in oral cancer.

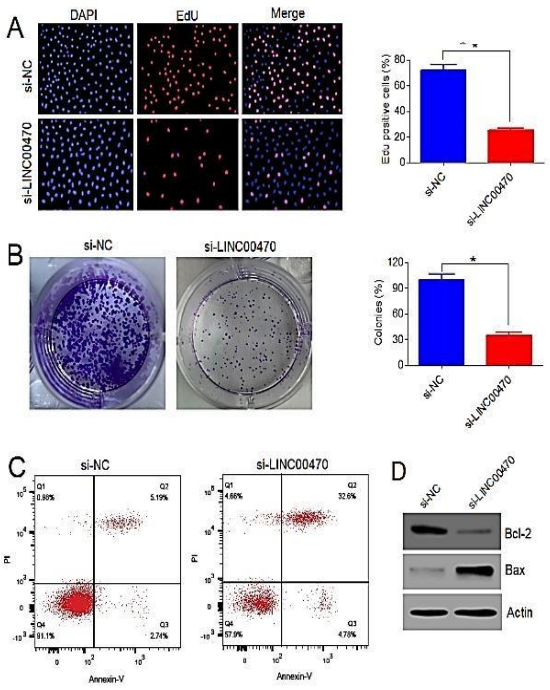

Figure 2: LINC00470 silencing induced apoptosis in CAL27 oral cancer cells. (A) EdU staining showing proliferation of CAL27 cells transfected with si-NC and si-LINC00470. (B) Colony-formation assay showing colony formation. (C) Annexin V/PI staining showing apoptosis of CAL27 cells transfected with si-NC and si-LINC00470. (D) Western blotting showing protein expressions of Bax and Bcl-2 in CAL27 cells transfected with either si-NC or si-LINC00470. The experiments were performed in three replicates, and the data are presented as mean ± SD (*p < 0.05)

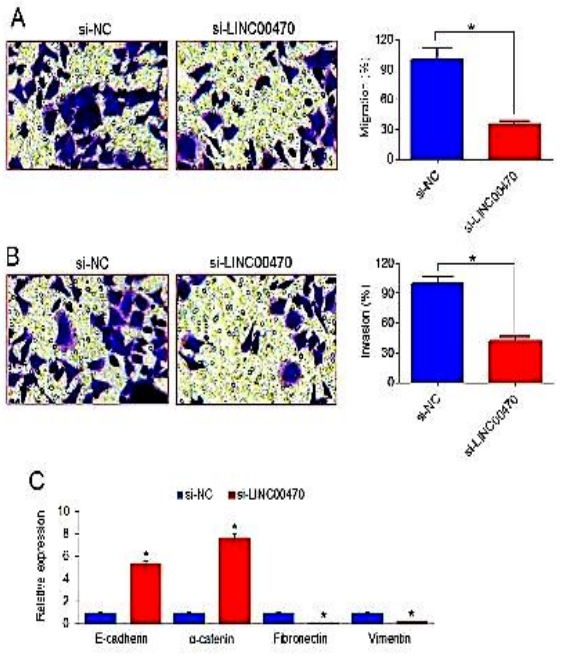

Figure 3: LINC00470 regulated migration and invasion of CAL27 oral cancer cells. Transwell assays showing (A) migration, and (B) invasion of CAL27 cells transfected with si-NC and si-LINC00470. (C) Expressions of epithelial and mesenchymal markers in CAL27 cells transfected with si-NC and si-LINC00470. The experiments were performed in three replicates, and the data are presented as mean ± SD (*p < 0.05)

DISCUSSION

Recent advancements in sequencing technology and revolution in the field of molecular biology have increased the understanding of scientists on the intricacies of the human genome. A major portion of the human genome previously considered to comprise "functionless or junk DNA" has been shown to be actively transcribed to different types of RNAs which do not code for proteins but are functionally very crucial [10]. Among these non-coding RNAs, the micro-RNAs (miRs) and long non-coding RNAs (lncRNAs) have, in particular, attracted immense research focus for their involvement in diverse cellular pathways of growth, development and differentiation [11]. Transcribed by RNA polymerase II, lncRNAs exhibit tissue-specific expression pattern, and play crucial roles in human biological and physiological processes [12]. Deviation from the programmed transcriptional build-up of lncRNAs has been linked to human disorders, including cancer [13].

A study has revealed that oral cancer is associated with aberrant expression levels of specific lncRNAs [14]. Moreover, researchers have elucidated the prognostic and therapeutic applicability of a number of lncRNAs in human oral cancer [15]. The present study elucidated the role of lncRNA LINC00470 in regulating the growth and progression of oral cancer cells. There was significant up-regulation of LINC00470 in human cancer cells. This result is in agreement with a previous report showing significant over-expression of LINC00470 in oral cancer tissues and cell lines [16]. The *in vitro* loss of cell viability and suppression of oral cancer growth by LINC00470 silencing is reflective of its already established oncogenic regulatory role [17]. Moreover, LINC00470 enhances the proliferation and survival of malignant tumor cells. In the present study, LINC00470 was shown to negatively regulate apoptosis: silencing of LINC00470 significantly induced apoptosis. The silencing of LINC00470 led to significant up-regulation of Bax protein expression and down-regulation of Bcl2 protein expression, thereby increasing Bax/Bcl-2 ratio in favor of induction of apoptosis in oral cancer cells [18].

The anti-cancer effect of LINC00470 silencing on oral cancer cells was also evident in the significant suppression of cell migration and invasion *in vitro*, which reflects the pro-metastatic role of LINC00470. Furthermore, it was found that silencing of LINC00470 in oral cancer cells reversed EMT by enhancing the expressions of epithelial markers and down-regulating the expressions of mesenchymal markers.

CONCLUSION

This study demonstrates that LINC00470 is significantly up-regulated in oral cancer, and its transcriptional silencing inhibits the proliferation of cancer cells via induction of apoptosis. The oral cancer cells show a reduction in the rate of migration and invasion *in vitro* under LINC00470 silencing. Furthermore, LINC00470 silencing inhibits EMT in oral cancer cells. These results indicate the key regulatory effects of LINC00470 in oral cancer growth and metastasis, thereby suggesting its prognostic and therapeutic potentials.

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

The authors 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 them.

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