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Original Article

Kindlin-2-mediated upregulation of ZEB2 facilitates migration and invasion of oral squamous cell carcinoma in a miR-200b-dependent manner

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Abstract: The miR-200 family suppresses epithelial-mesenchymal transition by inhibiting ZEB1 and ZEB2 mRNA translation in several types of cancers. Kindlin-2 is a target gene of miR-200b and its expression level correlates positively to ZEB2 in oral squamous cell carcinoma (OSCC). Whether Kindlin-2 and ZEB2 share a competitive endogenous RNAs regulatory network in OSCC remains unclear. Here, we studied the expression levels of miR-200b, Kindlin-2, and ZEB2 and found direct interaction between miR-200b, ZEB2, and Kindlin-2 mRNA in OSCC. A series of experiments was performed to elucidate the role of miR-200b and Kindlin-2 in OSCC cells. To further investigate whether Kindlin-2 regulates ZEB2 as a "ceRNA", we utilized pools of siRNAs to deplete Kindlin-2 or ZEB2 in Tca-8113 cells. Significantly elevated expression levels of Kindlin-2 and ZEB2, down-regulated mRNA levels of miR-200b, and a positive correlation between Kindlin-2 and ZEB2 were found in OSCC cells. Additional results suggest that miR-200b directly targets ZEB2 and that Kindlin-2 3'UTR miR-200b repressed both the migration and invasive functionality of Tca-8113. Kindlin-2 and ZEB2 are involved in accelerated migration and invasion of Tca-113 cells *in vitro* and Kindlin-2 controlled ZEB2 expression. However, Kindlin-2-mediated ZEB2 regulation did not depend on miRNAs. These results indicate that Kindlin-2 does not act as ZEB2 ceRNA and modify the migration of Tca-8113 cells. Our results improve our understanding of the underlying molecular and cellular mechanisms of oral cancer metastasis.

Keywords: Oral squamous cell carcinoma, OSCC, ZEB2, Kindlin-2, miR-200b, ceRNA

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common head and neck cancers globally, with increasing incidence and more than 500,000 new cases annually [1]. The 5-year survival has not been significantly improved over the last two decades, despite notable improvements in treatment [2, 3]. To date however, the exact pathological mechanism remains poorly understood. Thus, related and detailed research is required to improve prevention, diagnosis, and therapy of OSCC.

MicroRNAs (miRNAs) are endogenous and non-coding RNA molecules with the ability to bind to messenger RNAs (mRNAs). These contain miRNA response elements (MREs) and either decrease the stability of target RNAs or at least limit their translation [4, 5]. Epithelial-mesenchymal transition (EMT) is typical for cancer cells and promotes a more advanced state of tumors progression [6]. Numerous studies indicated that miRNAs regulate cancer metastasis (both positively and negatively) by influencing the process of EMT [7]. The miR-200 family has been shown to suppress EMT by

inhibiting the translation of ZEB1 and ZEB2 mRNAs in several types of cancers [8, 9]. However, data about the exact role of the miR-200 family in oral cancer is still missing.

Theoretically, each miRNA can target several mRNAs and several miRNAs can potentially target one specific mRNA that contains multiple MREs. Thus, different mRNAs can co-adjust via binding of common MREs to the same miRNAs and acting as competitive endogenous RNAs (ceRNA) [10]. Several studies confirmed that mRNA serving as ceRNA contributes to disease progression [11-13]. However, it remains unclear whether ZEB2 significantly interacts with its ceRNAs and their common miRNAs in OSCC. Kindlin-2 is a member of the kindlin family, which includes structurally similar and evolutionarily conserved proteins, and regulates cell-matrix adhesion and integrin signaling. Kindlin-2 has also been reported to be up-regulated in breast cancer cell lines [14], gastric cancer cell lines [15], and human uterine leiomyomas [16]. Kindlin-2 has been suggested to functionally promote tumor cell proliferation, migration, invasion, and adhesion [14, 17, 18]. Furthermore, Zhang et al. reported that miR-200b regulates the cytoskeletal and adhesive machinery and suppresses invasiveness via targeting Kindlin-2 [19]. These results suggestKindlin-2 could act as ceRNA of ZEB2.

We found that miR-200b directly targeted ZEB2 and Kindlin-2 3'UTRs, consequently suppressing migration and invasion of OSCC cells. Moreover, we found a positive correlation between Kindlin-2 and ZEB2 expression levels. These findings provide mechanistic insight for the occurrence of OSCC and suggest both Kindlin-2 and ZEB2 as promising targets for OSCC therapy.

Methods

Clinical specimens

Formaldehyde-fixed, paraffin-embedded (FFPE) samples of fresh tissue specimens, including eight cases of carcinoma and paracancerous tissue, were collected from January 2009 to June 2015 from the Stomatology Hospital of the Xi'an Jiaotong University/the Affiliated Hospital of Qingdao University. None of the patients received relevant chemotherapy prior to surgery and samples were immediately frozen in liquid nitrogen after surgery and constantly remained at -80°C. All histological diagnoses for normal or OSCC tissues were performed by two qualified pathologists. The Medical Ethical Committee of the College of Medicine approved access to patient tumor samples and informed consent was obtained from all patients prior to the experiment.

Cell culture and transfection

The human tongue squamous cell line Tca-8113 was established in the Ninth People's Hospital, Shanghai Second Medical University in 1981, cultured in RPMI 1640 medium (HyClone, America) with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY), and obtained from KeyGEN Biotech (Nanjing, China). Cal-27 and SCC-15 were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS. All cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Cell transfection with plasmids and/or oligonucleotides was conducted using the Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. Cells were trypsinized, counted, and seeded in plates, the day prior to transfection to ensure appropriate cell density on the day of transfection. Oligonucleotides were used at a final concentration of 37.5 nM and the plasmids were used at a concentration of 1.25 ng/uL. Both were maintained in antibiotic-free RPMI 1640 medium. Transfection efficiency of the plasmids and oligonucleotides was verified via the pcDNA™ 6.2-GW/EmGFP-miR plasmid and FAM-labeled oligonucleotides, respectively, and was found to be above 60% (Supplementary Figure 1). All transfections were repeated in three independent experiments.

Plasmids/siRNA

To construct the pcDNA6.2/pre-miR-200b expression vector (pre-miR-200b), the stem-loop sequence of miR-200b was synthesized at Shanghai Sangon Biotech Co. Ltd. and cloned into the pcDNA6.2-GW vector at EcoR Iand Hind III sites (Promega). The complimentary sites for miR-200b in the 3'UTR of ZEB2 and Kindlin-2 were produced (Shanghai Sangon Biotech Co. Ltd.) and inserted into the pmirGLO vector at Sac Iand Xho I sites (Promega). Furthermore, mutated 3'UTR sequences of ZEB2 and Kind-

lin-2 were cloned and named ZEB2-3'UTR-MT and Kindlin-2-3'UTR-MT, respectively (Supplementary Table 1). The sequences of the constructed plasmids were validated via DNA sequencing (Sangon Biotech, Shanghai, China). The pcDNA6.2-GW vector (miR-200b-ctrl), anti-miR-200b-ctrl, and negative control (NC) (GenePharma, Shanghai, China) were all considered as negative controls of pre-miR-200b, anti-miR-200b, and small interfering RNA (siRNA) against Kindlin-2/ZEB2/Dicer. The sequences of siRNA are listed in Supplementary Table 2.

Total RNA extraction and quantitative real time PCR (qRT-PCR)

The total RNA of oral cancer cells or frozen tissue samples was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. The total RNA was subjected to qRT-PCR and all reagents for qRT-PCR were purchased from Takara (Dalian, China). RNA concentrations were detected with NanoDrop (NanoDrop Technologies, Inc.). To obtain mature miRNAs, 500 ng of total RNA extracted from the samples was reverse transcribed into cDNA using miRNA-specific primers and the resulting cDNA was then used to amplify mature miR-200b and an endogenous control (U6) via PCR. To analyze protein-coding genes, first-strand cDNA was generated using the PrimeScript® RT reagent Kit following the manufacturer's instructions. cDNA was used to amplify ZEB2 and Kindlin-2 genes, as well as an endogenous control (the β-actin gene) via PCR. The corresponding PCR primers are listed in Supplementary Table 3. The following PCR conditions were applied to amplify miRNA or protein-coding genes: initial denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 20 s, 55°C for 40 s, and 72°C for 40 s. Quantitative PCR was performed with the SYBR Premix Ex TaqTM II (TaKaRa, Otsu, Shiga, Japan) with the iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Comparative real-time PCR was performed in triplicate and relative expression was obtained using the $2^{-\Delta\Delta Ct}$ method.

Luciferase reporter assays

To verify direct interaction between miR-200b and ZEB2 or Kindlin-2 mRNA, Tca-8113 cells were inoculated in 96-well plates (5,000 cells per well) the day before transfection. All samples were transiently co-transfected with the fluorescent reporter plasmids pre-miR-200b, miR-200b-ctrl, anti-miR-200b, or anti-miR-200b-ctrl. Luciferase activity was measured 24 h after transfection using the Dual-Glo Luciferase Assay kit (Promega), following the manufactures' instructions. Firefly luciferase levels were normalized to Renilla luciferase levels. For each transfection, the average luciferase activity of three replicates was calculated.

Cell proliferation assay

The cell proliferation capacity was documented via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Exponentially growing cells were seeded in 96-well plates prior to transfection. Subsequent to transfection, cells were cultured for 24, 48, or 72 h and stained at indicated time points using 100 μL sterile MTT dye (0.5 mg/ml, Sigma, St. Louis, MO, USA) for 4 h at 37°C. Subsequently, the culture medium was removed and 100 μl of DMSO was added to each well to dissolve the crystals for 10 min at room temperature. Cell proliferation was assessed daily for three consecutive days. The absorbance was measured at 492 nm, using a microplate reader. All experiments were performed in biological triplicate.

Wound healing assays

Wound-healing assays were conducted after causing a linear wound, scraping a pipette tip across the confluent cell monolayer. Cells were then washed with PBS and cultured in RPMI 1640 supplemented with 10% FBS. Recovery of the disruption was observed for up to 48 h. Images were captured 0, 12, 24, and 48 h post-wounding. The cell migration motility of the repaired area was measured.

Transwell invasion and migration assay

Cell migration and invasion assays were performed using Transwell chambers (24-well insert, 8 μm pore size, BD Biosciences, USA). For the migration assay, 5×10^4 cells in 200 μl serum-free RPMI-1640 medium were plated in the uncoated top chamber. For the invasion assay, 2×10^5 cells in 200 μl serum-free RPMI-1640 medium were plated in the top chamber, which had been coated with 1:10 diluted Matrigel (BD Biosciences, San Jose, CA, USA). In the lower chamber, 500 μl medium, supplemented with 10% serum, was used as a che-

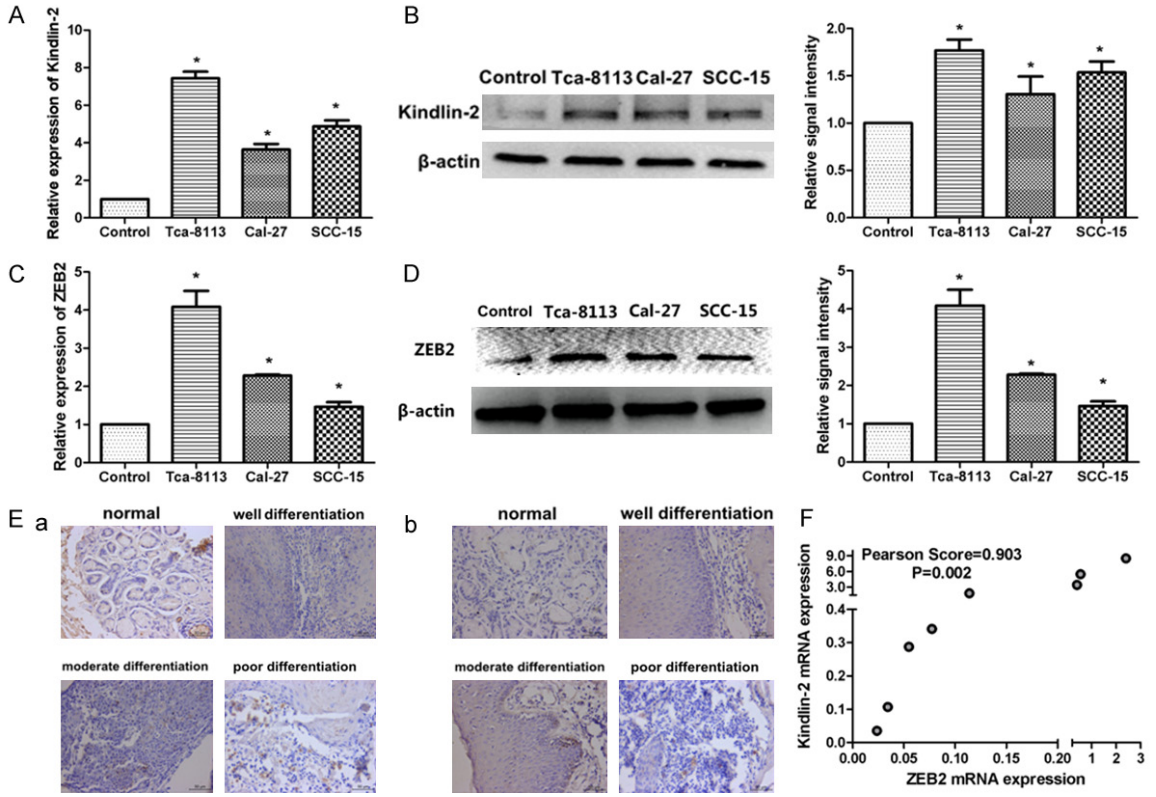

Figure 1. Kindlin-2 expression is positively correlated with ZEB2 in OSCC. A. qRT-PCR analysis of Kindlin-2 mRNA levels. B. Kindlin-2 protein expression measured via Western blot and quantitative data of densitometric analyses. C. qRT-PCR analysis of ZEB2 mRNA levels. D. ZEB2 protein expression. E. Immunostaining of ZEB2 (a) and Kindlin-2 (b). Representative pictures of OSCC samples are presented with normal, good, moderate, and poor differentiation. F. Pearson's correlation coefficients were calculated for mRNA expression of Kindlin-2 and ZEB2 (*P < 0.05 via Student's t-test).

moattractant. After the chamber was incubated in medium for 24 h (migration assay) or 48 h (invasion assay), cells that failed to migrate or invade through the pores were scraped out via wiping with a cotton swab. Cells that migrated to the bottom surface of the filter membrane were fixed with methanol, stained with 0.1% crystal violet, and photographed. The cell number was counted in three randomly selected fields using an inverted microscope.

Western blot analysis

The total protein of tissue samples and transfected cells was extracted with RIPA buffer (Sigma-Aldrich) according to the manufacturer's instructions. Identical amounts of protein lysates were resolved on 7-10% SDS-PAGE and then electrophoretically transferred to a methanol-activated polyvinylidene difluoride (PVDF) membrane (Millipore, Beijing, China). The membrane was then blocked with 5% non-fat milk in Tris-buffered saline Tween-20 (TBST) for 2 h after which it was probed with a primary monoclonal antibody overnight at 4°C, followed by incubation with horseradish peroxidase (HRP)-labeled goat-anti rabbitor anti-mouse IgG. The following primary antibodies were used in this study: anti-ZEB2, anti-Kindlin-2, and anti-β-actin. The antibodies to Kindlin-2, ZEB2, and β-actin were purchased from Millipore (1:3000 dilution, USA), GeneTex (1:800 dilution, USA), and Santa Cruz Biotechnology (1:5000 dilution, USA), respectively. Secondary antibodies were purchased from Santa Cruz Biotechnology (1:8000 dilution, USA). Protein expression was visualized via the ECL detection system.

Statistical analysis

All experiments were repeated thrice at least. SPSS 19.0 (SPSS, Chicago, IL) was used to perform statistical analyses. The differences in expression levels between groups were deter-

mined with the Student's t-test, while Pearson's correlation coefficients were calculated for mRNA expression of Kindlin-2 and ZEB2. All obtained *P*-values were double-sided and the null hypothesis was rejected at the $P < 0.05$ level, which is indicated with asterisks as described in figure legends.

Results

Kindlin-2 expression positively correlated with ZEB2

We investigated the expression of ZEB2 and Kindlin-2 in eight OSCC tissues, and a panel of three OSCC cell lines, including Tca-8113, Cal-27, and SCC-15. Western blotting and qRT-PCR were utilized to detect RNA and protein expression levels of both Kindlin-2 and ZEB2 and the results indicated that their expression levels were significantly higher in the three OSCC lines than in control (**Figure 1A-D**). The qRT-PCR result showed that the expression of miR-200b was down-regulated in OSCC cells (Supplementary Figure 2). These results suggest a negative correlation between the expressions of Kindlin-2 or ZEB2 and miR-200b. In fresh clinical samples, a positive correlation was found between the expressions of Kindlin-2 and ZEB2 (**Figure 1F**). Furthermore, we performed immunohistochemical staining of 24 OSCC tissues and four normal oral mucosa tissues. Of these 24 OSCC tissues, 21 (87.5%) cases tested positive for Kindlin-2 and 19 (79.2%) cases tested positive for ZEB2 (**Figure 1E**); however, none of the normal oral mucosa tissues expressed Kindlin-2 or ZEB2. To explore the clinical significance of the observed Kindlin-2 and ZEB2 accumulation during OSCC progression, we investigated the correlation between Kindlin-2 and ZEB2 levels depending on the stage of the disease. The results showed severely increased expression levels of Kindlin-2 and ZEB2 during OSCC progression, indicating that their upregulation was clearly associated with the OSCC stage.

miR-200b directly binds to Kindlin-2 and ZEB2 3'UTRs

To investigate a potential link between miR-200b and genes that are related to OSCC progression, candidate target genes of miR-200b were investigated with TargetScan. Kindlin-2 (GenBank Accession No. NM_006832) was predicted as a potential target of miR-200b. Kindlin-2 was found to contain a putative binding site at its 3'UTR for the seed region of miR-200b (**Figure 2A**). To verify direct targeting of Kindlin-2 by miR-200b, reporter plasmids were constructed by cloning wild-type 3'UTR or mutant 3'UTR (bearing a three-base mutation in the seed sequence of Kindlin-2) into a pmirGLO vector and the effect of the resulting miR-200b on luciferase activity was determined. Co-transfection of wild-type reporter plasmid and pre-miR-200b in Tca-8113 cells decreased the luciferase signal compared to miR-200b-ctrl. Blocking miR-200b via antisense oligomers significantly increased the luciferase signal. In contrast, the luciferase signal of the mutant reporter plasmid (containing the mutated Kindlin-2 3'UTR) was not affected by expression of miR-200b or by miR-200b knockdown (**Figure 2B**). To confirm that this targeted binding was functional, qRT-PCR analysis and Western blot analyses were conducted for the cell lysis of pre-miR-200b, anti-miR-200b, and negative control transfected cells. The results showed that the mRNA expression level of Kindlin-2 was not affected when Tca-8113 cells were transfected with pre-miR-200b or anti-miR-200b (**Figure 2C**). However, Kindlin-2 protein expression in these cells was either decreased or increased in response to pre-miR-200b or anti-miR-200b transfection, respectively (**Figure 2D**). These results suggest that miR-200b regulated Kindlin-2 expression via translational inhibition instead of the induction of degradation of its mRNA in OSCC cells.

Furthermore, the results showed that the 3' UTR of ZEB2 was directly targeted by miR-200b in Tca-8113 cells. **Figure 2E** shows that 3'UTR of ZEB2 mRNA included complementary sites for the seed region of miR-200b. Overexpression of miR-200b in Tca-8113 cells inhibited wild-typeZEB2 3'UTR reporter activity, which was not detected with the luciferase assay in the 3'UTR reporter of the mutant-type ZEB2. Furthermore, knocking down miR-200b enhanced luciferase activity in wild-type ZEB2 (**Figure 2F**). In contrast to Kindlin-2 regulation by miR-200b, miR-200b suppressed ZEB2 expression in OSCC cells via mRNA degradation and translational inhibition (**Figure 2G** and **2H**). Therefore, miR-200b simultaneously targeted Kindlin-2 and ZEB2. These results indicate a competitive endogenous RNA regulatory network between Kindlin-2 and ZEB2.

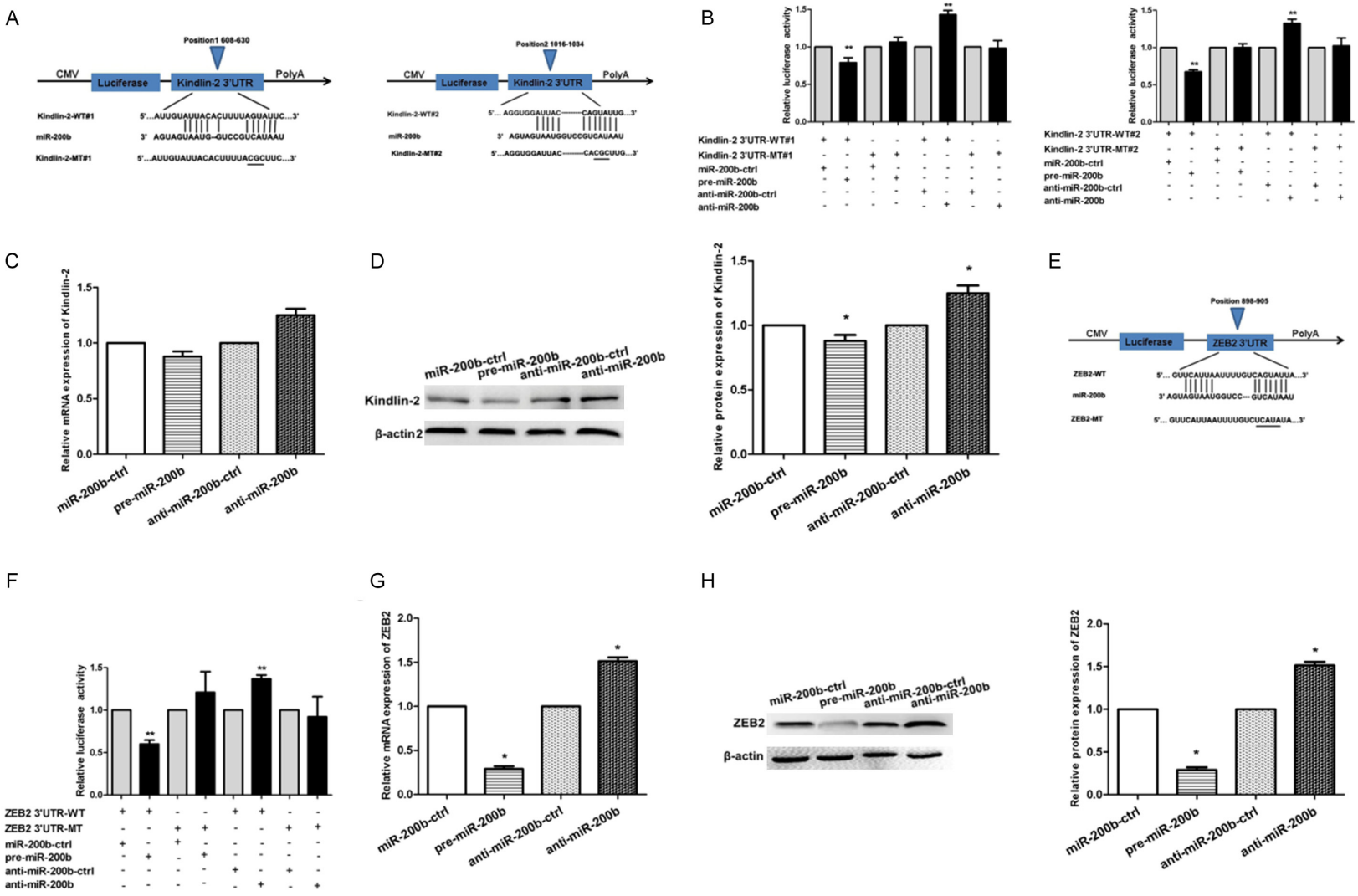

Figure 2. miR-200b directly targets Kindlin-2 and ZEB2 3'UTRs. A. Consequential pairing of the target region between miR-200b and 3'UTR of Kindlin-2 predicted via computational analysis. B. Luciferase activities of wild-type or mutant Kindlin-2 reporters in Tca-8113 cells, pre-miR-200b, or anti-miR-200b that were co-transfected with wild-type or mutant Kindlin-2 reporter in the cells. C. Relative mRNA expression of Kindlin-2 in Tca-8113 expressing pre-miR-200b or anti-miR-200b. D. Western blot shows Kindlin-2 protein and β-actin from Tca-8113 cells transfected with pre-miR-200b, anti-miR-200b, and negative controls. E. Consequential pairing of the target region between miR-200b and 3'UTR of ZEB2 predicted via computation analysis. F. Luciferase activities of wild-type or mutant ZEB2 reporters in Tca-8113 cells, pre-miR-200b, or anti-miR-200b that were co-transfected with the reporter in the cells. G. Relative mRNA expression of ZEB2 in Tca-8113 expressing pre-miR-200b or anti-miR-200b. H. Western blotting detected ZEB2 protein and β-actin from Tca-8113 cells transfected with pre-miR-200b, anti-miR-200b, and negative controls (*P < 0.05, **P < 0.01).

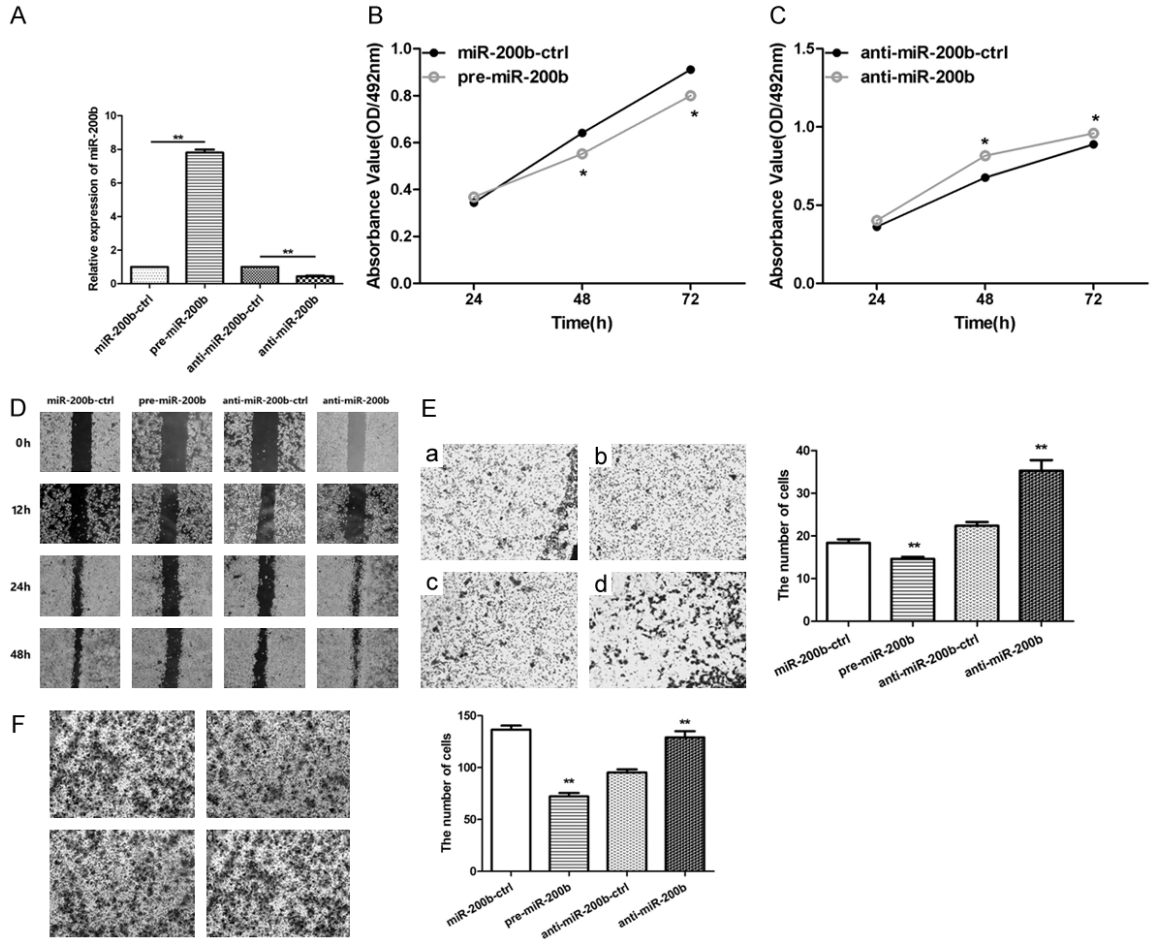

Figure 3. miR-200b repressed migration and invasion function of OSCC cells. A. miR-200b expression was examined in Tca-8113 cells that were transfected with miR-200b-ctrl, pre-miR-200b, anti-miR-200b-ctrl, or anti-miR-200b. B. The effects of pre-miR-200b on cell proliferation in Tca-8113 cells. C. The effects of anti-miR-200b on cell proliferation in Tca-8113 cells. D. Effects of miR-200b on Tca-8113 cell migration *in vitro*, detected via wound healing. E. Effects of miR-200b on invasion of Tca-8113 cells *in vitro*, detected via transwell assay. F. Effects of miR-200b on migration of Tca-8113 cells *in vitro*, detected via transwell assay (*P < 0.05, **P < 0.01).

miR-200b repressed migration and invasion function of Tca-8113 cells in vitro

To further elucidate the role of miR-200b in OSCC progression, a series of experiments were performed. Cells transfected with pre-miR-200b consistently displayed an increment of approximately 700% in miR-200b levels compared to pre-miR-200b-ctrl, while cells cultured in the presence of anti-miR-200b showed reduction of approximately 60% in miR-200b levels compared to anti-miR-200b-ctrl (**Figure 3A**). We performed an MTT assay and found that Tca-8113 cells with miR-200b over-expression significantly reduced Tca-8113 cell proliferation (**Figure 3B**). In contrast, the proliferation of Tca-8113 cells was promoted when endogenous miR-200b was knocked down via antisense oligonucleotides (**Figure 3C**). The results of transwell migration and matrigel invasion assays indicated that forced expression of miR-200b significantly decreased the migration and suppressed the invasion of Tca-8113 cells in transwell assays with matrigel. In contrast, knock-down of endogenous miR-200b significantly promoted migration and invasion of Tca-8113 cells (**Figure 3D**, **3E**). Similarly, wound-healing assay showed that overexpression and deletion of miR-200b displayed a notable slower or quicker recovery, respectively, compared to negative control cells after transient transfection of pre-miR-200b expression plasmid or anti-miR-200b (**Figure 3F**). In summary, these results demonstrate that miR-200b effectively

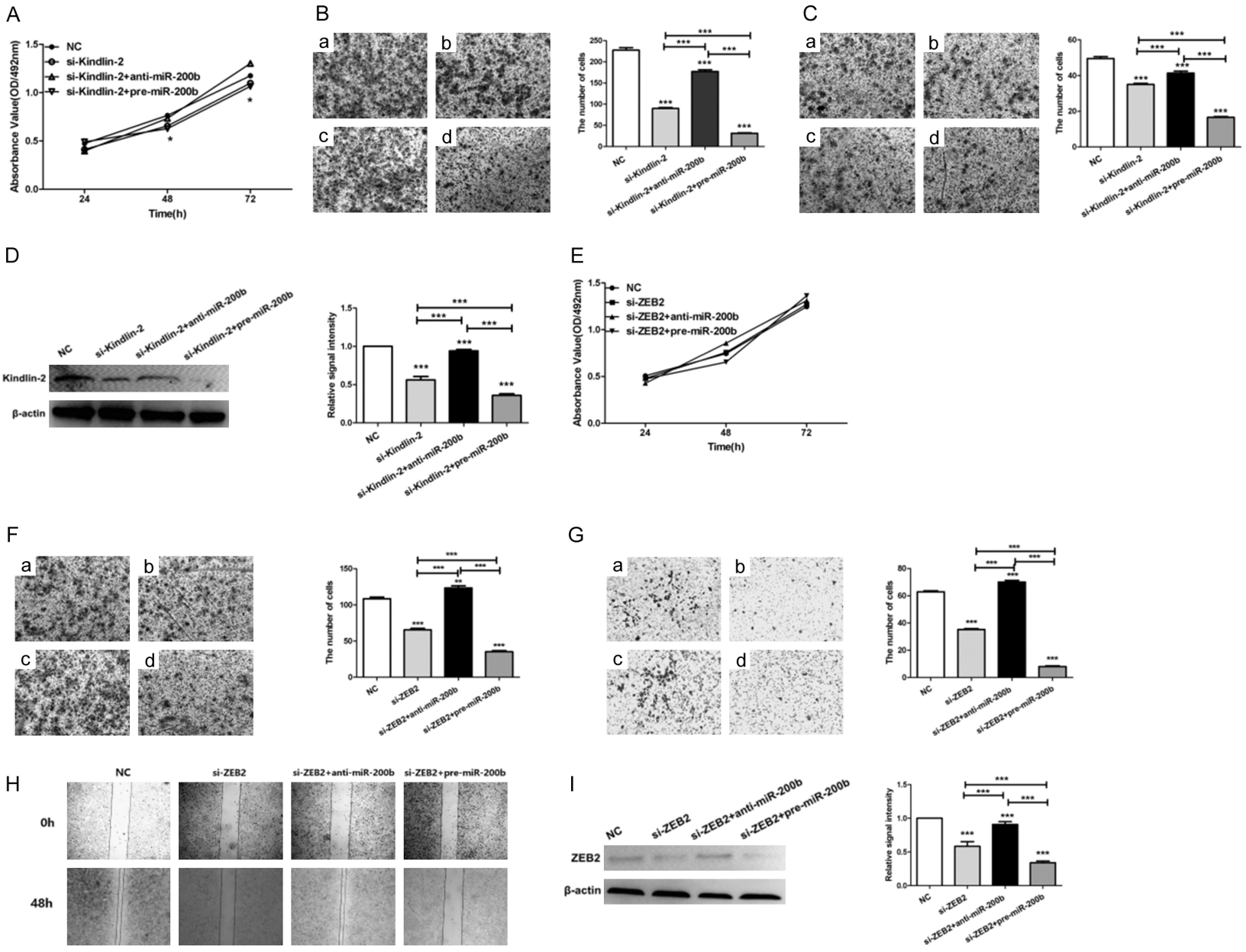
A
NC
si-Kindlin-2
si-Kindlin-2+anti-miR-200b
si-Kindlin-2+pre-miR-200b
Absorbance Value(OD/492nm)
Time(h)
B
C
The number of cells
D
Kindlin-2
β-actin
Relative signal intensity
E
si-ZEB2
si-ZEB2+anti-miR-200b
si-ZEB2+pre-miR-200b
F
G
H
0h
48h
I
ZEB2

Figure 4. Kindlin-2 and ZEB2 accelerated migration and invasion of Tca-113 cells *in vitro*. A. The effect of si-Kindlin-2 on the proliferation of Tca-113 cells was suppressed via inhibition of miR-200b *in vitro*. B. The effect of si-Kindlin-2 on the migration of Tca-113 cells was suppressed via transwell migration assays. C. The effect of si-Kindlin-2 on the invasion of Tca-113 cells was suppressed via matrigel invasion assays. D. Western blot detected Kindlin-2 protein and β-actin and quantitative data of densitometric analyses from Tca-8113 cells. E. The effect of si-ZEB2 on the proliferation of Tca-113 cells was suppressed via inhibition of miR-200b*in vitro*. F. The effect of si-ZEB2 on migration and invasion of Tca-8113 cells was suppressed via transwell migration. G. The effect of si-ZEB2 on migration and invasion of Tca-8113 cells was suppressed via matrigel invasion. H. The effect of si-ZEB2 on migration of Tca-8113 cells was suppressed via wound healing assays. I. Western blot detected ZEB-2 protein and β-actin and quantitative data of densitometric analyses of Tca-8113 cells (*P < 0.05, ***P < 0.001).

stopped proliferation, migration, and invasion of OSCC cells *in vitro*.

Kindlin-2 and ZEB2 accelerated migration and invasion of Tca-113 cells in vitro

We either transfected Tca-8113 cells with NC, Kindlin-2 siRNA, Kindlin-2 siRNA, and anti-miR-200b, or Kindlin-2 siRNA and pre-miR-200b. Kindlin-2 knockdown significantly suppressed proliferation, migration, and invasion of Tca-8113 cells. In addition, simultaneous knockdown of miR-200b sufficed to reverse the effects of Kindlin-2 siRNA on proliferation, migration, and invasion, which was assessed via MTT, transwell migration, and matrigel invasion assays (**Figure 4A-C**). This coincided with a pronounced fluctuation of Kindlin-2 protein levels (**Figure 4D**). Hence, these results suggest a converse effect between miR-200b and Kindlin-2. Moreover, the effect of silencing Kindlin-2 was, at least partly, dependent on the loss of miR-200b functional inhibition.

A small interfering RNA (siRNA) was used for ZEB2 knockdown and the resulting interference efficiency was verified via Western blot (Supplementary Figure 3). MTT assays indicated no remarkable impact of ZEB2 on Tca-8113 cell proliferation (**Figure 4E**). Otherwise, cell migration and invasion were clearly inhibited when ZEB2 was effectively silenced. Furthermore, the effect of ZEB2 siRNA on both migration and invasion of Tca-8113 cells was eliminated by miR-200b inhibition as indicated by transwell migration, matrigel invasion, and wound healing assays (**Figure 4F-H**). Western blot analyses confirmed that ZEB2 protein level decreased upon ZEB2 knockdown, which could be rescued via anti-miR-200b transfection (**Figure 4I**). In summary, these results suggest that the effect of ZEB2 silencing was dependent on the loss of miR-200b function suppression.

Kindlin-2 3'UTR could not act as a ceRNA and indirectly regulated the expression of ZEB2, thus influencing migration and invasion of Tca-8113 cells

To further examine whether Kindlin-2 regulated ZEB2 as a "ceRNA", we used pools of siRNAs to either deplete Kindlin-2 or ZEB2 in Tca-8113 cells. Kindlin-2 knockdown reduced ZEB2 protein levels by approximately 60% (**Figure 5A**). Kindlin-2 expression was slightly attenuated by si-ZEB2 (**Figure 5A**), which matched our expectations. Since Dicer is required for microRNA (including miR-200b processing), we determined its role in Kindlin-2 functionality. We treated cells to Dicer knockdown to evaluate whether Kindlin-2 controls ZEB2 levels in a miRNA-dependent manner. In these cells, siRNA against Kindlin-2 lowered ZEB2 levels. Likewise, Kindlin-2 depletion significantly reduced ZEB2 expression after Dicer knockdown in Tca-8113 cells (**Figure 5B**). Moreover, ZEB2 silencing resulted in diminishing Kindlin-2 levels, which remained unaffected in Tca-8113 cells after Dicer knockdown. Furthermore, there were no apparent differences in cell proliferation between different treatment groups (**Figure 5C**). Transwell migration and wound healing assays showed that the cell migration ability was significantly inhibited after Dicer knockdown, but not in response to Kindlin-2 silencing with siRNA (**Figure 5D**, **5E**). Kindlin-2 controlled ZEB2 expression; however, this regulation was not dependent on miRNAs. This indicates that Kindlin-2 could not act as ZEB2 ceRNA and thus could not modify Tca-8113 cell migration.

Discussion

Currently, the molecular mechanisms responsible for OSCC development and progression remain poorly understood. Thus, exploring the OSCC pathogenesis still remains an urgent requirement. Results of preliminarily immunohistochemical staining suggested that Kindlin-

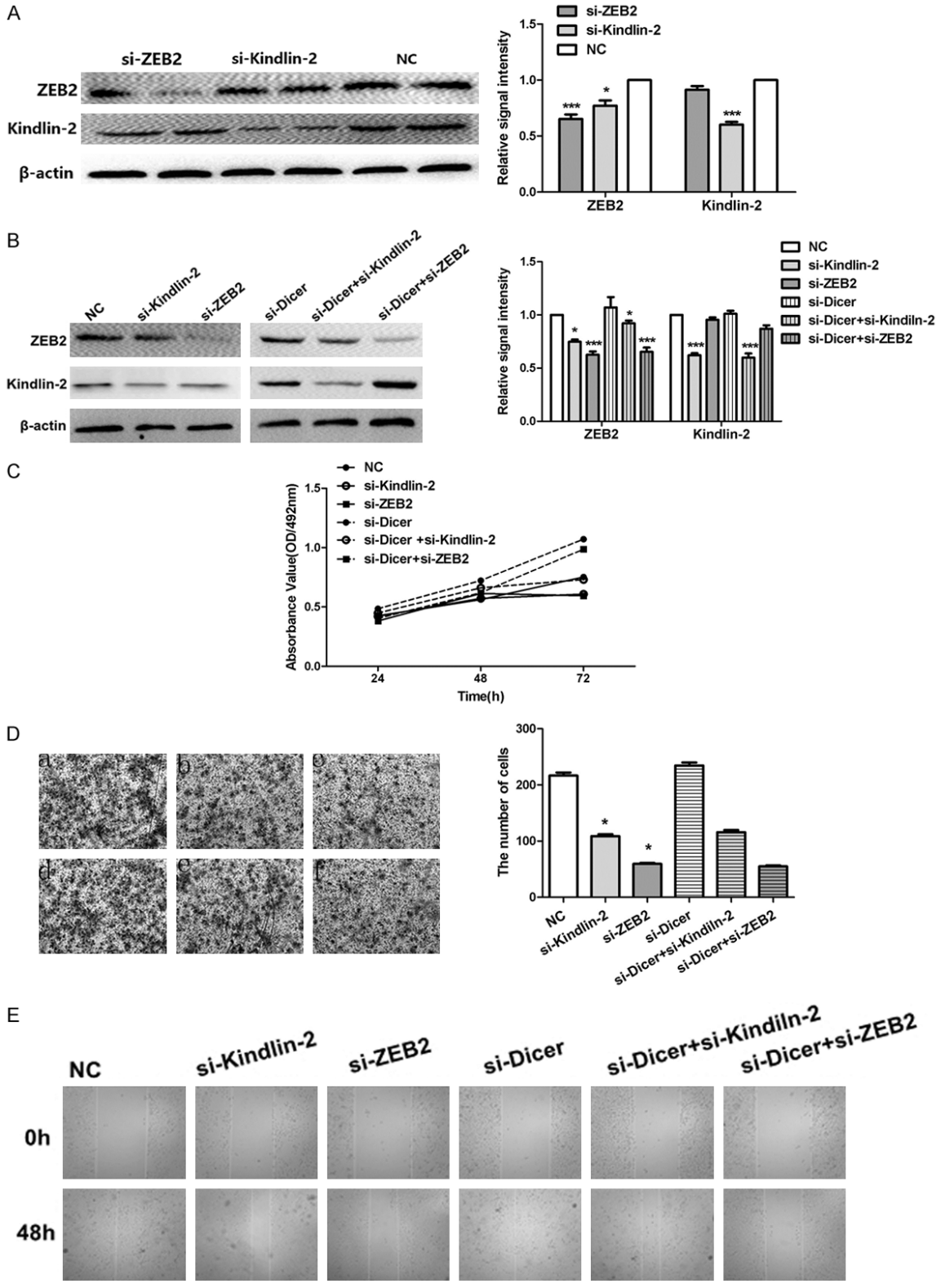

Figure 5. Kindlin-2 3'UTR could not act as a ceRNA to regulate the expression of ZEB2, and influence migration and invasion of Tca-8113 cells. A. Kindlin-2 silencing lowered ZEB2 protein levels in Tca-8113 cells. Western blot analysis (left) and quantification (right). B. Kindlin-2 controlled ZEB2 levels in a miRNA-independent manner. C. Cell proliferation curves are displayed for NC, si-Kindlin-2, si-ZEB2, and si-Dicer co-transfected cells. D. Migration. E. Wound healing (*P < 0.05, ***P < 0.001).

2 and ZEB2 upregulation could be related to a lower OSCC differentiation degree. However, this speculation required further confirmation due to the small sample size. Still, this was the first result to confirm the clinical significance of Kindlin-2 in OSCC.

The Kindlin-2 protein contains a FERM domain and has been reported to change the cell shape by connecting the cytoskeleton to cell-extracellular matrix adhesions [20, 21]. Kindlin-2 belongs to the Kindlin protein family, mediating the inside-out activation of integrin signaling pathways [22]. The central role of Kindlin-2 in malignant tumor progression has been recognized, e.g., Kindlin-2 levels were found to be higher in malignant mesothelioma cell lines and enforced expression contributed to tumor progression [18]. However, in OSCC, both expression and biological significance of Kindlin-2 remain unclear. In agreement with previous concepts, the results of our study show that in OSCC, the expression of miR-200b was down-regulated, while the expression of Kindlin-2 was up-regulated. Kindlin-2 has been shown to be one of the targets of miR-200b. A functional study further revealed that miR-200b effectively abolished migration and invasion of oral cancer cells *in vitro*, whereas Kindlin-2 (as an oncogene) positively correlated with the ability of a cell to migrate and invade. A recent report showed that Kindlin-2 knockdown phenocopied the functions of miR-200b for ESCC cell migration and the suppression of invasiveness, whereas overexpression of Kindlin-2 rescued these phenotypic effects of miR-200b [19], which is similar to our results for OSCC. In summary, the combination of previous literature and our results suggests that miR-200b can inhibit tumor progression in most tumors. If this hypothesis can be confirmed, a novel and promising therapeutic target for tumor intervention would have been identified.

In 2011, Salmena et al. presented the "competing endogenous RNA" (ceRNA) hypothesis, indicating that transcribed pseudogenes, messenger RNAs, and long non-coding RNAs "talk" to each other, using microRNA response elements (MREs) as letters of a new "language" [10]. Soon, this hypothesis has been widely confirmed in disease processes. It has been demonstrated that both mRNAs, VAPA, and CNOT6L mediated the protagonist PTEN via ceRNA interplay in DU145 cells and HCT116 cells, antagonizing PI3K/AKT signaling and thus resulting in anti-proliferation effects [13]. Furthermore, Hansen et al. reported a circRNA that acted as a miR-7 sponge and named it circular RNA sponge for miR-7 (ciRS-7). This includes more than 70 selectively conserved MREs and clearly inhibits miR-7 activity, thus resulting in an increased level of miR-7 targets [23]. To date, no ceRNA report for oral cancer has been published. Here, the relationship between Kindlin-2 and ZEB2 was explored, as well as their coordination to regulate the biological function of OSCC. We found that Kindin-2 expression positively correlated with ZEB2, and that miR-200b could regulate migration and invasion of Tca-8113 cells by directly targeting both. However, kindlin-2 could not function as miR-200b sponge to inhibit free miR-200b activity, thus repressing migration and invasion of OSCC cells. Several reasons are likely: (i) Specificity of OSCC cells. Although the roles of ceRNA in other cancer types have been defined (such as prostate cancer [24], liver cancer [25], breast cancer [26], lung cancer [27], endometrial cancer [28], melanoma [12], glioblastoma [29], and renal cancer [30]), we found no reports about ceRNA networks in OSCC. Therefore, it is not surprising that Kindlin-2 could not act as ceRNA and mediate the biological behavior of OSCC due to disease-specific expression patterns. (ii) Other ZEB2 ceRNAs are involved in the regulation. ceRNA regulation follows a highly complex mechanism. Likely, a ceRNA (denoted ceRNA1) connects Kindlin-2 with ZEB2. Kindlin-2 directly interacts with ceRNA1 via a pool of miRNAs, and ceRNA1 directly interacts with ZEB2 via an additional pool of miRNAs. Kindlin-2 would then indirectly interact with ZEB2 miRNAs-ceRNA1-miRNAs. Ala et al. presented compelling evidence that such an indirect ceRNA interaction was exceptionally effective and that ceRNAs were very sensitive to changes of other ceRNAs even without shared miRNAs [31]. Thus, Kindlin-2 could regulate the expression of ZEB2, thus facilitating migration and invasion of OSCC, but could not function as ceRNA of ZEB2. (iii) Other signaling pathways. As indicated by our results, Kindlin-2 did not act as ZEB2 ceRNA; however, Kindlin-2 regulatedZEB2. One plausible explanation may be that Kidnlin-2 was involved in another signaling pathway and indirectly affected the level of ZEB2. (iv) No equilibrium phenomenon between miR-200b and Kindlin-2 or a smaller number of shared miR-

NAs: The best ceRNA interaction with one ceRNA has been demonstrated to have a noticeable effect on its interacting ceRNA partners. This has been proposed to occur when miRNA and ceRNA levels were near equimolarity [32, 33]. Therefore, this imbalance between miR-200b and Kindlin-2 could have weakened the ceRNA effect, thus could not be examined in our study. However, the number of shared miRNAs among RNA transcripts played a key role in determining the effectiveness of ceRNA interaction [31, 32, 34]. Consequently, the smaller number of shared miRNAs may explain why Kindlin-2 cannot act as ceRNA of ZEB2.

Conclusion

The reported results clearly show the ability of miR-200b to functionality repress migration and invasion via directly targeting both Kindlin-2 and ZEB2. Furthermore, Kindlin-2 3'UTR did not act as ceRNA and indirectly regulated the expression of ZEB2, thus influencing migration and invasion of Tca-8113 cells. Despite this limitation, our results improve our understanding of the underlying molecular and cellular mechanisms of oral cancer metastasis. These genes may be targets for the development of novel therapeutics that will eventually improve both prevention and treatment of oral cancer progression. Currently, exploration and studies of ceRNAs in OSCC are at a primary stage. However, we envision the ceRNA network to eventually be fully understood and thus, present novel treatment opportunities for oral cancer.

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Disclosure of conflict of interest

None.

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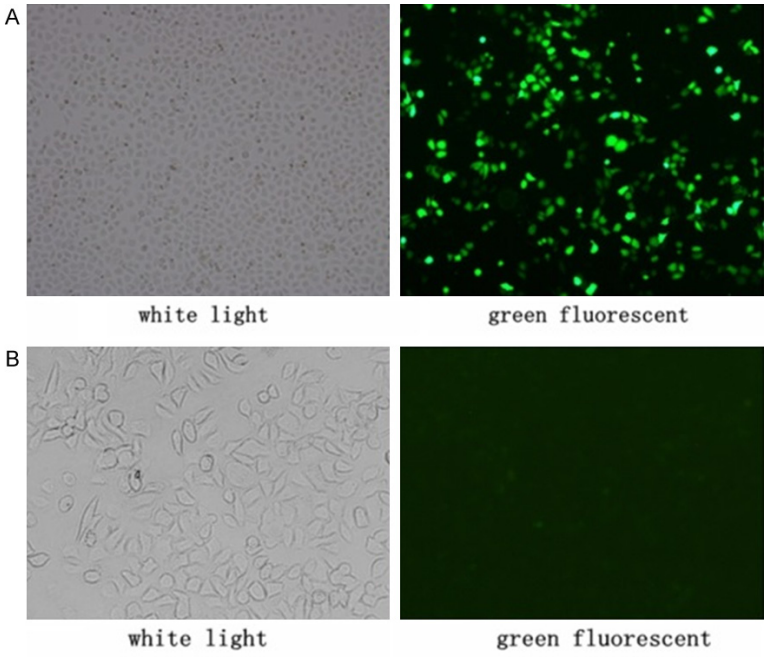


Supplementary Figure 1. Fluorescent microscopy confirmed the transfection efficiecy of pre-miR-200b or si-FAM. pre-miR-200b (A) and si-FAM (NC) (B) were transfected into Tca-8113 cells. Light pictures and fluorescent images were captured at 48 h after transfection.

Supplementary Table 1. Nucleotide sequences used for the cloning of expression vector

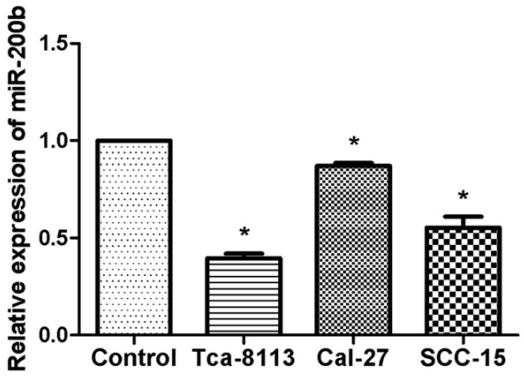
Name	Sequence (5'-3')
miR-200b-sence	AATTCCCAGCTCGGGCAGCCGTGGCCATCTTACTGGGCAGCATTGGATGGAGTCAGGTCTCTAATACTGCCTGGTAATGATGACGGCGGAGCCCTGCACGA
miR-200b-antisense	AGCTTCGTGCAGGGCTCCGCCGTCATCATTACCAGGCAGTATTAGAGACCTGACTCCATCCAATGCTGCCCAGTAAGATGGCCACGGCTGCCCGAGCTGGG
ZEB2-Wild-sense	CGTTCATTAATTTTGTCAGTATTAC
ZEB2-Wild-antisense	TCGAGTAATACTGACAAAATTAATGAACGAGCT
ZEB2-Mutant-sense	CGTTCATTAATTTTGTCTCATATAC
ZEB2-Mutant-antisense	TCGAGTATATGAGACAAAATTAATGAACGAGCT
Kindlin-2-Wild-sense #1	CATTGTATTACACTTTTAGTATTCC
Kindlin-2-Wild-antisense #1	TCGAGGAATACTAAAAGTGTAATACAAT GAGCT
Kindlin-2-Mutant-sense #1	CATTGTATTACACTTTTACGCTTCC
Kindlin-2-Mutant-antisense #1	TCGAGGAAGCGTAAAAGTGTAATACAATGAGCT
Kindlin-2-Wild-sense #2	CAGGTGGATTACCAGTATTGC
Kindlin-2-Wild-antisense #2	TCGAGCAATACTGGTAATCCACCTGAGCT
Kindlin-2-Mutant-sense #2	CAGGTGGATTACCACGCTTGC
Kindlin-2-Mutant-antisense #2	TCGAGCAAGCGTGGTAATCCACCTGAGCT

Supplementary Table 2. The sequences of anti-miR-200b and siRNA for Kindlin-2/ZEB2/ Dicer

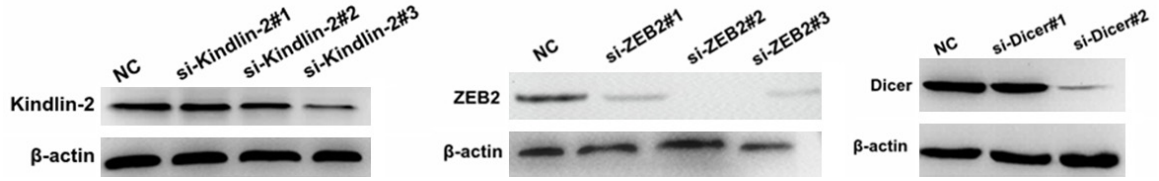
Name	Sequence (5'-3')
anti-miR-200b	UCAUCAUUACCAGGCAGUAUUA
si-ZEB2 #1	CCUCUUGUCAUCUGUACUUUU
	AAGUACAGAUGACAAGAGGUU
si-ZEB2 #2	GCAUGUAUGCAUGUGACUUUU
	AAGUCACAUGCAUACAUGCUU
si-ZEB2 #3	CCUCUUGUCAUCUGUACUUUU
	AAGUACAGAUGACAAGAGGUU
si-Kindlin #1	GCUUAAGCUGGUGGAGAAATT
	UUUCUCCACCAGCUUAAGCTT
si-Kindlin #2	CCAAUCACGUCACCAGAAATT
	UUUCUGGUGACGUGAUUGGTT
si-Kindlin #3	GCAGAAGGCAUGAAUGAAATT
	UUUCAUUCAUGCCUUCUGCTT
si-Dicer #1	AGAGGUACUUAGGAAAUUU
Si-Dicer #2	GGAGCUUGAUUUGCAUGAU

Supplementary Table 3. Primers used for qRT-PCR

Name	Sequence (5'-3')
miR-200b-RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGAC TCATCAT
miR-200b-F	ATCCAGTGCGTGTCGTG
miR-200b-R	TGCTTAATACTGCCTGGTA
U6-RT	CGCTTCACGAATTTGCGTGTCAT
U6-F	GCTTCGGCAGCACATATACTAAAAT
U6-R	CGCTTCACGAATTTGCGTGTCAT
ZEB2-F	CAAGAGGCGCAAACAAGC
ZEB2-R	GGTTGGCAATACCGTCATCC
Kindlin-2-F	GGATCCATGGCTCTGGACGGGATAAGGATG
Kindlin-2-R	CTCGAGCAGTATTCCTATTCACACCCAACC
β-actin-F	CCAACCGCGAGAAGATGA
β-actin-R	CCAGAGGCGTACAGGGATAG



Supplementary Figure 2. Expression of miR-200b in a panel of oral cancer cell lines measured by qRT-PCR.



Supplementary Figure 3. Western blottings indicated the expression of Kindlin-2, ZEB2, and Dicer with different siRNAs.