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MiR-1254 Functions as a Tumor Suppressor in Oral Squamous Cell Carcinoma by Targeting CD36

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Abstract

Oral squamous cell carcinoma is one of the most common cancers around the world. The patients with oral squamous cell carcinoma are often diagnosed at late stages, leading to unfavorable prognosis. MicroRNAs might function as oncogenes or tumor suppressor genes in the tumorigenesis of cancer. This study aimed to explore the role of miR-1254 in oral squamous cell carcinoma. We examined the expression levels of miR-1254 in oral squamous cell carcinoma tissue samples and cell line.Proliferation and invasion assays were performed in oral squamous cell carcinoma cells with miR-1254 overexpression or underexpression. The potential regulatory mechanisms were also explored. We found that miR-1254 was significantly reduced in oral squamous cell carcinoma tissues and cell lines. In addition, downregulation of miR-1254 in oral squamous cell carcinoma tumor tissues was closely associated with cancer staging and lymph node metastasis. Enforced expression of miR-1254 significantly inhibited proliferation and invasion in oral cancer cells, and downregulation of miR-1254 promoted the oncogenic activities of oral cancer cells. CD36 was identified as a direct downstream target of miR-1254 by the luciferase reporter assay. Overexpression of CD36 partially restored the proliferation and invasion capacity inhibited by miR-1254. CD36 expression was inversely correlated with miR-1254 expression in the oral squamous cell carcinoma tissues. Taken together, our study provided the compelling evidence that miR-1254 might inhibit the progression of OSCC by partially downregulating CD36, and restoration of miR-1254 may represent an effective strategy for treating oral squamous cell carcinoma.

Keywords

miR-1254, oral squamous cell carcinoma, CD36, proliferation, invasion

Abbreviations

ATCC, American Type Culture Collection; cDNA, complementary DNA; LNM, lymph node metastasis; mRNAs, messenger RNAs; miRNA, microRNA; OD, optical density; OSCC, oral squamous cell carcinoma; RT-PCR, real-time polymerase chain reaction; UTR, untranslated region

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Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide, accounting for around 90% of malignant oral neoplasms.[1,2] Despite diagnostic and therapeutic advances in the past few decades, the 5-year overall survival rate of OSCC remained at approximately 50%.[3] A high percentage of patients with OSCC are diagnosed at the advanced stage, with positive LMN or even with distant metastasis, which contribute to unfavorable clinical outcome of the disease.[4] Herein, it is extremely important to elucidate the molecular mechanisms underlying the initiation and progression of OSCC.

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MicroRNAs (miRNAs) is a class of endogenous noncoding RNAs with 17 to 25 nucleotides in length.[5] It can negatively regulate gene expression at the post-transcriptional level by binding to 3′-untranslated regions (UTRs) of target messenger RNAs (mRNAs), resulting in direct mRNA degradation or translational repression.[6] It has been demonstrated that miRNAs regulate various biological processes such as proliferation, differentiation, and development. As deregulation of a single miRNA might affect hundreds or even thousands of downstream targets, accumulating evidences show that aberrant expression of miRNAs is closely associated with the development and progression of most cancers including OSCC.[7] For instance, miR-16 was downregulated in OSCC tissues and acted as a tumor suppressor gene in OSCC by suppressing the activity of tousled-like kinase 1.[8] On the contrary, the expression level of miR-182-5p was upregulated in OSCC tissues and cell lines. In addition, ectopic expression of miR-182-5p promoted the proliferative capacity of oral cancer cells by inhibiting CAMK2N1,[9] suggesting that miR-182-5p functions as an oncomiR in OSCC.

CD36 belongs to the B-class scavenger receptor family and is an 88-kDa glycosylated transmembrane protein.[10] Recent studies have suggested that CD36 is closely involved in the occurrence and development of cancer[11-12] Pascual *et al* reported that upregulation of CD36 significantly promoted the LMN of oral cancer cells. In addition, blocking CD36 led to almost complete inhibition of metastasis in the animal model,[13] indicating that CD36 played a key role in regulating the metastasis of OSCC.

Deregulated miR-1254 has been reported in various types of cancer, such as gastric cancer, breast cancer, and non-small cell lung carcinoma.[14-16] However, its role in OSCC was poorly known. In this study, we aimed to investigate the expression pattern of miR-1254 in OSCC tissues and cell lines. Then, the effects of miR-1254 overexpression or underexpression on the malignant behaviors of oral cancer cells were explored. Finally, the potential regulatory mechanisms responsible for the tumor suppressive role of miR-1254 were further explored.

Materials and Methods

Tissue Samples

The study was approved by the Ethical Committee of College and Hospital of Stomatology, Hebei Medical University. This research was performed in accordance with the ethical guidelines of the Declaration of Helsinki and written informed consent was obtained from patients or their relatives. Seventy surgical OSCC specimens and 30 adjacent normal tissues were collected. The specimens were pathologically confirmed to be OSCC and staged based on the seventh edition of the American Joint Committee on Cancer staging system. The specimens were snap-frozen in liquid nitrogen and stored at −80°C until further analysis.

Cell Culture

The normal human oral keratinocytes, human embryonic kidney HEK-293T cells, human OSCC cell lines CAL-27, HSC3, HSC4, SCC9, and SCC25 were obtained from American Type Culture Collection (ATCC; Manassas, Virginia) and the Cell Bank of the Chinese Academy of Science (Shanghai, China). All cells were cultured in appropriate media with 10% fetal bovine serum (Invitrogen, Carlsbad, California), 1% penicillin G, and streptomycin (Invitrogen) in a 5% CO_2 incubator at 37°C.

Cell Transfection

The miR-1254 mimic #1, miR-1254 mimic #2, control mimic, miR-1254 inhibitor, and control inhibitor were purchased from Shanghai GenePharma Co, Ltd (Shanghai, China). Then, the OSCC cells were transfected with 100 nM of either miR-1254 mimic #1, miR-1254 mimic #2, or control mimic using Lipofectamine 2000 (Invitrogen) according to the manufactures' instructions. The transfection concentration for either miR-1254 inhibitor or control inhibitor was 50 nM.

Lentivirus Construction and Infection

The full-length human CD36 complementary DNA (cDNA) was cloned into the GV341 plasmid. Then, the lentiviruses overexpressing CD36 were generated and produced by Shanghai GeneChem (Shanghai Genechem Co, Ltd). CD36 overexpression lentiviruses and control lentiviruses were transfected into the oral cancer cells, respectively. The multiplicity of infection was 30.

Quantitative Real-Time Polymerase Chain Reaction

TRIzol reagent (Invitrogen) was used to extract the total RNA from tissues or cells based on the manufacture's protocol. The cDNA was synthesized by PrimeScript RT reagent (TaKaRa Biotech Corporation, Dalian, China). The expression levels of miRNA and mRNA were examined by SYBR Green Master Mix (TaKaRa Biotech Corporation) on an ABI Prism7500 fast real-time polymerase chain reaction (RT-PCR) system (Applied Biosystems, Foster City, California). U6 and β-actin were used as internal controls for miR-1254 and CD36, respectively. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative fold of gene expression. The following primers were used: miR-1254–specific forward primer was purchased (ABM, Richmond, British Columbia, Canada), U6-F:5′-CTCGC TTCGGCAGCACA-3′, U6-R: 5′-AACGCTT CACGAATTTGCGT-3′, CD36-F: 5′-TGTGCAAAATCCA CAGGAAG-3′, CD36-R: 5′-GCCACAGCCA GATTGA-G AAC-3′, β-actin-F: 5′-CTCACCATGGATGATGATATCGC-3′, and β-actin-R: 5′'-AGGAAT-CCTTCTGACCCATGC-3′.

Western Blot

Equal amount of protein samples was loaded and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel (Invitrogen). Then, the blots on the gels were transferred onto

a nitrocellulose membrane. Follow by blocking with 5% non-fat milk in Tris buffered saline with Tween-20 (TBST), the membranes were probed with CD36 primary antibody (Santa Cruz Biotechnology, Inc, Dallas, Texas) overnight at 4°C. Then the membranes were washed with TBST for 3 times and incubated with secondary antibody at room temperature for 1 hour. The signal was detected using ECL-Plus Western blotting reagent kit (GE Healthcare Chicago, Illinois).

Cell Count

The miR-1254 overexpressed or miR-1254 silenced cancer cells and the corresponding control cells were harvested and plated into 12-well plates at a density of 1×10^5 cells/well. After 24, 48, 72, and 96 hours of incubation, the number of viable cells was counted with a Vi-CELL XR cell counter (Beckman Coulter, Inc, Fullerton, California).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)

The transfected cells (2000 cells/well) were cultured for 24, 48, 72, and 96 hours. Then, 20 μL MTT (Sigma-Aldrich, St. Louis, Missouri) was added to each well and cultured for 4 hours. The supernatant was removed and 150 μL dimethyl sulfoxide was added to dissolve the formazan. The absorbance was measured at 570 nm using a microplate reader (BioTek, Winooski, Vermont) and a proliferation curves were calculated.

AlamarBlue Assay

The AlamarBlue assay was performed based on the manufacturer's protocol. A total of 2000 cells/well were seeded in 96-well plates. 10 μL (10%, vol/vol) AlamarBlue dye (Invitrogen) was added into each well and incubated for 4 hours. The absorbance was measured at 570 nm using a microplate reader (BioTek).

Matrigel Invasion Assay

The effects of miR-1254 on the invasive capacity of oral cancer cells were measured with the Transwell Matrigel Invasion Chambers (BD Biosciences, Bedford, Massachusetts). Briefly, the miR-1254 overexpressed or miR-1254 silenced cancer cells and the corresponding control cells were added to upper chamber in serum-free Dulbecco's modified Eagle medium. The lower chamber was filled with 500 μL complete culture medium. Following 24 hours of incubation, the cancer cells remaining in the upper chamber were removed. The cells that had invaded through the membrane were fixed with 4% paraformaldehyde for 30 minutes and stained for 30 minutes with crystal violet. Then, the invaded cells were photographed and counted in at least 4 randomly selected fields.

Luciferase Reporter Assay

Both pMIR-CD36-3′-UTR wild-type vectors and pMIR-CD36-3′-UTR mutant vectors were synthesized by Shanghai GenePharma Co, Ltd. The miR-1254 overexpressed oral cancer cells and corresponding control cells were transfected with pMIR-CD36-3′-UTR wild-type vectors or pMIR-CD36-3′-UTR mutant vectors using Lipofectamine 2000 (Invitrogen), respectively. After 48 hours of post-transfection, the cells were lysed, and the relative luciferase activity was analyzed with Dual-Luciferase Assay kit (Promega, Madison, Wisconsin).

Statistically Analysis

All results are expressed as the mean (standard deviation). GraphPad 7.0 (GraphPad Software Inc, La Jolla, California) were used for statistical analysis. Two-tailed Student *t* test and 1-way analysis of variance were used to analyze the differences among 2 groups or multiple groups. The linear correlation coefficient (Pearson *r*) was calculated to estimate the correlation between miR-1254 and CD36 in OSCC tissues. $P < .05$ was considered statistically significant.

Results

MiR-1254 Level was Reduced in OSCC Tissues and Cell Lines

We first compared the expression pattern of miR-1254 in 70 OSCC tissues and 30 adjacent normal tissues. Our results showed that miR-1254 levels were significantly lower in the OSCC tissues compared with the normal tissues (***$P < .001$, Figure 1A). In addition, patients with OSCC in the advanced stage (III-IV) had lower miR-1254 levels than those in the early stage (I-II; ***$P < .001$, Figure 1B). The expression level of miR-1254 was lower in the patients with OSCC with lymph node metastasis (LNM) than those without LNM (**$P < .01$, Figure 1C). Moreover, the miR-1254 expression levels were markedly reduced in all the OSCC cell lines compared to the normal oral epithelial cell (***$P < .001$, Figure 1D).

Upregulation or Downregulation of MiR-1254 in Oral Cancer Cells

The miR-1254 mimic #1, miR-1254 mimic #2 and control mimic, or miR-1254 inhibitor and control inhibitor were transfected into CAL-27 and SCC-25. Our quantitative RT-PCR results showed that the expression level of miR-1254 was significantly higher in miR-1254 mimic #1 or miR-1254 mimic #2 treated cells than the miR-1254 mimic control treated cells (***$P < .001$, Figure 2A). On the contrary, miR-1254 inhibitor suppressed the expression level of miR-1254 in CAL-27 and SCC-25 (***$P < .001$, Figure 2B).

Upregulation of MiR-1254 Suppressed the Proliferation and Invasion of Oral Cancer Cells

The cell counting assay showed that the number of cancer cells was significantly lower in miR-1254 mimics group than the control group (*$P < .05$, **$P < .01$, ***$P < .001$, Figure 3A). Similarly, the MTT assay revealed that the optical density (OD) values were lower in the miR-1254 mimics transfected cells

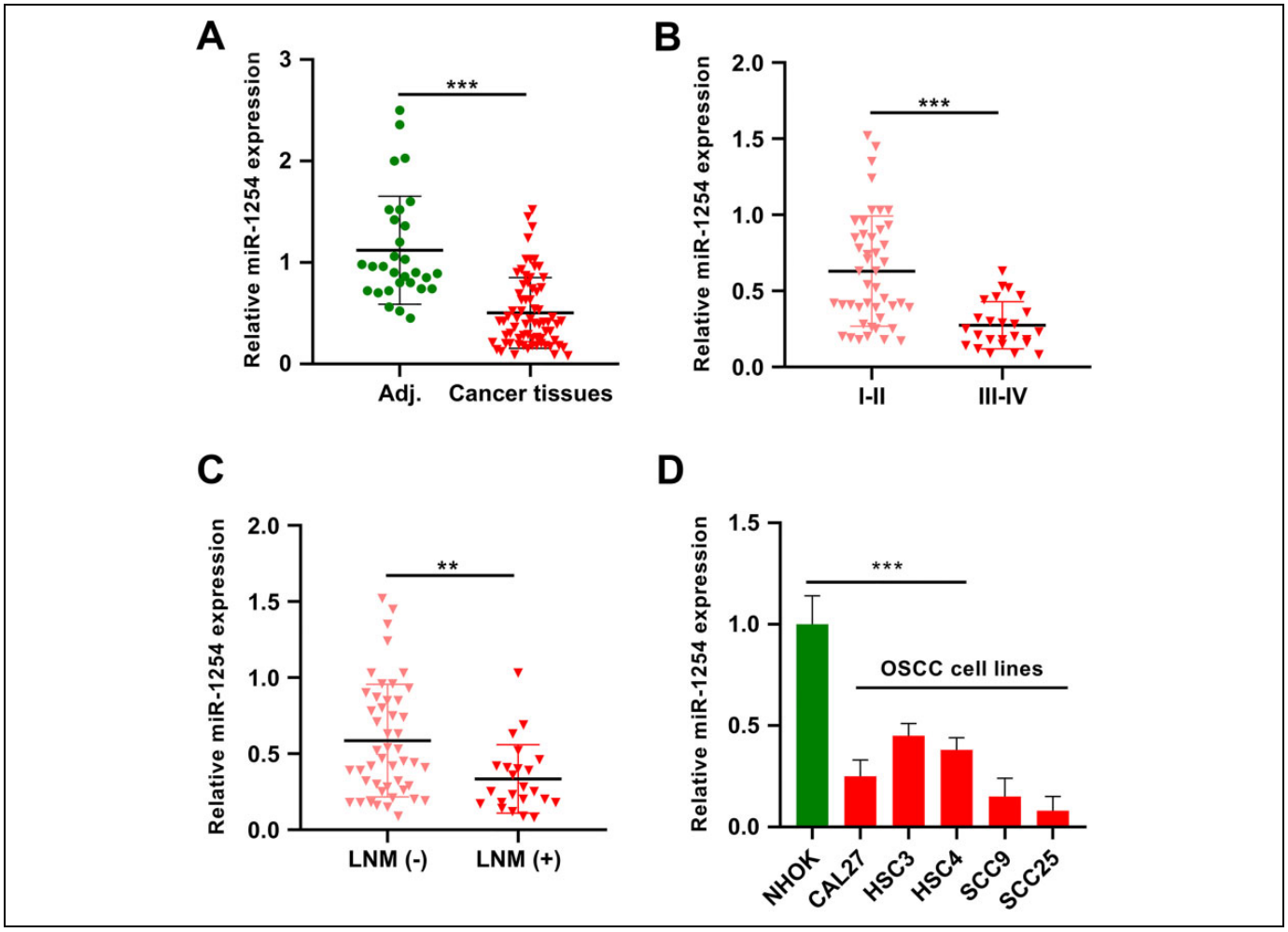

Figure 1. MiR-1254 was reduced in OSCC tissues and cell lines. A, The expression level of miR-1254 was significantly lower in OSCC tissues (***$P < .001$). B, MiR-1254 level was lower in advanced stage OSCC (***$P < .001$). C, MiR-1254 level was lower in OSCC with LNM ($P = .0038$). D, The expression level of miR-1254 was significantly lower in OSCC cell lines compared to the normal control cells (*** $P < .001$). OSCC indicates oral squamous cell carcinoma; LNM, lymph node metastasis.

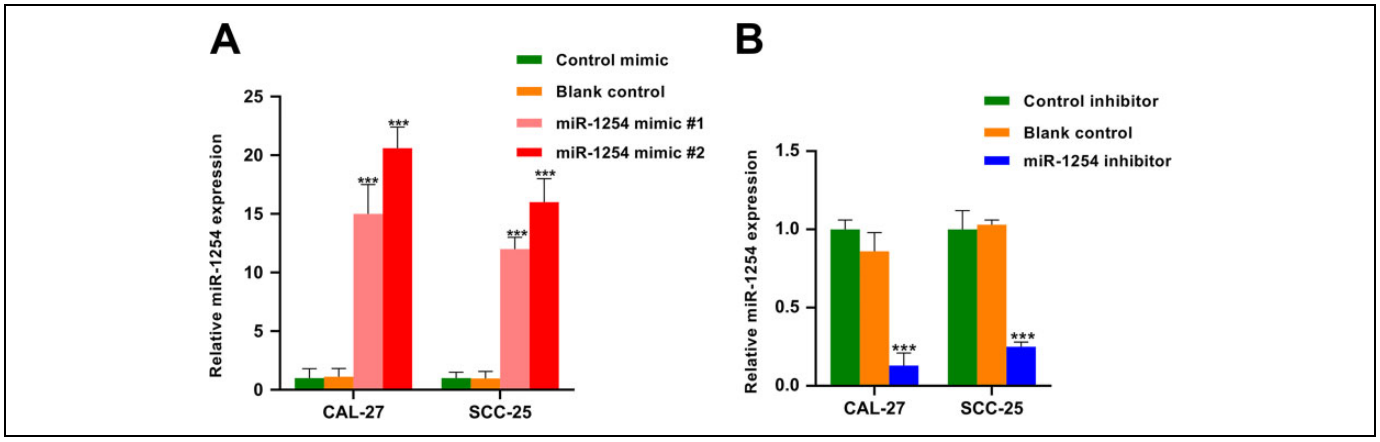

Figure 2. Upregulation or downregulation of miR-1254 in oral cancer cell lines. A, The expression levels of miR-1254 were significantly higher in miR-1254 mimic transfected oral cancer cells compared to control cells (***$P < .001$). B, The expression level of miR-1254 was markedly lower in miR-1254 inhibitor transfected oral cancer cells compared to control cells (***$P < .001$).

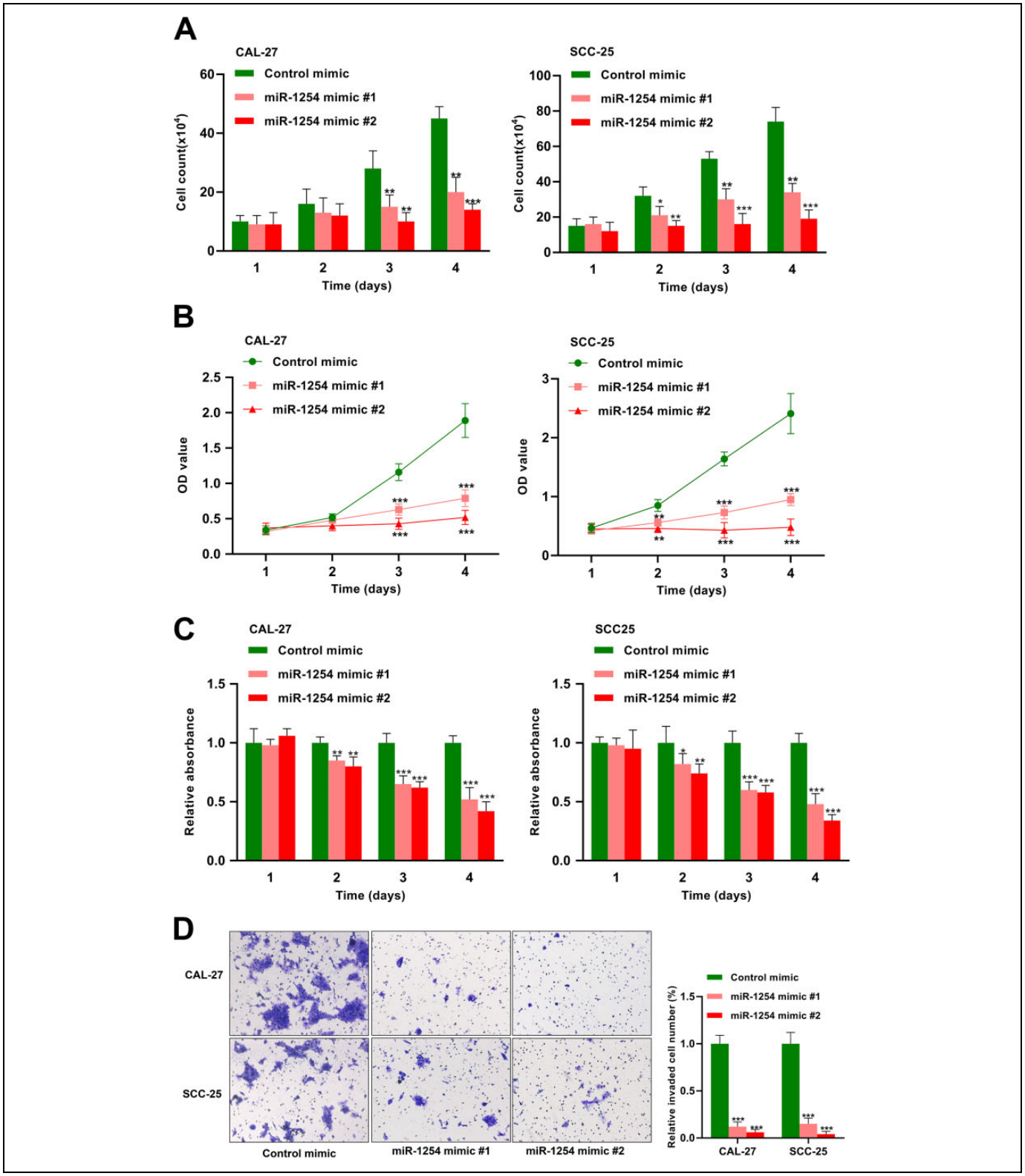

Figure 3. MiR-1254 overexpression suppressed the proliferation and invasion of oral cancer cells. A, The cell count assay showed that the number of viable cells was lower in miR-1254 mimic transfected oral cancer cells compared to control cells (*$P < .05$, **$P < .01$, ***$P < .001$). B, The MTT assay revealed that the OD values were lower in miR-1254 mimic transfected oral cancer cells (**$P < .01$, ***$P < .001$). C, The AlamarBlue assay showed the relative absorbance was dramatically lower in miR-1254 mimic treated group (*$P < .05$, **$P < .01$, *** $P < .001$). D, The invasion assay showed that the miR-1254 mimic transfected oral cancer cells have less invasive capacity than the control cells (***$P < .001$). MTT indicates; OD, optical density.

compared to the miR-1254 mimic control transfected cells (**P < .01, ***P < .001, Figure 3B). The AlamarBlue assay showed that the relative absorbance was lower in the miR-1254 mimic group (***P < .001, Figure 3C). The Matrigel invasion assay showed that the number of cells that invade through the membrane was dramatically lower in miR-1254 mimics group than the control group (***P < .001, Figure 3C).

Downregulation of MiR-1254 Promoted the Proliferation and Invasion of Oral Cancer Cells

The cell counting assay revealed that the cell number was significantly higher in miR-1254 inhibitor group than the control group (**P < .01, Figure 4A). Similarly, the MTT assay revealed that the OD values were higher in the miR-1254 inhibitor group compared to the control group (**P < .01, ***P < .001, Figure 4B). The AlamarBlue assay showed that the relative absorbance was higher in the miR-1254 inhibitor group (***P < .001, Figure 4C). The Matrigel invasion assay demonstrated that the number of cells that invade through the membrane was dramatically higher in miR-1254 inhibitor group than the control group (***P < .001, Figure 4D).

CD36 was the Direct Downstream Target of MiR-1254

The potential downstream target of miR-1254 was retrieved from TargetScan7.2 (Figure 5A). Then, protein–protein interaction analysis revealed the central genes of the downstream targets (Figure 5B). We found that the 3′-UTR of CD36 was highly complementary to the seed sequence of miR-1254 (Figure 5C). In addition, for the wild-type CD36 vector, our luciferase reporter assay showed that the relative luciferase activity was lower in the miR-1254 inhibitor group than the control group (***P < .001, Figure 5D), indicating that CD36 was the direct downstream target of miR-1254. The relative luciferase reporter activity was not affected by miR-1254 mimic when transfections were performed with the pMIR-CD36-3′-UTR mutant vector. Moreover, overexpression of miR-1254 significantly suppressed the expression level of CD36 in CAL-27 and SCC-25 (***P < .001, Figure 5E), and downregulation of miR-1254 increased CD36 expression level in oral cancer cells (***P < .001, Figure 5F).

Overexpression of CD36 Partially Rescues the Tumor Suppressive Effects of MiR-1254 in Oral Cancer Cells

The expression level of CD36 protein was significantly lower in miR-1254 mimic transfected cells compared with the control cells (Figure 6A). The CD36 overexpression lentiviruses were able to enhance CD36 level in miR-1254 mimic transfected cells (Figure 6B). The cell count assay, MTT assay, and AlamarBlue assay showed that ectopic expression of CD36 partially restored the proliferation capacity of miR-1254 mimic transfected cells (**P < .01, ***P < .001, Figure 6C-E). The invasion assay revealed that overexpression of CD36 partially restored the invasion capacity of miR-1254 mimic transfected cancer cells (***P < .001, Figure 6F).

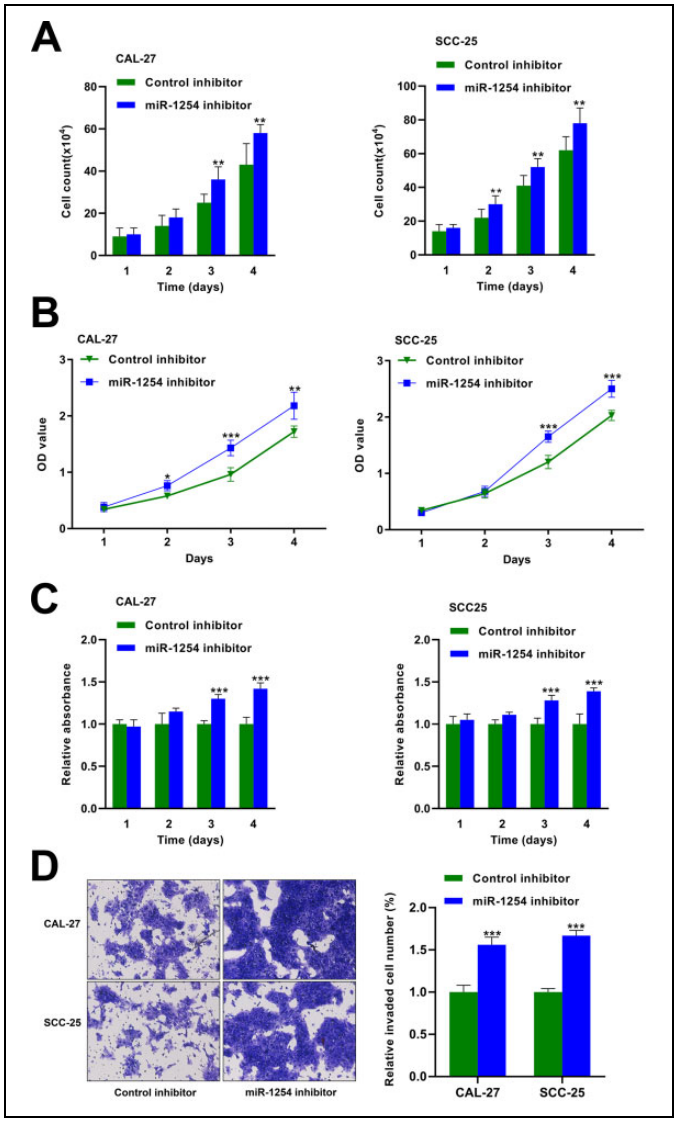

Figure 4. MiR-1254 inhibition promoted the proliferation and invasion of oral cancer cells. A-C, The cell count assay, MTT assay, and AlamarBlue assay showed that miR-1254 inhibitor transfected cells had higher proliferation capacity than the control cells (**P < .01, ***P < .001). D, The invasion assay revealed that the miR-1254 inhibitor transfected cells have higher invasive capacity than the control cells (***P < .001).

CD36 Expression Level was Negatively Associated With MiR-1254 in OSCC Tissues

The potential correlation between CD36 and miR-1254 expression in OSCC tissues was explored. Our results showed that a negative correlation was found between miR-1254 and CD36 expression in the OSCC tissues (Figure 7).

Discussion

In the present study, our results showed that the expression level of miR-1254 was remarkably downregulated in OSCC tissue samples. Reduced miR-1254 was strongly associated with LMN and tumor stage. In addition, miR-1254 levels were

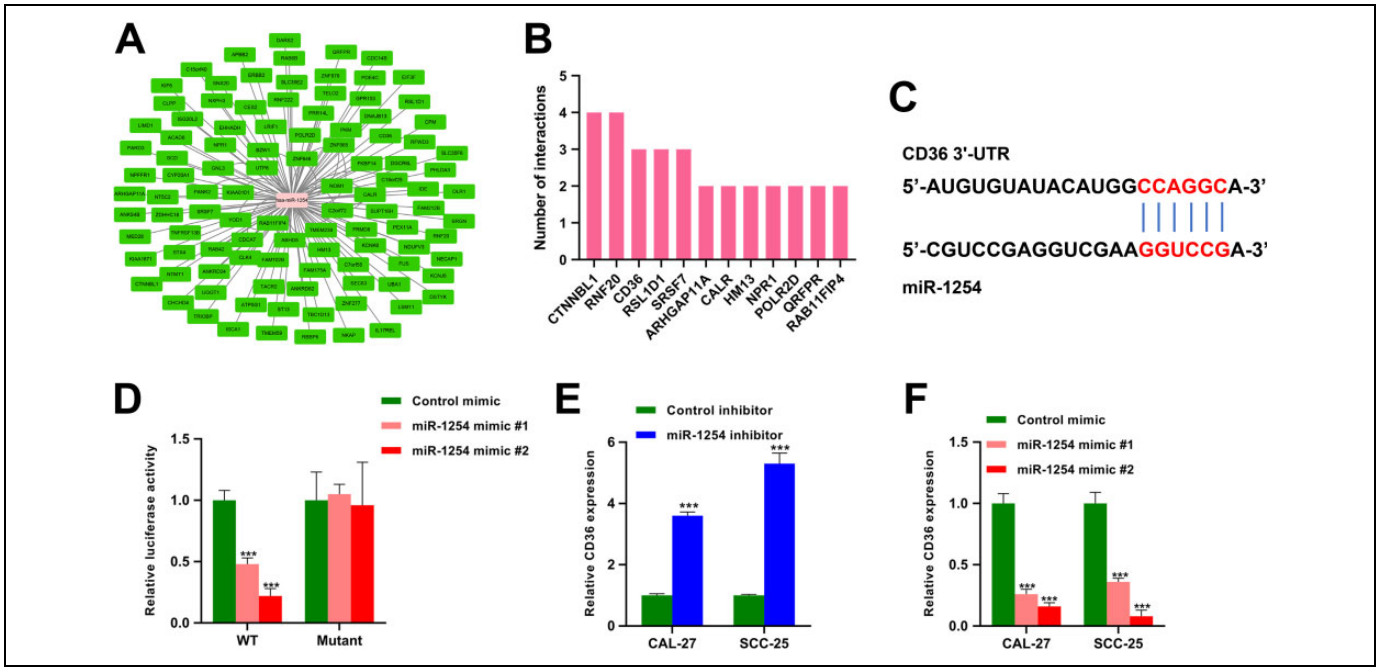

Figure 5. CD36 was a direct downstream target of miR-1254. A, The potential downstream target genes of miR-1254. B, PPI analysis revealed the top central nodes in the potential downstream target genes of miR-1254. C, The 3′-UTR of CD36 was highly complementary to the seed sequence of miR-1254. D, For the WT vector, the relative luciferase activity was lower in miR-1254 mimic transfected cells (***$P < .001$). However, no significant difference was found for the mutant vector. E, The expression level of CD36 mRNA was lower in miR-1254 mimic transfected cells (***$P < .001$). mRNAs indicates messenger RNAs; PPI, protein–protein interaction; UTR, untranslated region; WT, wild type.

also reduced in oral cancer cell lines. Upregulation or downregulation of miR-1254 suppressed or promoted the proliferation and invasion capacity of oral cancer cells *in vitro*. Moreover, the luciferase reporter assay revealed that miR-1254 could directly regulate CD36. Overexpression of CD36 partially restored the proliferation and invasion capacity in miR-1254 overexpression oral cancer cells. Furthermore, CD36 expression level was negatively correlated with miR-1254 in OSCC tissues. All these data indicated that miR-1254 might play a tumor suppressive role in OSCC by partially downregulating CD36. Previous study has suggested that CD36 was a key molecule regulating the metastasis of OSCC.[13] This might partially explain our findings that patients with OSCC with lower miR-1254 had higher tendency to LMN, as CD36 is a direct target of miR-1254.

Consistent with the results in our study, miR-1254 has been shown to inhibit tumor progression in many types of cancers. For instance, the expression level of miR-1254 was significantly downregulated in colorectal cancer tissues. Enforced expression of miR-1254 suppressed the migration capacity of colorectal cancan cells by targeting PSMD10, which was an important regulator for epithelial–mesenchymal transition.[17] Similarly, miR-1254 expression was lower in cervical cancer tissues than in adjacent normal tissues. Reduced miR-1254 was positively associated with positive lymphatic invasion, distant metastasis, and advanced International Federation of Gynecology and Obstetrics stage. In addition, patients with cervical cancer with lower miR-1254 levels had shorter overall survival than those with higher miR-1254, and miR-1254 was demonstrated to be an independent prognosis factor for cervical cancer.[18] Upregulation of endogenous miR-1254 by CCAR1 5′-UTR sensitized tamoxifen-resistant breast cancer cells to tamoxifen, indicating modulating the miR-1254 level might contribute the treatment of breast cancer.[19] Jiang *et al* reported that ectopic expression of miR-1254 in gastric cancer cells inhibited the proliferation, migration, and invasion *in vitro* and suppressed tumorigenesis *in vivo*, and smurf1 was the direct target of miR-1254.[14]

However, miR-1254 might also function as an oncomiR in cancer. For instance, the expression level of miR-1254 was increased in lung cancer tissues and cell lines. Upregulation of miR-1254 enhances the proliferation capacity of lung cancer cells, and vice versa.[20] This finding suggested that miR-1254 played an oncogenic role in lung cancer. Another independent study showed that the expression of serum miR-1254 significantly increased in the early-stage non-small cell lung carcinoma compared to the controls.[21] The results of both above studies were contradictory with the findings reported by Pu *et al*.[16] It was possible that the role of miR-1254 in tumorigenesis might be closely associated with the tumor type and tumor microenvironment. The downstream targets affected by miR-1254 in the specific environment might determine the oncogenic or tumor suppressive role of miR-1254 in cancer.

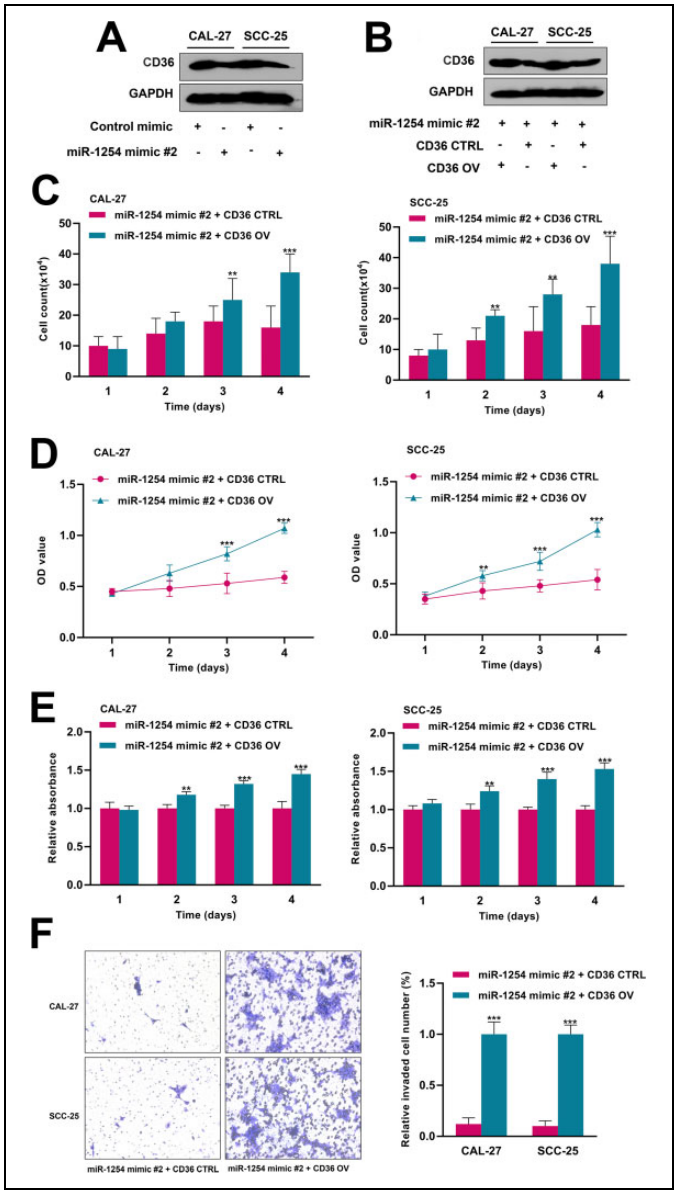

Figure 6. Overexpression of CD36 partially rescues the tumor suppressive effects of miR-1254 in oral cancer cells. A, The expression level of CD36 protein was lower in miR-1254 mimic transfected cells compared with the control cells. B, The CD36 overexpression lentivirus upregulated CD36 expression in miR-1254 mimic transfected cells. C-E, The cell count assay, MTT assay, and AlamarBlue assay showed that overexpression of CD36 partially restored the proliferation capacity of miR-1254 overexpression cancer cells (**$P < .01$, ***$P < .001$). D, The invasion assay revealed that overexpression of CD36 partially restored the invasion capacity of miR-1254 mimic transfected cancer cells (***$P < .001$). Note: OV and CTRL indicate overexpression and control, respectively.

In conclusion, the expression of miR-1254 is downregulated in OSCC tissues and cell lines. Reduced miR-1254 is associated with unfavorable clinicopathological parameters of OSCC. In addition, miR-1254 might suppress the proliferation and invasion capacity of oral cancer cells by partially targeting CD36, which suggests a potential therapeutic target for OSCC.

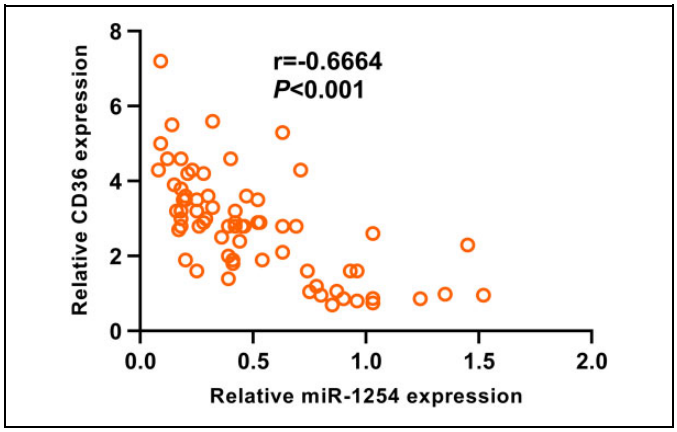

Figure 7. CD36 expression level was negatively associated with miR-1254 in OSCC tissues ($r = -0.6664$, ***$P < .001$). OSCC indicates oral squamous cell carcinoma.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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