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Research article

MicroRNA 142-5p promotes tumor growth in oral squamous cell carcinoma via the PI3K/AKT pathway by regulating PTEN

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ABSTRACT

MicroRNAs (miRNAs) play an important role in carcinogenesis and cancer progression. The purpose of this study was to identify miRNAs associated with carcinoma function in OSCC and to investigate the potential role of the specific miRNAs. First, a comprehensive microarray analysis was performed, and miR-142-5p was identified as a candidate miRNA involved in OSCC. miR-142-5p has been reported to show high expression levels in cancer patients and to be involved in tumor growth and metastasis. However, the expression and function of miR-142-5p in oral squamous cell carcinoma (OSCC) are not fully characterized. We evaluated miR-142-5p expression in both OSCC-derived cell lines and primary OSCC tissues and performed functional analysis of miR-142-5p in OSCC-derived cell lines using mimics and inhibitors. miR-142-5p expression was up-regulated in OSCC tissues and OSCC cell lines. Overexpression of miR-142-5p significantly promoted the proliferation and invasion of OSCC cells. Bioinformatics analysis was performed using TargetScan to predict potential target sites that match the seed region of miR-142-5p. Phosphatase and tensin homolog deleted on chromo-some 10 (PTEN) was identified as a potential target and selected for further analysis. PTEN expression levels were down-regulated and AKT expression levels were up-regulated in miR-142-5p-overexpressing cells. We have shown that miR-142-5p targets the PTEN gene and is involved in cancer progression. Our results suggest that miR-142-5p is involved in the progression of OSCC by controlling the phosphatidylinositol 3-kinase (PI3K)/AKT pathway by targeting the PTEN gene. Our findings suggest that miR-142-5p may be a new target for the treatment of OSCC.

1. Introduction

Oral cancer is a malignant neoplasm that develops in the oral cavity, and 90% or more of oral cancer is oral squamous cell carcinoma (OSCC) [1]. OSCC is the eighth most common cancer type in the world [2]. While extensive research on OSCC has been conducted, the survival rate for patients with OSCC has not significantly improved; the reported 5-year survival rate of OSCC patients is 50% or less [3].

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which is one of the most well-known tumor suppressors involved in squamous cell carcinoma, is a phosphatase whose main substrate is phosphatidylinositol 3,4,5-trisphosphate (PIP3) [4, 5]. Abnormalities in PTEN at the gene/protein level have been identified in various cancer types [6]. The phosphatidylinositol 3-kinase (PI3K)/AKT pathway is an important tumor suppressor pathway that is regulated by PTEN [7]. AKT is activated by phosphorylation and functions in the regulation of migration, invasion and apoptosis [8]. PTEN dephosphorylates PIP3 and converts it to phosphatidylinositol-4,5-bisphosphate (PIP2), which then de-phosphorylates, and thus inactivates, AKT. Therefore, carcinogenesis may be suppressed by controlling PTEN.

MicroRNA (miRNA) is a short non-coding RNA that complementarily binds to the 3′ untranslated region (UTR) of messenger RNA (mRNA) to regulate gene expression at the post-transcriptional level by inhibiting translation or promoting mRNA degradation [9]. Recent reports have also revealed the presence of miRNA binding sites in the 5′UTR and protein-coding of mRNAs [10]. Multiple studies have shown that miRNAs are involved in cell development, differentiation, proliferation, metabolism, and apoptosis [11, 12, 13, 14], as well as in cancer. For example,

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miR-10a and miR-133a-3p are also involved in cancer promotion and suppression in OSCC [15, 16]. Similarly, miR-142-5p has been reported as an oncogenic miRNA (oncomiR) that promotes cancer in colorectal cancer and renal cell carcinoma [17, 18]. Some reports have shown that miR-142-5p regulates PTEN and is involved in tumor progression and cell function in several cancer types [19, 20]. However, the relationship between OSCC and miR-142-5p has not been reported.

The purpose of this study was to comprehensively investigate the expression of miRNAs in OSCC and to clarify the mechanism by which specific miRNAs function in tumor progression in OSCC.

2. Materials and methods

2.1. OSCC clinical specimens

We obtained 70 primary OSCC tissue samples from 70 untreated OSCC patients who visited the University of Tsukuba Hospital (Tsukuba, Japan) from February 2008 to November 2010. The tissue was taken at the time of biopsy. Normal gingival tissue samples were obtained for comparison from five patients who did not have cancer. Samples were prepared for formalin fixed paraffin embedded (FFPE) histology using standard procedures. In addition, tissues were collected from nine untreated patients with OSCC and four cancer-free patients between July 2015 and November 2015, and frozen specimens for microarray analysis of miRNA expression in OSCC. Patients with cancer other than OSCC were excluded from this study. OSCC was diagnosed and classified based on the International Cancer Control Union (UICC 7th edition) Tumor Lymph Node Metastasis (TNM) system. All OSCC cases were clinically diagnosed and histologically confirmed by pathologists. This study was approved by the University of Tsukuba Ethics Committee Hospital (approval No. 215). All patients provided informed consent prior to enrollment.

2.2. Microarray analysis of miRNA

Total RNA was extracted from frozen samples using the miRNEasy Mini Kit (Qiagen, cat# 217004, Venlo, The Netherlands). The microarray was performed by Toray Industries (Osaka, Japan), and the expression of 2565 miRNAs was analyzed.

2.3. Cell lines

OSCC cell lines (SAS, HSC-M3) were obtained from Japan Research Biological Resource Collection (Osaka, Japan). The human benign epidermal keratinocyte cell line (HaCaT) was used as a control. Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Nichirei Bioscience, Tokyo, Japan) and 1% penicillin-streptomycin at 37 °C, 5% CO2 and 95% air in humidified conditions.

2.4. TaqMan-based quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assays of miRNA expression

Total RNA was extracted using the miRNeasy FFPE kit (Qiagen, cat# 217504) for FFPE tissues and the miRNeasy Mini kit (Qiagen, cat# 217004) for cell lines. Reverse transcription was performed using the TaqMan MicroRNA Reverse transcription kit (Applied Biosystems, cat# 4366596, Foster City, CA, USA). Expression of miR-142-5p was measured using the TaqMan MicroRNAassay system (Applied Biosystems, cat# 4427975) according to the manufacturer's instructions. PCR reactions were performed using the CFX384 Real-Time system (Bio-Rad Laboratories, Pleasanton, CA, USA).

2.5. TaqMan-based qRT-PCR assays of mRNA expression

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, cat# 74104) and reverse transcribed using the PrimeScript RT Reagent Kit (Takara, cat# RR037A, Shiga, Japan). PCR reactions were performed using the CFX384 Real-Time system (Bio-Rad, CA, USA). Relative mRNA expression was normalized to GAPDH mRNA. The relative expression of PTEN mRNA was analyzed using the standard $2-\Delta\Delta Ct$ method [21]. Mean Ct values were calculated for all samples, and the ΔCt value was calculated by subtracting Ct values of GAPDH mRNA from the Ct values of PTEN mRNA. The results were expressed as fold-change in cells transfected with miRNA142-5p mimic or inhibitor compared with control cells. PCR primer sequences are as follows: PTEN forward, 5′-TGGATTCGACTTAGACTTGACCT-3′ and reverse, 5′-GGTGGGTTATGGTCTTCAAAAGG-3'; and GAPDH forward, 5′-CAAGGTCATCCATGACAACTTTG-3′
and reverse, 5′-GTCCACCACCCTGTTGCTGTAG-3'.

2.6. Transfections with miRNA142-5p mimic or inhibitor or small interfering RNA (siRNA)

Cells were grown in 12-well plates to 70%–80% confluence and transfected with 50 nM miR-142-5p mimics or inhibitors (Invitrogen, cat# 4464066, 4464084, Carlsbad, CA, USA). As controls, 50 nM miRNA Mimic Negative Control and miRNA Inhibitor Negative Control (Invitrogen, cat# 4464058, 4464076) were used. To knock down PTEN, cells were transfected with 50 nM PTEN siRNA; as controls, cells were transfected with 50 nM control siRNA (Invitrogen, cat# AM16708,4390843). Lipofectamine 3000 (Invitrogen, cat# L3000015) was used for transfection according to the manufacturer's instructions. After transfection, cells were incubated at 37 °C for 24 h; the medium was then replaced with FBS-free medium, and cells were incubated for an additional 24 h. Both the transfected cells and culture media were used for assays and analyses, as described below.

2.7. Cell proliferation assay

Cell culture was performed as previously described [22]. Cell proliferation was determined using the MTT Cell Count Kit (Nacalai Tesque, cat# 23506-80, Kyoto, Japan). Cells were transfected with miR-142-5p mimic or inhibitor and seeded in a 96-well plate at 1×10^3 cells/well. After 24 h, cell proliferation was measured using the MTT cell count kit.

2.8. Cell migration and invasion assays

Cell migration and invasion assays were performed as described in a previous study [23, 24]. Cell migration was also assessed by wound-healing assays using a Culture-Insert 2-well in an Ibidi μ-Dish (cat# 81176, Martinsried, Germany). After transfection, cells were seeded at a density of 1×10^5 cells per well. After cells were cultured overnight and adhered, the silicone well was removed, washed with PBS to remove non-adhered cells, and then filled with fresh DMEM medium. Fluorescence microscopy was performed at 0 h and 8 h to capture two different fields at each point in each plate. The photographs were acquired by fixed-point observation with a BZ X700 microscope (Keyence, Osaka, Japan) at 4× magnification. The area was quantified using the image-analysis software BZX-Analyzer (Keyence).

Cellular invasion was assessed by 3D Matrigel culture as described [25, 26]. A total of 5×10^5 transfected cells were suspended in Cell Matrix Type IV (NittaGelatin, cat# KP-7000, Osaka, Japan). Next, a 150-μL Matrigel mixture containing the transfected cells was placed on the bottom of a 6-well microplate; the wells were filled with 2 mL DMEM medium and incubated for 48 h. Images were captured at 0 h, 24 h, and 48 h with a fluorescence microscope, and the images were acquired by fixed-point observation using a BZ-X700 microscope (Keyence) at 40× magnification. The BZ-X Analyzer was used to analyze changes in the infiltrated cell area from the collagen gel over time.

Table 1. MiRNAs that was highly expressed in OSCC tissue specimens based on the results of microarray analysis.

miRNA	expression (carcinoma/ normal)	P-value	miRNA	expression (carcinoma/ normal)	P-Value
miR-524-5p	4.99	0.50	miR-142-5p	4.00	0.04
miR-455-5p	4.98	0.22	miR-223-3p	3.93	0.14
miR-526a	4.54	0.51	miR-4690-3p	3.90	0.36
miR-146b-5p	4.17	0.05	miR-29b-1-5p	3.73	0.04
miR-301a-3p	4.14	0.26	miR-187-3p	3.69	0.30
miR-142-3p	4.07	0.03	miR-454-3p	3.56	0.21

2.9. Identification of candidate genes regulated by miR-142-5p

The target gene of miR-142-5p was predicted in silico by bioinformatics analysis using the TargetScan (http://www.targetscan.org/vert_72/) algorithm. The potential binding site of miR 142-5p in the PTEN mRNA 3′UTR was predicted.

2.10. Western blot analysis

After transfection, the cells were incubated for 48 h. Total proteins were extracted using Laemmli sample buffer containing phosphatase and protease inhibitors. Protein lysates were separated on a Mini-PROTEAN TGX gel (Bio-Rad, cat# 4561096) and transferred to Trans-Blot Turbo Mini PVDF membrane (Bio-Rad, cat# 1704156). Membrane blocking was performed using blocking one (Nakalai Tesque, cat# 03953-66), and the membrane was incubated with primary antibody overnight at 4 °C. The following primary antibodies were used: anti-PTEN antibody (1:1000, Cell Signaling Technology, cat#9556, Danvers, MA, USA), anti-Akt antibody (1:1000, Cell Signaling Technology, cat# 9271), anti-pan-Akt antibody (phospho T308; 1:1000, abcam, cat# ab8805, Cambridge, UK), and anti-GAPDH antibody-Loading Control (1:1000, abcam, cat# 8245). The next day, the membrane was incubated with the appropriate secondary antibody for 1 h at room temperature. Proteins were detected by chemiluminescence using Chemi Lumi One Ultra (Nakalai Tesque, cat# 11644-40) and band images were acquired with the ChemiDoc XRS Plus system (Bio-Rad).

2.11. Statistical analysis

Statistical comparisons between two data samples were compared with the Mann–Whitney U test. The cut-off value of miR-142-5p in OSCC tissue specimens was evaluated by receiver operating characteristic (ROC) curve analysis. Survival data were analyzed using the log-rank test with the Kaplan–Meier curve. JMP version 11 software was used to perform the statistical analysis. $P < 0.05$ indicated statistical significance.

3. Results

3.1. Identification of highly expressed miRNAs in OSCC in microarray results

The expression of 2565 miRNAs in OSCC tissues was examined by comprehensive analysis using a microarray, and the results showed that miR-142-5p expression was up-regulated in OSCC tissues compared with healthy control tissues. The miRNAs that showed three times or more expression in OSCC tissues than in normal gingival tissue were selected as potential candidates for involvement in OSCC (Table 1). Each candidate miRNA was statistically processed and targeted miR-142-5p, which had the small statistically difference (Figure 1A).

3.2. miR-142-5p is overexpressed in OSCC

To determine the clinical significance of miR-142-5p expression, we examined the expression levels of miR-142-5p in OSCC tissue specimens and two OSCC cell lines: SAS and HSC3-M3. MiR-142-5p expression was

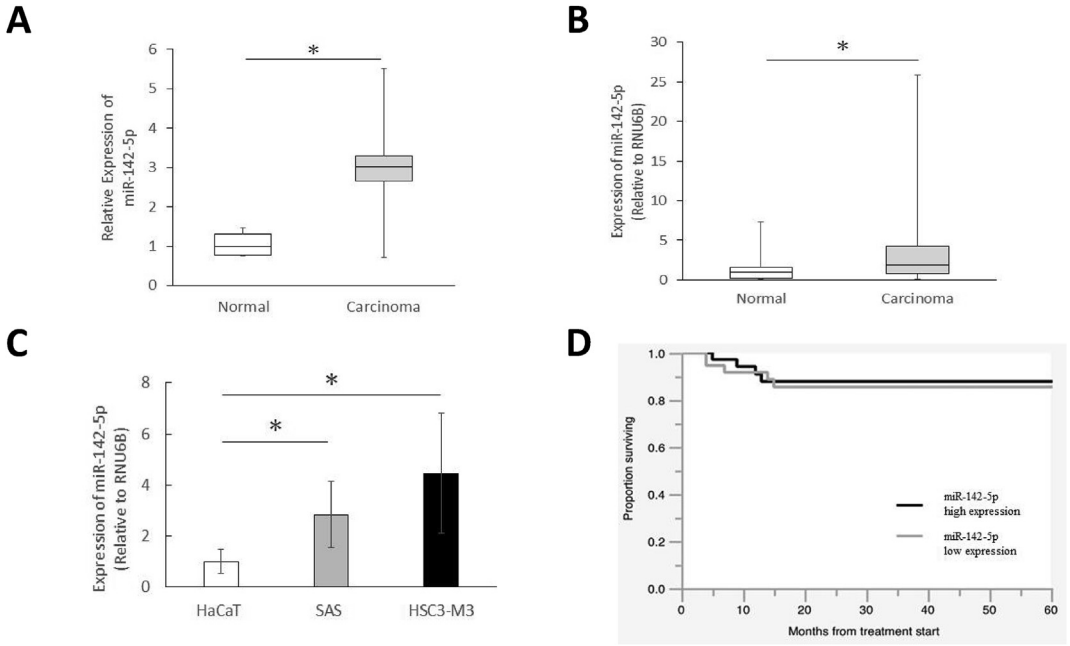

Figure 1. Comparison of miR-142-5p expression on microarray analysis. Frozen tumor samples of OSCC patients (n = 9) and normal gingival tissues of patients without cancer (n = 4) were used. (A) miRNA expression was analyzed using microarray analysis. Box-and-whisker plots show miR-142-5p expression in OSCC and non-cancer groups. *P < 0.05. MiR-142-5p expression in OSCC clinical specimens and cell lines. (B and C) Clinical specimens included normal gingiva tissue (n = 5) and primary tumor tissue of OSCC patients (n = 70). The SAS and HSC3-M3 cell lines were used to analyze miRNA expression. HACAT was used as a non-cancer cell line. RNU6B was used for normalization. Box-and-whisker plots represent data from clinical specimens. *P < 0.05. (D) The 70 OSCC patients were divided into miR-142-5p high and low expression groups according to miR-142-5p expression in primary OSCC tissues. Kaplan–Meier curves for overall survival of 38 cases with high miR-142-5p expression and 32 cases with low miR-142-5p expression are shown. The difference between these two groups was determined by the log-rank test. *P = 0.25. These experiments were performed in duplicate.

up-regulated in OSCC tissue specimens and OSCC cell lines compared with RNU6B (Figure 1B and C). We then investigated potential correlations between miR-142-5p levels and patient clinical characteristics (Table 2). However, no association was found between any of the clinical characteristics and miR-142-5p expression levels.

To investigate the association of miR-142-5p with prognosis, the cut-off value of miR-142-5p in OSCC tissue specimens was set by ROC analysis. The 5-year survival rate was analyzed using the Kaplan–Meier method and compared using the log-rank test. No significant difference was observed between the miR-142-5p high expression group and miR-142-5p low expression group among OSCC patients (Figure 1D).

3.3. Effect of miR-142-5p mimic or inhibitor on cell proliferation, migration, and invasion

miR-142-5p mimic–transfected SAS and HSC3-M3 cells showed markedly increased proliferation compared with the negative control cells (Figure 2A). Conversely, the proliferation of miR-142-5p inhibitor–transfected HSC3-M3 cells was significantly decreased compared with control cells; however, there was no significant difference in SAS cells (Figure 2B). In cell migration assays, there was no difference between the miR-142-5p mimic or inhibitor–transfected SAS and HSC3-M3 cells compared with control cells with regard to migration (Figure 2C and D). Cell invasion was greatly promoted in miR-142-5p mimic–transfected SAS and HSC3-M3 cells compared with control cells (Figure 2E). In miR-142-5p inhibitor–transfected SAS, cell invasion was significantly suppressed; however, there was no significant change in HSC3-M3 cells (Figure 2F).

3.4. Identification of target genes of miR-142-5p

miRNAs regulate the expression of multiple mRNAs, forming a complex regulatory network. The TargetScan database was used to search for potential target genes for miR-142-5p. The results identified PTEN as a candidate target for miR-142-5p (Figure 3A). qRT-PCR and Western blot analyses were performed to assess the expression level of PTEN in SAS and HSC3-M3 cells. PTEN mRNA expression in miR-142-5p mimic–-transfected cells was significantly lower than in control cells; conversely,

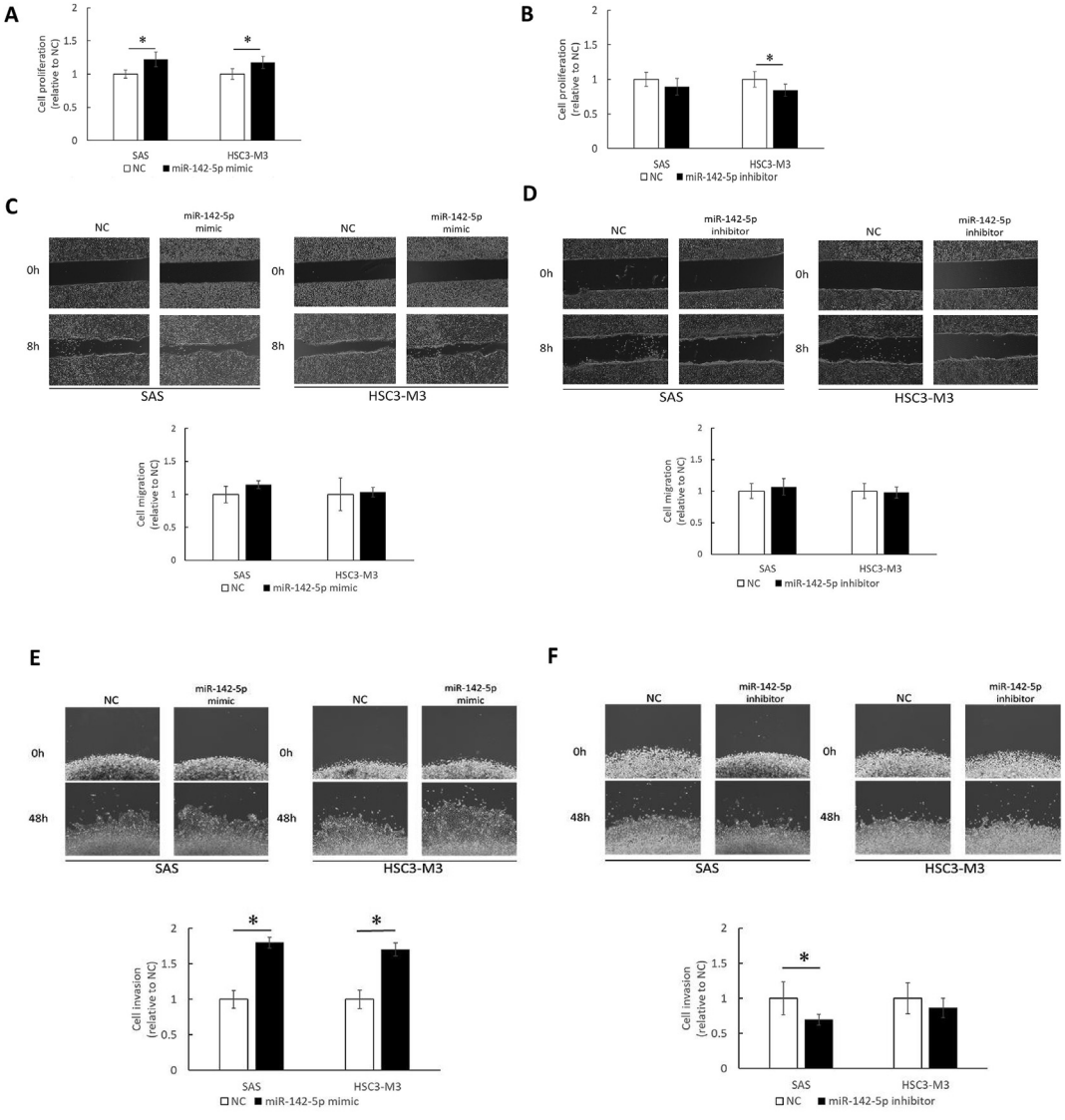

Figure 2. Effect of miR-142-5p on the proliferation, migration, and invasiveness of OSCC cell lines. (A) SAS and HSC3-M3 cells were transfected with 50 nM miR-142-5p mimic or inhibitor or a negative control. Cells were cultured for 24 h after transfection and then assayed for proliferation. (A and B) Migration assay was performed by wound healing assay as shown in the Materials and methods. (C and D) The invasion assay was performed after transfection by the 3D culture method. (E and F) The value for the negative control (NC) was set to 1. Error bars indicate the means ± SD. *P < 0.05. Experiments were performed in triplicate.

Table 2. Clinical characteristics of patients with oral squamous cell carcinoma.

Patient characteristics	Number of patients	miR-142-5p expression		P-value
	(n = 70)	High (n = 38)	Low (n = 32)	
Age				
< 60	18	10	8	0.59
≥60	52	28	24	
Gender				
Male	46	27	19	0.36
Female	24	11	13	
T-primary tumor				
T1-T2	38	23	15	0.78
T3-T4	32	15	17	
pTNM stage				
I–II	29	16	13	0.82
III–IV	41	21	20	
Differentiation				
Well	34	15	19	0.53
Moderate, Poor	36	19	17	

PTEN levels in miR-142-5p inhibitor–transfected cells were significantly increased compared with control cells ($P < 0.05$) (Figure 3B). PTEN protein levels in miR-142-5p mimic–transfected cells were also decreased compared with control cells. While there was no noticeable difference in AKT protein expression in miR-142-5p mimic–transfected cells compared with controls, we detected a difference in p-AKT levels in miR-142-5p mimic–transfected cells compared with controls (Figure 3C).

3.5. PTEN silencing in OSCC cell lines

We next performed functional analyses of PTEN in OSCC cells using siRNA. Suppression of PTEN expression with siRNA significantly inhibited cellular proliferation and invasion compared with the control cells; however, there was no significant difference in cellular migration (Figure 4A, B, and C).

4. Discussion

miRNAs are involved in the regulation of gene expression by inhibiting the translation of mRNA, thereby suppressing the expression of various genes [27]. Previous studies have shown that a single mRNA can be regulated by multiple miRNAs [28]. Thus, miRNA and mRNA form a complex gene expression control network.

miR-142-5p has been reported as a cancer-promoting miRNA in various cancer types [17, 18, 19]. In this study, our microarray analysis revealed that miR-142-5p was one of the up-regulated miRNAs in OSCC. We therefore performed experiments to evaluate the potential of miR-142-5p as a cancer-promoting oncomiR in OSCC. Our results confirmed that miR-142-5p was highly expressed in both OSCC tissue specimens and OSCC cell lines. In the cellular functional analyses, promotion of miR-142-5p expression with miR-142-5p mimic significantly activated cellular proliferation and invasion compared with the control, while suppression of miR-142-5p expression with miR-142-5p inhibitor suppressed cellular function in only one cell line. This may be because of the specific properties of mimics and inhibitors. While mimics and inhibitors both act on miRNAs, mimics greatly increase the expression level during transfection [29], while inhibitors block the action of miRNAs using a method using an antisense nucleic acid molecule. Therefore, when mimics are transfected, their intracellular expression is amplified thousands of times. However, when an inhibitor is transfected, the transfected amount is small, so the absolute amount does not change as much as for mimic [30] (supplementary figure). Therefore, experiments with mimics may be more likely to show a significant difference than experiments with inhibitors.

The tumor suppressor PTEN is involved in cancer suppression through its regulation of the PI3K/AKT pathway [7]. Previous studies reported that miRNAs such as miR-19a-3p and miR-224-5p down-regulate PTEN expression and are involved in cancer progression [31, 32]. Based on these previous studies and in silico bioinformatics analysis, we investigated the potential of miR-142-5p in functioning as an oncomiR in OSCC. Several reports have identified KLF6, BTG3, and PTEN genes as target genes of miR-142-5p [17, 18, 20]. Our results show that miR-142-5p targets PTEN and increases the level of activated AKT (p-AKT). This confirms that PTEN actively regulates the PI3K/AKT pathway and inhibits PIP3 dephosphorylation to promote cancer progression (Fig. 4D). Our results showed that PTEN expression was down-regulated at both the mRNA and protein levels in miR-142-5p mimic–transfected OSCC cells, which supported the hypothesis. While p-AKT increased in response to miR-142-5p, the protein expression level of AKT did not change; however this was in the state where p-AKT was phosphorylated and activated, whereas in the state where AKT was inactivated [33, 34]. Furthermore, suppression of PTEN expression with siRNA significantly inhibited cellular proliferation and invasion compared with the controls. Several

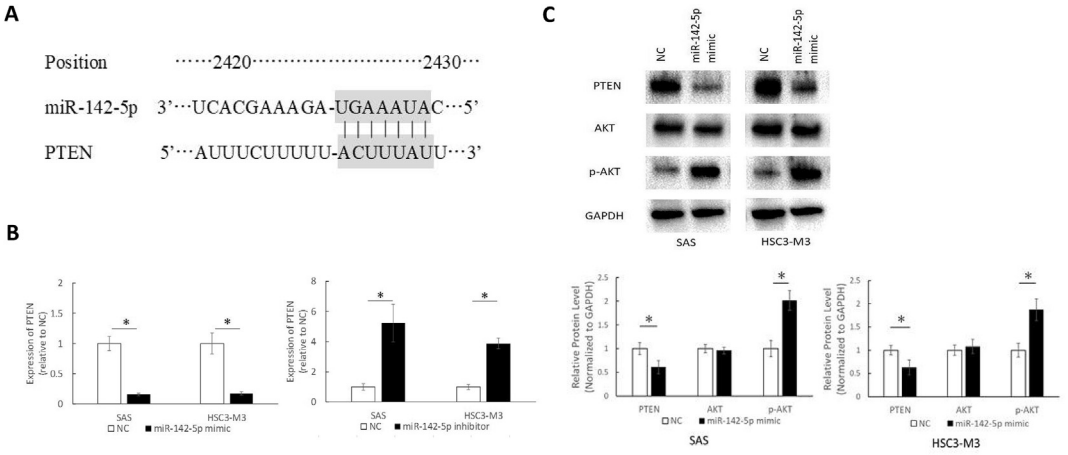

Figure 3. PTEN is a direct target of miR-142-5p. (A) Schematic showing the putative miR-142-5p binding site in the 3′UTR of PTEN mRNA. (B) PTEN mRNA expression levels in SAS and HSC3-M3 cells transfected with miR-142-5p mimic or negative control. PTEN mRNA level was normalized to GAPDH mRNA level. Negative control (NC) expression was set to 1 and error bars indicate the means ± SD. *$P < 0.05$. (C) Western blot showing the expressions of PTEN, AKT, and p-AKT in SAS and HSC3-M3 cells transfected with miR-142-5p mimic. GAPDH was used as a loading control. Bands obtained by Western blotting were quantified with ImageJ. Experiments were performed in triplicate. Full, non-adjusted images of Figure 3C is provided as supplementary material.

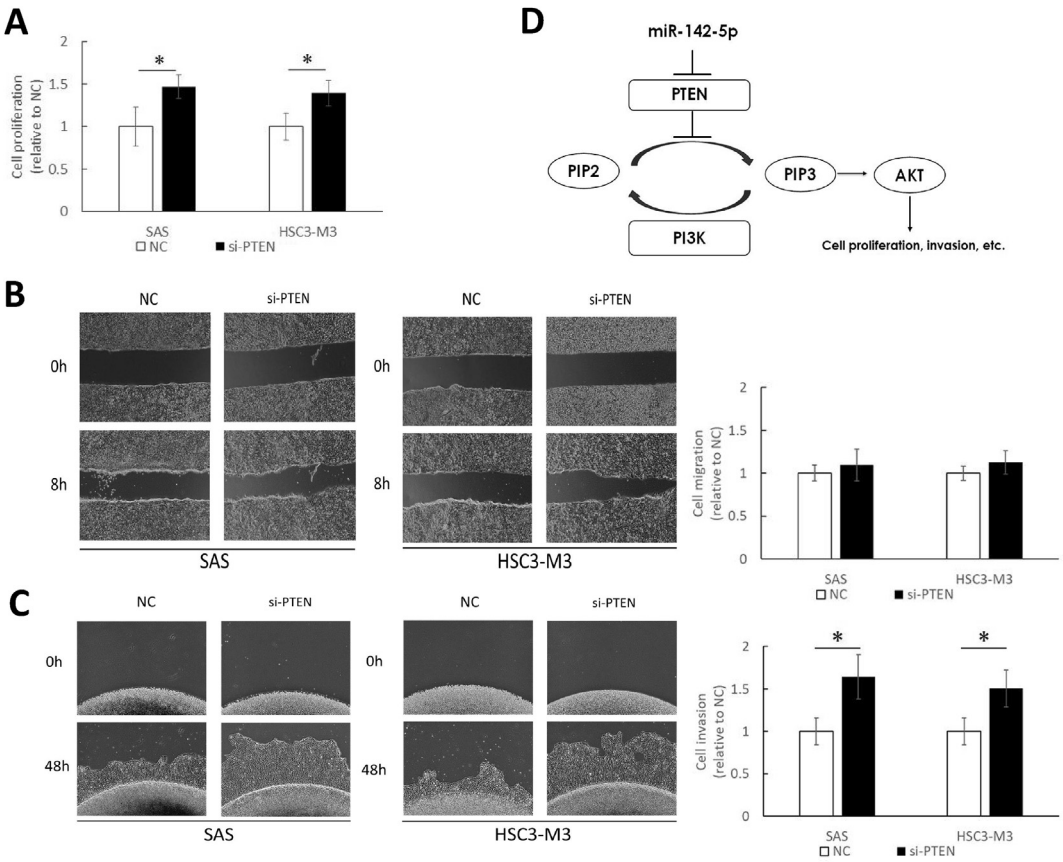

Figure 4. Effect of PTEN siRNA transfection on OSCC cell lines. (A) Proliferation assay in SAS cells and HSC3M3 cells transfected with si-PTEN (50 nM) or Negative control (NC). (B and C) Migration assay and invasion assays were performed in cells transfected with si-PTEN or NC. (D) The pathway by which miR-142-5p down-regulates PTEN and promotes cancer activation. miR-142-5p suppress the expression of PTEN and inhibits dephosphorylation of PIP3. This activates various downstream intracellular signal transduction molecules such as AKT to participate in cell responses such as cell proliferation and invasion. The value for the NC was set to 1 and error bars indicate the means ± SD. *P < 0.05. Experiments were performed in triplicate.

miRNAs increase the invasion and proliferation of various cancer types by targeting the PTEN/AKT pathway [35]. Other studies showed that up-regulation of miR-655 expression inhibits the activation of the PTEN/AKT pathway in OSCC cells and inhibits the proliferation and invasion of OSCC [36, 37]. These results indicate that direct down-regulation of PTEN changes the cellular function in the same manner as with miR-142-5p mimic transfection.

Our results showed that PTEN is a direct target of miR-142-5p in OSCC. We also found that overexpression of miR-142-5p was associated with down-regulation of PTEN in OSCC cells and led to promotion of cancer cell growth and invasion. Furthermore, OSCC cells with PTEN down-regulation showed the same activities as when miR-142-5p mimic was introduced. Therefore, our data revealed that miR-142-5p might be involved in the progression of oral cancer by regulating PTEN expression in OSCC. We have shown that miR-142-5p may be an important therapeutic target for OSCC.

Declarations

Author contribution statement

Seiichiro Iizumi: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Fumihiko Uchida: Analyzed and interpreted the data; Wrote the paper.

Hiroki Nagai: Performed the experiments; Analyzed and interpreted the data.

Shohei Takaoka: Performed the experiments.

Satoshi Fukuzawa, Naomi Ishibashi Kanno, Kenji Yamagata: Contributed reagents, materials, analysis tools or data.

Toru Yanagawa, Hiroki Bukawa: Conceived and designed the experiments.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2021.e08086.

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