[UCI]I804:24011-200000266275

2017년 8월
박사학위논문

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조선대학교 대학원

치의학과

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2017년 8월 25일

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2017년 4월

조선대학교 대학원

치의학과

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2017년 6월

조선대학교 대학원

TABLE OF CONTENT

LIST OF FIGURES

ABSTRACT in KOREAN

Sema6A 표적작용을 통한 microRNA-203의 구강암세포 성장 억제

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MicroRNA(miRNA)는 21-25개의 염기서열로 구성된 noncoding RNA 분자로, 표적 유전자의 3'-UTR 부위에 상보적인 염기서열을 가지고 그 표적유전자의 단백합성을 억제시키거나 촉진시킨다. Oncogenic miRNA는 종양과 관련된 miRNA로 최근 여러 종양에서 oncogenic miRNA 발현 분석을 통해 다양한 종양의 조기진단, 예후측정에 응용가능성을 시사하고 있다. 본 연구에서는 구강암세포에서 miRNA들의 발현을 비교·분석하여 구강암 특이 miRNA를 확립하고, miRNA-203 제어에 의한 종양유전

자 *Sema6A* 표적작용을 통한 구강암세포 성장억제의 분자적 기전을 밝히고자 하였다.

구강암세포에서 miRNA-203 제어에 의한 구강암세포 성장억제 작용기전을 규명하기 위해 사람 구강암세포주 YD-38에 miRNA-203을 처리한 후, miRNA microarray 분석, PCR 분석, 세포증식 분석, 세포사멸 분석, immunoblotting 및 luciferase activity 분석 등을 시행하여 다음과 같은 결과를 얻었다.

1. 정상 사람 구강각화세포 NHOK에 비해 YD-38 구강암세포에서 miRNA-203의 발현이 현저히 감소하였다.
2. YD-38 세포에서 miRNA-203은 세포의 성장을 뚜렷이 억제시켰다.
3. YD-38 세포에서 miRNA-203은 세포 apoptosis를 유도하였다.
4. YD-38 세포에서 miRNA-203은 종양유전자 *Sema6A*의 발현을 억제하였다.
5. YD-38 세포에서 miRNA-203은 종양유전자 *Sema6A*를 억제함으로써 luciferase 활성을 감소시켰다.

본 연구의 결과로서 사람 구강암세포주 YD-38에서 miRNA-203은 종양억제 miRNA로써 기능을 하여 종양유전자 *Sema6A*의 발현을 감소시켜 구강암세포 증식억제 및 세포 apoptosis를 유도하는 것으로 사료된다.

중심어: miRNA-203, 종양유전자, 구강암, *Sema6A*, 세포사멸

I. INTRODUCTION

MicroRNAs (miRNAs) are endogenous 19 - 25 nucleotide non-coding sequences [1] and are important post-transcriptional, sequence-specific regulators of gene expression that act on the 3'-untranslated regions (UTR) of mRNA target genes, causing translation inhibition and/or mRNA degradation [2]. miRNAs have been profiled according to their typical mechanism of transcriptional regulation in a variety of cells and cellular systems [1]. They may regulate >90% of human genes [3] and control various cellular biological processes such as cell development, proliferation and differentiation, and apoptosis [4]. A special sequence component in miRNAs constituting the RNA-induced silencing complex (RISC) hybridizes to the target mRNA to inhibit transcription and reduces the stability of the target mRNA, resulting in mRNA degradation and translation inhibition [5,6]. The interaction between the miRNA-RISC complex and its target mRNA requires only partial base-pairing (typically 6 - 8 nucleotides) of the miRNA, known as the seed sequence [5,6]. Therefore, miRNA may target hundreds or thousands of mRNAs, allowing each small RNA to substantially or weakly suppress the expression of a range of genes depending on the characteristics of the target sites within the 3'-UTR of the target mRNA [7,8]. These regulatory small RNA molecules have been implicated in cancer biology [9]. A large number of studies have demonstrated alterations in miRNA function as oncogenes or suppressive genes and that their aberrant expression contributes to human diseases such as cancer [10]. Therefore, recent studies have focused on the utility of miRNA as diagnostic and prognostic

tools as well as potential therapeutic targets [10]. However, their biological functions and cellular mechanisms remain largely unknown.

Many studies have examined the biological functions of miRNA-203 (miR-203) as a tumor suppressor in carcinogenesis. Saini *et al.* [11] reported that miR-203 suppresses prostate cancer cell progression and metastasis by targeting a cohort of pro-metastatic genes. Wang *et al.* [12] and Jin *et al.* [13] reported that miR-203 inhibits cell proliferation and migration by targeting *PKCa* or *survivin* expression in lung cancer cells. In another study, Bueno *et al.* [14] demonstrated that genetic and epigenetic silencing of miR-203 enhanced expression of the oncogenes *ABL1* and *BCR-ABL1* in specific hematopoietic malignancies. In contrast, the anti-tumorigenesis function and signaling pathway of miR-203 in oral cancer cells remain unclear.

Semaphorins are secreted or transmembrane proteins initially identified based on their role in axon guidance and regulation of cell motility, immune response, cell differentiation, morphology, angiogenesis, and tumor progression [15]. Semaphorins contain a conserved extracellular domain of ~500 amino acids (Sema domain) that is shared with their receptors plexins [16]. In the mammalian system, they are subdivided into five different subfamilies (semaphorins 3 - 7) based on specific structural properties [17]. Emerging evidence suggests that the expression of specific semaphorins is important in tumor development or progression [17]. However, some studies found that semaporins are over-expressed in cancer, whereas others are downregulated [18 - 21].

The transmembrane protein Sema6A, the first identified class 6 semaphorin [22], is a membrane-bound repellent and functions as a guidance cue for nerve

axons and migrating cells in the developing central nervous system [17]. It was shown that Sema6A regulates the formation of lamina-specific axon projections in the hippocampus [23] as well as formation of the cortical spinal tract [24]. Class 6 semaphorins have also been implicated in cancer development [17]. One study showed that in melanoma cells, depletion of *Sema6A* alters the cytoskeleton, impairs anchorage-independent growth, and affects motility and invasive activities [25]. In addition, *Sema6A* showed increased mRNA expression levels in several renal tumor tissue samples and renal cell carcinoma cell lines, whereas its recombinant soluble extracellular domain inhibited angiogenesis induced by growth factors and tumor cell lines [15,26]. However, the mechanism underlying *Sema6A* regulation in cancer cells is largely unknown.

Oral cancer is a cancer that can develop in any part of the oral cavity, including the tongue, gums, skin lining the mouth, or lips. According to global cancer statistics, an estimated 263,900 new cases and 128,000 deaths from oral cavity cancer occurred in 2008 worldwide [27]. Furthermore, despite therapeutic improvements, the survival rate of oral cancer has not improved significantly over the past few decades, with a 5-year survival rate slightly above 50% [28]. Pathophysiological studies have shown that the development of oral cancer is linked to modifiable behaviors such as tobacco use and excessive alcohol consumption [29]. Other factors include poor oral hygiene, irritation caused by ill-fitting dentures and other rough surfaces on the teeth, poor nutrition, and some chronic infections caused by bacteria or viruses [27]. Although numerous studies examining the pathophysiological etiologies of oral cancer are being conducted worldwide, the molecular mechanism of oral cancer is not fully understood yet [28,29]. Therefore,

multi-clinical studies, those examining including surgical excision, radiation therapy, and chemotherapy, have been performed to develop novel methods for treating oral cancer.

Here, we examined the biological function of miR-203 as a tumor suppressor in YD-38 oral cancer cells. Furthermore, the molecular mechanism underlying the miR-203-mediated effect on *Sema6A* expression in YD-38 oral cancer cells was investigated.

II. MATERIALS AND METHODS

1. Materials

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich (St. Louis, MO, USA). Anti-cleaved caspase-3, anti-cleaved caspase-9, anti-cleaved poly (ADP-ribose) polymerase (PARP), anti-Bak, anti-Bax, and anti-β-actin antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). All other analytical reagents purchased were of analytical grade.

2. Cell line and cell cultures

Normal human oral keratinocytes (NHOKs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and maintained according to the manufacturer's instructions. The human oral cancer cell line derived from Korean, YD-38 cells, was provided by the Korean Cell Line Bank (Seoul, Korea) and cultured as according to the cell culture instructions provided by the Korean Cell Line Bank. Briefly, YD-38 human oral cancer cells were grown in RPMI 1640 containing 10% fetal bovine serum. The cells were maintained as monolayers in plastic culture plates at 37°C in a humidified atmosphere containing 5% CO_2.

3. Affymetrix miRNA array and data pre-processing

Total RNAs from both YD-38 cells and NHOKs were isolated with a miRNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The miRNA array was scanned using an Affymetrix GeneChip Platform (Affymetrix, Santa Clara, CA, USA). Briefly, for each sample, total RNA was subjected to a tailing reaction (2.5 mM $MnCl_2$, ATP, and polyA polymerase; incubation for 15 min at 37°C), followed by ligation of the biotinylated signal molecule to the target RNA sample (16 Flash Tag ligation mix biotin, T4 DNA ligase; incubation for 30 min at room temperature) and the addition of stop solution. Each sample was hybridized to a GeneChipH miRNA Array at 48°C and 60 rpm for 16 h, washed, stained on a Fluidics Station 450, and scanned on a GeneChip® Scanner3000 7G (Affymetrix, Santa Clara, CA, USA). Image data were analyzed with miRNA QC Tool software for quality control.

4. Identification of miR-203 target genes

DNA samples isolated from YD-38 cells over-expressing miR-203 were run on the DMET microarray (Affymetrix Inc., Santa Clara, CA, USA) using the DMET Plus Premier Pack kits according to the protocol described in the DMET Plus Premier Pack User Guide. Hybridized DMET arrays were washed and stained in Affymetrix fluidic stations and scanned with the Affymetrix GeneChip® Scanner 3000 7G. Data were generated with Affymetrix GeneChip® Command console software and analyzed with DMET Console software.

5. Plasmid construction

To induce the over-expression of miR-203, we generated the pSuper-miR-203 construct as previously described [30]. Briefly, microRNA expression vector pSuper was obtained from OligoEngine (Seattle, WA, USA). The miR-203 gene was amplified from human genomic DNA and the primers for miR-1 amplification were 5'-GAAGATCTGUGUUGGGGACUCGCGCGCUGGGUCCAGUGGUUCUUAACAGUU CAACAGUUCUGUAG-3' (forward) and 5'-CCGCTCGAGTCGCTGTCGCCGCGC CCGCCGGTCTAGTGGTCCTAAACATTTCACAATTGCGCTACAGAACTGTTGA-3' (reverse). The PCR products were digested with *Bgl*II and *Xho*I. The digested PCR products were phenol-extracted and inserted into the restriction enzyme site of the pSuper vector to generate pSuper-miR203. The clones were confirmed by sequencing.

To verify miR-203-induced *Sema6A* gene expression using a luciferase assay, the 3'-UTR of *Sema6A* containing the predicted target sites of miR-203 was amplified from human genomic DNA by proof reading the Phusion High-Fidelity PCR master mix with the following primers: *Sema6A*-3'-UTR-F, 5'-CGAGCCCGGGACTCCCAGGGGGAGGGGGTCAGGT-3'; *Sema6A*-3'-UTR-R, 5'-TCAGCGGCCGCTCGTTTTTTCCCTTTTTTTCT-3'. Amplified *Sema6A*-3'-UTR 2,997 base pairs in size, was digested with *SmaI* and *Not*I. The digested *Sema6A*-3'-UTR was inserted into the multiple cloning region downstream of the *hRluc* gene in psiCHECK^{TM}-1. The clones were confirmed by sequencing.

6. Transfection and cell viability assay

The MTT assay was applied to estimate the effect of miR-203 on YD-38 oral cancer cell proliferation. The cells were plated at a density of 5 x 10^3 cells/well in 96-well plates and allowed to attach to the wells overnight. After incubation, the cultured cells were transfected with pSuper-miR-203 at 2, 20 or 200 ng/ml for 24 or 48 h at 37℃ in a dose-dependency test using Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In addition, 200 ng/ml of pSuper-miR-203 was transfected into the YD-38 cells and incubated for 24 or 48 h as a time-dependency test. Next, the cells were incubated for an additional 4 h in 20 μl of 5 mg/ml MTT. To dissolve the formazan crystals transformed from MTT, the cells were resuspended in 150 μl dimethyl sulfoxide and the optical density (OD) of the solution was determined using a spectrometer at a wavelength of 495 nm. The experiments were repeated at least four times.

7. Evaluation and quantification of apoptosis

To evaluate chromatic condensation as an apoptotic phenomenon in YD-38 cells transfected with miR-203, cells were seeded at a density of 2 x 10^3 cells/well in a 4-well chamber slide and incubated overnight. Next, 200 ng/ml of pSuper-miR-203 was transfected into the cultured YD-38 cells using Lipotectamine™2000 for 48 h. After incubation, the cells were fixed with 1%

paraformaldehyde and washed 3 times with phosphate buffered saline (PBS). For nuclear staining, YD-38 cells were incubated with 10 ㎍/ml of 4'-, 6'-diamidino-2-phenlyindole dihydrochloride (DAPI, Roche Diagnostics, Madison, WI, USA) for 15 min. Apoptotic cells were quantified by assessing the characteristic nuclear changes of apoptosis using fluorescence microscopy (Eclipse TE200; Nikon Instruments, Melville, NY, USA).

8. Quantitative real-time PCR (qRT-PCR) and quantitative PCR (qPCR)

Total RNA, including mature miRNA, were isolated using an miRNeasy mini kit (Qiagen, Valencia, CA, USA), and the RNA was quantified by spectrophotometry (Nanodrop 2000, Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Reverse transcription of miRNA was performed using a miScript Reverse Transcription kit (Qiagen, Valencia, CA, USA) with 1 ㎍ of total RNA. TaqMan miRNA assays kits (Life Technologies, Grand Isalnd, NY, USA) were used to examine miR-203 (5'-GTGAAATGTTTAGGACCACTA-3') expression by qRT-PCR according to the manufacturer's instructions. The qRT-PCR results, which were recorded as threshold cycle numbers (Ct), were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (hGAPDH-forward primer sequences: 5'-CTTTGGTATCGTGGAAGGACTC-3'; hGAPDH-reverse primer sequences: 5'-AGTAGAGGCAGG GATGATGT-3') used as an internal control, and the comparative threshold cycle method ($^{\Delta\Delta}$Ct) was used to determine miRNA

expression levels. Furthermore, the level of *Sema6A* (*Sema6A*-forward primer sequences: 5'-TGCCCATATGCCAAACATGCC-3'; *Sema6A*-reverse primer sequences: 5'-TCTCCGTAATCCACGGCTTGAACA-3') induction was measured by PCR. The deviations in the samples were determined from four separate experiments.

To perform qPCR, total RNA was isolated from YD-38 cells using TRIzol reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's protocol. Reverse transcription was carried out with 1 μg of total RNA and Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA). The expression levels of Sema6A were measured by qPCR and visualized by DNA agarose gel electrophoresis. Differences in expression were presented as a histogram after densitometric analysis using a VersaDoc^{TM} imaging system (BioRad, Hercules, CA, USA). GAPDH was used as an internal standard.

9. Immunoblotting

To determine the levels of activated Sema6A, cleaved caspase-3, cleaved caspase-9, cleaved-PARP, Bak, and Bax in the YD-38 cells transfected with pSuper-miR-203, the proteins were extracted as previously described [31]. Briefly, YD-38 cells were cultured in 60-cm dishes. After the cells reached confluence, they were transfected with 200 ng/ml of pSuper-miR-203 for 48 h. After incubation, the cells were washed twice with ice-cold PBS and lysed in RIPA buffer for protein extraction according to the manufacturer's instructions. Total protein concentrations

were determined using the Bradford Assay (Bio-Rad, Hercules, CA, USA). An equal amount of protein was resolved by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes for immunoblotting. Membranes were blocked for 2 h in 5% non-fat dry milk in Tris-buffered saline containing Tween 20. Membranes were probed with primary antibodies against Sema6A, cleaved caspase-3, cleaved caspase-9, cleaved PARP, Bak, Bax, and β-actin overnight at 4℃ and then incubated with secondary antibody for 2 h at room temperature. Immunoactivity was visualized using an enhanced chemiluminescent system (Amersham Biosciences, Piscataway, NJ, USA) and single visual enhancer system (Pierce, Rockford, IL, USA) to magnify the signal.

10. Luciferase assays

miR-203 and scrambled miR-203 were purchased from Ambion (Austin, TX, USA). The miR-203, scrambled miR-203, and psiCHECK-1-promoter-*Sema6A*-3'-UTR were transfected into cultured YD-38 cells using Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The experimental group of YD-38 cells was treated with 1 ng/ml miRNAs for 2 days. Next, the cells were harvested and lysed for luciferase assays 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol. Renilla/firefly luciferase activity was calculated for each reaction.

11. Statistical analysis

All experiments were performed at least four times. The results are presented as the mean ± SEM. Statistical significance was analyzed by using Student's t-test for two groups and one way analysis of variance for multi-group comparisons. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Values of $p < 0.05$ were considered significant.

Ⅲ. RESULTS

1. miR-203 expression was significantly downregulated in human YD-38 oral cancer cells

To identify YD-38-related miRNAs, we performed an miRNA microarray on YD-38 cells and NHOK samples (n = 4 per cell line) using an Affymetrix Genechip and the data were analyzed as described previously [32]. Up- or down-regulated miRNAs differentially expressed in YD-38 cells and NHOKs were identified (Fig. 1A). We focused on miR-203 because its expression was significantly downregulated up to approximately 24-fold in YD-38 cells compared to in NHOKs. The microRNA array results were verified by examining the expression pattern of miR-203 by miRNA qRT-PCR using miRNA isolated from both YD-38 cells and NHOKs (Fig. 1B). qRT-PCR analysis showed that miR-203 expression was significantly downregulated in YD-38 cells by up to 95% compared to in NHOKs (Fig. 1C). Therefore, miR-203 expression was significantly downregulated in YD-38 oral cancer cells compared to in NHOKs.

2. Over-expressed miR-203 inhibits cell proliferation and induces apoptosis of YD-38 cells

To determine whether over-expression of miR-203 affects the cell proliferation of YD-38 cells, 200 ng/ml of pSuper-miR-203 was transfected into YD-38 cells

using Lipofectamine^{TM}2000. After 48 h, cell proliferation was assessed in an MTT assay. As shown in Fig. 2A, upregulation of miR-203 did not alter the viability of NHOKs. In contrast, the proliferation of YD-38 cells transfected with 200 ng/ml of pSuper-miR-203 gradually decreased at each time points and was decreased by approximately 30% compared to in cells treated with either the non-transfected or empty vector only (Fig. 2B). Moreover, the cell proliferation of YD-38 cells decreased with increasing concentrations of pSuper-miR-203, as shown in Fig. 2C.

Cell morphology was also assessed by DAPI staining, which detects nucleus condensation. The number of cells with the typical morphology of apoptosis was increased when miR-203 was over-expressed in YD-38 cells by at least 3.5-fold more than either the un-transfected or empty vector cells, as shown in Fig. 3. Otherwise, cells remained intact with no chromatin condensation in either the un-transfected control or YD-38 cells transfected with the empty vector.

Next, to evaluate the apoptotic signaling pathways involved in over-expressed miR-203-induced apoptosis in YD-38 cells, we measured the expression of apoptotic signaling pathway-related proteins by western blotting in YD-38 cells treated with 200 ng/ml of pSuper-miR-203 for 48 h. Expression of the pro-apoptotic factors Bax, Bak, and cleaved caspase-9 was significantly increased when miR-203 was over-expressed (Fig. 4). Caspase-3 is a target molecule of mitochondria-dependent intrinsic apoptosis [33,34]. Therefore, we analyzed the expression of cleaved caspase-3 and its downstream target molecule PARP. As shown in Fig. 4, cleaved caspase-3 was significantly upregulated in YD-38 cells treated with miR-203. Subsequently, the level of cleaved PARP, which functions downstream of caspase-3 [33,34], was significantly increased by miR-203 treatment

in YD-38 cells (Fig. 4). Taken together, these data suggested that over-expression of miR-203 inhibited cell proliferation and induced the apoptosis of YD-38 oral cancer cells.

3. Over-expressed miR-203 regulates the expression of *Sema6A* in YD-38 cells

To identify miR-203 target genes in YD-38 cells, a target gene array was performed using DMET Plus Premier Pack kits (Affymetrix) according to the manufacturer's protocol. Briefly, target genes containing the miR-203 binding site were preferentially amplified by highly selective molecular inversion probe amplification. The resulting target DNA was then labeled and hybridized to the DMET Plus Array to detect altered genes using a single-color detection format. Finally, the array results were translated using DMET Console software and the genetic profiles were converted into a more conventional format.

As shown in Fig. 5, miR-203 over-expression induced downregulation of *Sema6A* (semaphorin 6A), *ABCE1* (ATP-binding cassette sub-family E member 1), and *MLLT4* (myeloid/lymphoid or mixed-lineage leukemia translocated to 4), whereas *IL24* (interleukin 24), *SEL1L* (sel-1 suppressor of lin-12-like), and *ADAMTS6* (ADAM metallopeptidase with thrombospondin type 1 motif 6) were significantly upregulated by miR-203 over-expression in YD-38 cells. This suggests that *ABCE1* plays an essential role in lung cancer progression and metastasis [35], and that *MLLT4* regulates cancer cell migration in colorectal

carcinogenesis [36]. In addition, *IL24* functions as a tumor suppressor gene in breast cancer [37]. Additionally, over-expression of *SEL1L* decreases tumor growth in breast cancer, suggesting that *SEL1L* has a tumor-suppressive role [38]. Furthermore, *ADAMTS6* suppresses tumorigenesis by inhibiting cell migration and invasion via the ERK pathway in breast cancer [39]. Expression alterations of these genes may be associated with tumorogenesis in various tissue, and thus we performed gene functional analysis of *Sema6A* [40] associated with miR-203 in the present study. We are currently evaluating alterations in the expression of other genes associated with miR-203. The mRNA expression of *Sema6A* is elevated in several renal tumor tissue samples, suggesting that *Sema6A* is a promising biomarker for the detection and diagnosis of cancer [15,26]. Therefore, we hypothesized that over-expression of miR-203 induces apoptosis via the downregulation of *Sema6A* in YD-38 cells. To further confirm that miR-203 inhibits *Sema6A* expression in YD-38 cells, pSuper-miR-203 constructs were transfected into YD-38 cells and the mRNA and protein levels of *Sema6A* were analyzed.

As shown in Fig. 6A and B, both the qPCR and qRT-PCR results showed that *Sema6A* was significantly decreased by approximately 40% in YD-38 cells transfected with 200 ng/ml of pSuper-miR-203 compared to in empty vector only cells.

In addition, Sema6A protein expression was notably decreased by miR-203, as shown in Fig. 7. A histogram after densitometric analysis normalized to β-actin showed that the level of Sema6A protein expression was decreased compared to that in empty vector-transfected cells. Together, these results suggest that

miR-203 decreased the expression of *Sema6A*, indicating that it is a downstream target gene in YD-38 cells.

4. *Sema6A* is directly targeted by miR-203

To determine the mechanisms of miR-203-mediated downregulation of *Sema6A* in YD-38 cells, we performed target gene scanning using TargetScan (http://www.targetscan.org/) and miRBase (http://www.mirbase.org/) and found that *Sema6A* was a predicted target gene. Furthermore, *Sema6A* contains a potential complementary binding sequence located from nucleotide 1,155 to 1,161 in its 3'-UTR, indicating that miR-203 can directly target this site. Therefore, we cloned 2,997 base pairs (full-length) of the *Sema6A* 3'-UTR into the firefly luciferase based-psiCHECK-1 vector. In addition, a psiCHECK-1-empty vector was transfected into YD-38 cells to assess the basal level of luciferase activity (Fig. 8A). As shown in Fig. 8B, the relative luciferase activity of the reporter containing the 3'-UTR of *Sema6A* was notably decreased in miR-203 by approximately 1.9-fold compared to control in YD-38 cells. In contrast, the psiCHECK-1-empty vector plus pSuper-miR-203 and psiCHECK-1-*Sema6A* plus pSuper-empty vector showed similar basal levels of luciferase activity. These results suggest that *Sema6A* is a novel and specific target gene of miR-203 and miR-203 may regulate *Sema6A* expression in YD-38 cells.

Ⅳ. DISCUSSION

miRNAs play important roles as gene regulators, which has prompted numerous studies of their functions [41]. miRNA studies have become increasingly important in human cancer research because of the roles of miRNAs as oncogenes or tumor suppressors [42]. However, the precise cellular mechanisms associated with the tumorigenesis or anti-oncogenesis functions of miRNAs remain largely unclear. Recent studies have shown that the dys-regulation of miRNAs is closely associated with oncogenesis in various cancer cells. In particular, downregulation of tumor suppressor miRNAs such as miR-34a [43], miR-145 [44], and miR-133a/b [44] enhanced the oncogenic effects in various cancer cells. Aberrant miR-203 acts as a tumor suppressor, and silencing has been reported in various tissues, such as in oral cancer [45] and hepatocellular carcinoma [46]. Moreover, Mathe et al. [47] demonstrated that miR-203 expression was reduced in cancerous tissues compared to in noncancerous tissues. Similar to a previous study examining the pathophysiological role of miR-203 in cancer cells, the expression of miR-203 was most significantly downregulated in YD-38 oral cancer cells compared to in NHOKs (normal tissues) in the present study (Fig. 1). Therefore, these data suggest that miR-203 is clinically useful for developing prognostic biomarkers. These data also suggest that downregulated expression of miR-203 is closely associated with oral cancer progression.

Recently, miRNA research associated with cancer therapies has suggested that over-expression of tumor suppressor miRNAs such as miR-124 (in gastric cancer)

[48], miR-494-3p (in prostate cancer) [49], miR-23a/b (in lymphoma) [50], and miR-205 (in oral cancer) [51] can inhibit proliferation and induce apoptosis of cancer cells. More recently, Tian *et al.* [52] reported that over-expression of miR-203 suppressed proliferation and induced apoptosis of laryngeal squamous cell carcinoma. Therefore, based on our results and those of previous studies, we hypothesized that over-expression of miR-203 could induce apoptosis of YD-38 cells by altering cellular responses. As shown in Fig. 2, cell viability gradually decreased in YD-38 cells transfected with miR-203 in both time- and dose-dependent manners. Furthermore, over-expression of miR-203 increased nuclear condensation in YD-38 cells, as shown by DAPI staining (Fig. 3). Moreover, miR-203 upregulated or activated the pro-apoptotic factors, such as Bax, Bak, and cleaved caspase-9 [33,34], as shown in Fig. 4. The levels of cleaved caspase-3 and cleaved PARP were significantly increased in YD-38 cells transfected with miR-203 (Fig. 4). Finally, as a typical phenomenon of apoptosis, activated PARP induced DNA fragmentation via single-strand DNA breakage in the nucleus of YD-38 cells [33,34]. These data suggest that miR-203 led to apoptotic cell death in YD-38 human oral cancer cells. However, the complete mechanisms of miR-203-induced apoptosis in YD-38 cells remain unclear. Further studies are needed to determine these cellular and molecular mechanisms.

miRNAs are thought to elicit their effects by base-pairing complementation between the seed sequence of miRNAs and 3'-UTR of their target mRNAs, which is a key determinant of miRNA-target recognition [53]. Therefore, we used an miR-203 target gene array to explore the molecular biological mechanism of miR-203-induced apoptosis in YD-38 cells. The results of the miR-203 target gene

array in YD-38 cells revealed that over-expression of miR-203 altered the expression patterns of some oncogenes. Although miR-203 changed the expression of some tumor suppressor genes, we focused on downregulated oncogenes in YD-38 cells transfected with miR-203 because miRNAs typically suppress protein expression by affecting mRNA translation and/or destabilization through interactions with the 3'-UTR of their target mRNAs [53]. As shown in Fig. 5, *Sema6A*, *ABCE1*, and *MLLT4* were significantly downregulated in YD-38 cells transfected with miR-203, whereas *IL24*, *SEL1L*, and *ADAMTS6* were significantly upregulated by miR-203 over-expression in YD-38 cells. *ABCE1* plays an essential role in lung cancer progression and metastasis [35] and *MLLT4* regulates cancer cell migration in colorectal carcinogenesis [36]. Additionally, *IL24* functions as a tumor suppressor gene in breast cancer [37] and *SEL1L* has a tumor-suppressive role in breast cancer [38]. Furthermore, *ADAMTS6* suppresses tumorigenesis by inhibiting cell migration and invasion in breast cancer [39]. However, their oncogenic or tumor-suppressive activities remain largely unclear in various cancer cells. Although we are still conducting functional studies for both *ABCE1* and *MLLT4* in YD-38 cells, the expression of *Sema6A* was most significantly downregulated by miR-203 in YD-38 cells (Fig. 5).

Semaphorins is a large family of conserved glycoproteins that regulate cell motility, immune response, cell differentiation, morphology, angiogenesis, and tumor progression [17]. Additionally, semaphorins and their receptors are involved in multiple functions during development and in the adult organism, particularly in the nervous system, immune system, and various pathological situations [54]. Sema6A is a membrane-bound repellent and functions as a guidance cue for nerve axons

and migrating cells in the developing central nervous system [17]. In addition, *Sema6A* shows elevated mRNA expression in several renal tumor tissues and renal cell carcinoma cells [15,26]. A recent study observed miRNA-mediated regulation of *Sema6A*. Wolter *et al.* [55] reported that *Sema6A* upregulation facilitated by miR-346 silencing may promote glioma growth. However, the mechanism underlying *Sema6A* regulation related to miRNAs in cancer cells is unclear. In the present study and based on the results of previous studies [15,26,55], we hypothesized that miR-203-induced apoptosis of YD-38 cells is related to the suppression of *Sema6A*. As shown in Fig. 6 and 7, both mRNA induction and protein expression of *Sema6A* were significantly suppressed by miR-203 transfection in YD-38 cells. These data suggest that *Sema6A* is a target oncogene of miR-203-induced apoptosis in YD-38 oral cancer cells.

However, miRNAs are thought to elicit their effects by silencing the expression of their target genes by directly interacting with their own regulatory region, such as the promoter [56] and/or 3'-UTR [53]. Therefore, to identify the miR-203 binding site in the *Sema6A* gene, we performed target gene scanning and identified potential complementary binding sequences from positions 1,155 to 1,161 in its 3'-UTR as shown in Fig. 8. Next, to verify whether miR-203 can suppress the expression of *Sema6A* in YD-38 cells, we generated the psiCHECK-1-*Sema6A* construct, which contained potential complementary binding sequences for miR-203 in the 3'-UTR of *Sema6A*. The miR-203 directly targets the 3'-UTR of *Sema6A*, as its over-expression was suppressed luciferase activity (Fig. 8). In contrast, the over-expressed mimic miR-203 did not affect *Sema6A* expression. These results suggest that miR-203 regulates the expression of *Sema6A* in YD-38 oral cancer

cells by downregulating *Sema6A* directly via targeting of its 3'-UTR.

In conclusion, we found that miR-203 functions as a tumor suppressor associated by suppressing *Sema6A* in YD-38 human oral cancer cells. Further analyses of the mechanism underlying the regulation of cancer progression by miR-203 will contribute to the development of new oral cancer treatments and provide a new marker for the diagnosis of cancer.

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Ⅵ. FIGURE LEGENDS

Fig. 1. Expression of miR-203 is downregulated in YD-38 cells. (A) Total RNA from both YD-38 cells and NHOKs was isolated, scanned using an Affymetrix GeneChip Platform, stained on Fluidics Station 450, and scanned on a GeneChip® Scanner3000 7G. Each absolute fold change value represents the mean of four independent experiments. (B) The relative expression of miR-203 in YD-38 cells and NHOKs was assessed by qRT-PCR, as described in the "MATERIALS AND METHODS". (C) The relative expression of miR-203 in YD-38 cells and NHOKs was assessed by qPCR and was demonstrated as shown in the histogram after densitometric analysis. Each data point represents the mean ± SEM of at least four independent experiments. $^{**}p < 0.01$ vs. control.

Fig. 2. Over-expressed miR-203 increased cell cytotoxicity in YD-38 cells. Cell viability of NHOKs (A) and YD-38 cells (B, C) was measured after miR-203 transfection over time and across different concentrations. Cell cytotoxicity was assessed by MTT assay after transfection into YD-38 cells following defined treatment condition. $^{*}p < 0.05$ vs. control and $^{**}p < 0.01$ vs. control.

Fig. 3. Over-expressed miR-203 changed the nuclear morphology of cells in YD-38 cells. (A) Nuclear morphology was observed by DAPI staining after

transfection with 200 ng/ml of pSuper-miR-203. (B) The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells. $^{**}p < 0.01$ vs. control.

Fig. 4. Over-expressed miR-203 induced cell apoptosis in YD-38 cells. YD-38 cells were transfected with 200 ng/ml of pSuper-miR-203. Cell lysate was prepared and analyzed by immunoblotting as described in "MATERIALS AND METHODS".

Fig. 5. *Sema6A* is a target gene of miR-203 in YD-38 cells. DNA samples isolated from YD-38 cells with over-expressed miR-203 were run on the DMET microarray, stained in the Affymetrix fluidic stations, and scanned with the Affymetrix GeneChip® Scanner 3000 7G. Data were generated with Affymetix GeneChip® Command console software and analyzed with the DMET Console software. Each absolute fold-change value represents the mean of four independent experiments. *Sema6A*; semaphorin 6A, *ABCE1*; ATP-binding cassette sub-family E member 1, *MLLT4*; myeloid/lymphoid or mixed-lineage leukemia translocated to 4, *IL24*; interleukin 24, *SEL1L*; sel-1 suppressor of lin-12-like, *ADAMTS6*; ADAM metallopeptidase with thrombospondin type 1 motif 6.

Fig. 6. Over-expressed miR-203 decreased expression of the oncogenic gene *Sema6A* in YD-38 cells. (A) The expression level of *Sema6A* was measured by qPCR after miR-203 transfection. The amplified PCR products

of *Sema6A* were electrophoresed on agarose gels and the data were presented on a histogram after densitometric analysis. (B) The expression level of *Sema6A* was assessed by qRT-PCR after miR-203 transfection into YD-38 cells. $^{*}p < 0.05$ vs. control and $^{**}p < 0.01$ vs. control.

Fig. 7. Over-expressed miR-203 decreased expression of Sema6A protein in YD-38 cells. Sema6A protein expression was quantified by western blotting using Sema6A-specific antibody. Each data point represents the mean ± SEM of at least four independent experiments. $^{*}p < 0.05$ vs. control.

Fig. 8. *Sema6A* is a direct target of miR-203 in YD-38 cells. (A) The sequence of the miR-203 target sites on the *Sema6A*-3'-UTR. (B) The luciferase activity of the *Sema6A* promoter against miR-203. psiCHECK-2-*Sema6A* was cloned as described in "MATERIALS AND METHODS". Luciferase activity was normalized to total protein. Each data point represents the mean ± SEM of at least four independent experiments. $^{*}p < 0.05$ vs. control.

Ⅶ. FIGURES

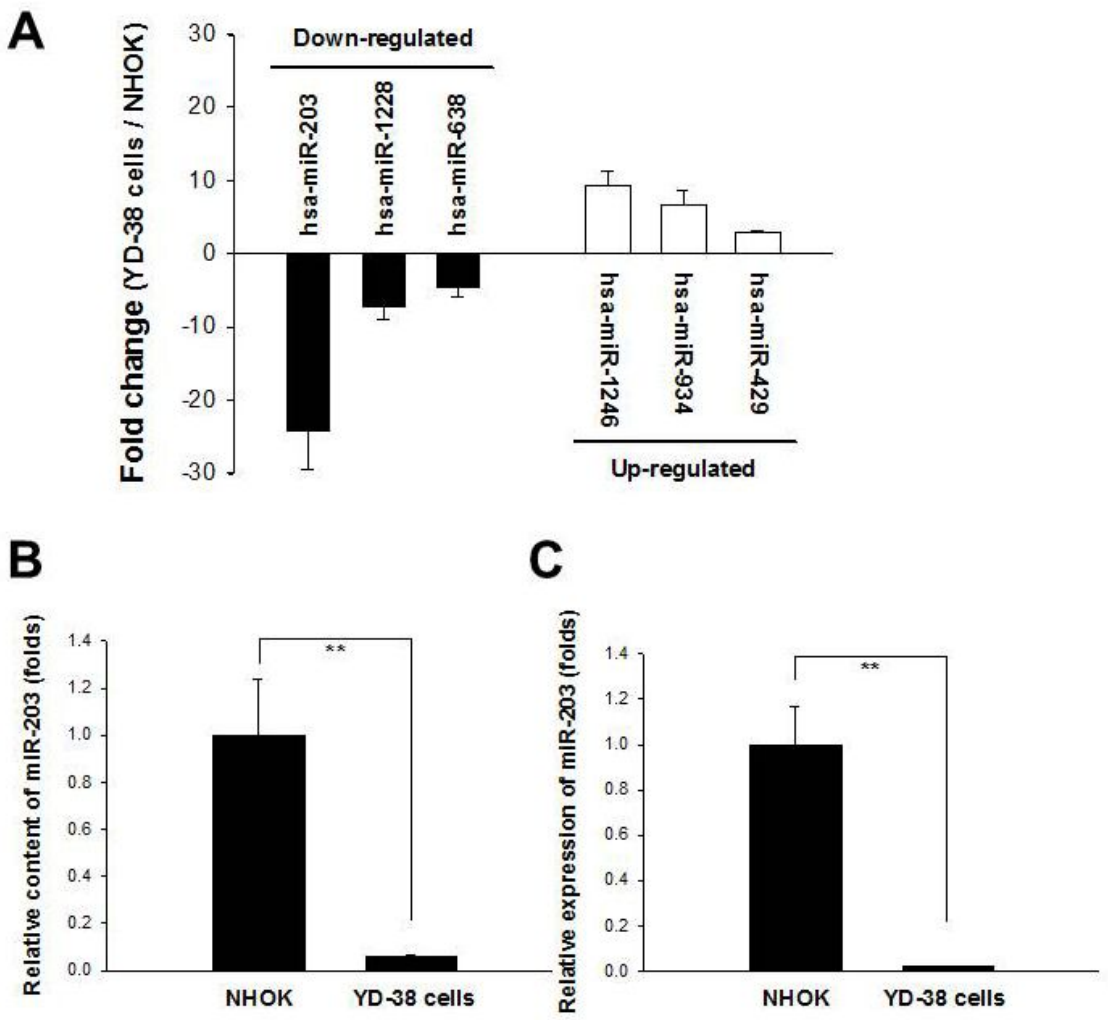

Fig. 1. Expression of miR-203 is downregulated in YD-38 cells.

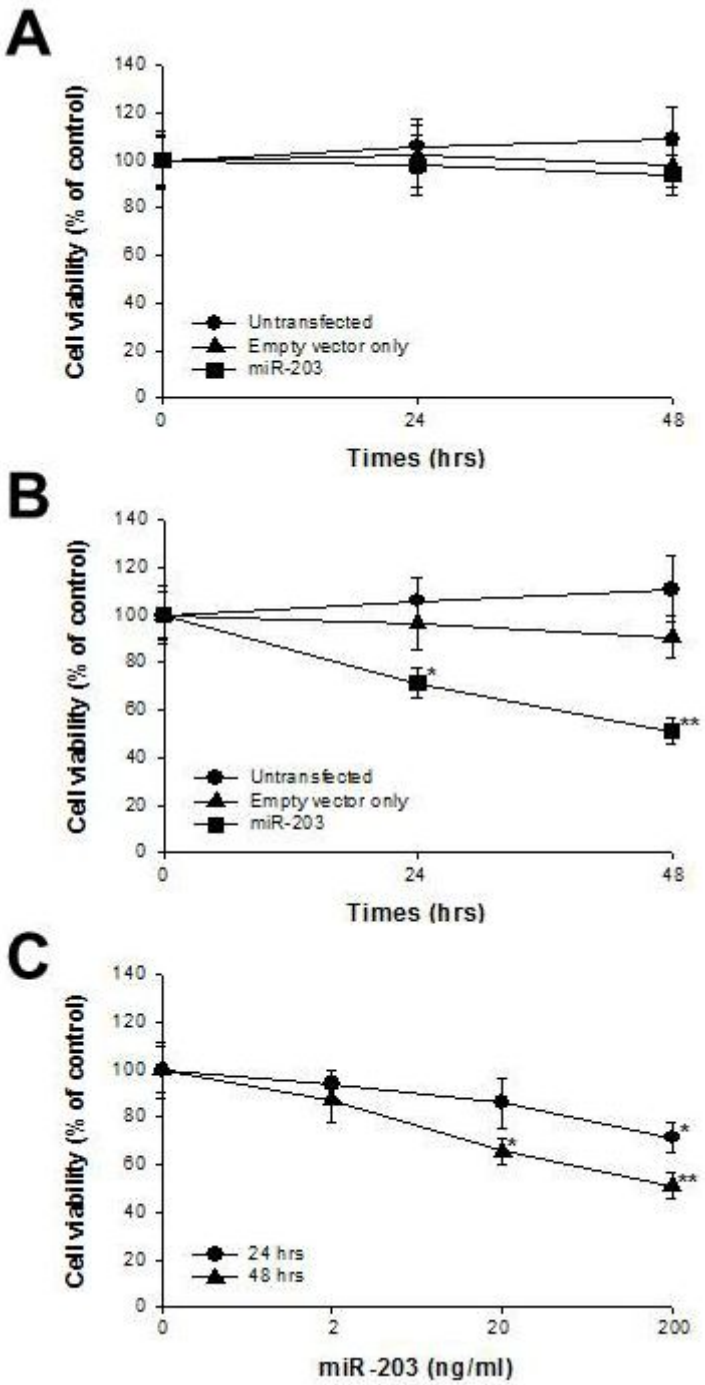

Fig. 2. Over-expressed miR-203 increased cell cytotoxicity in YD-38 cells.

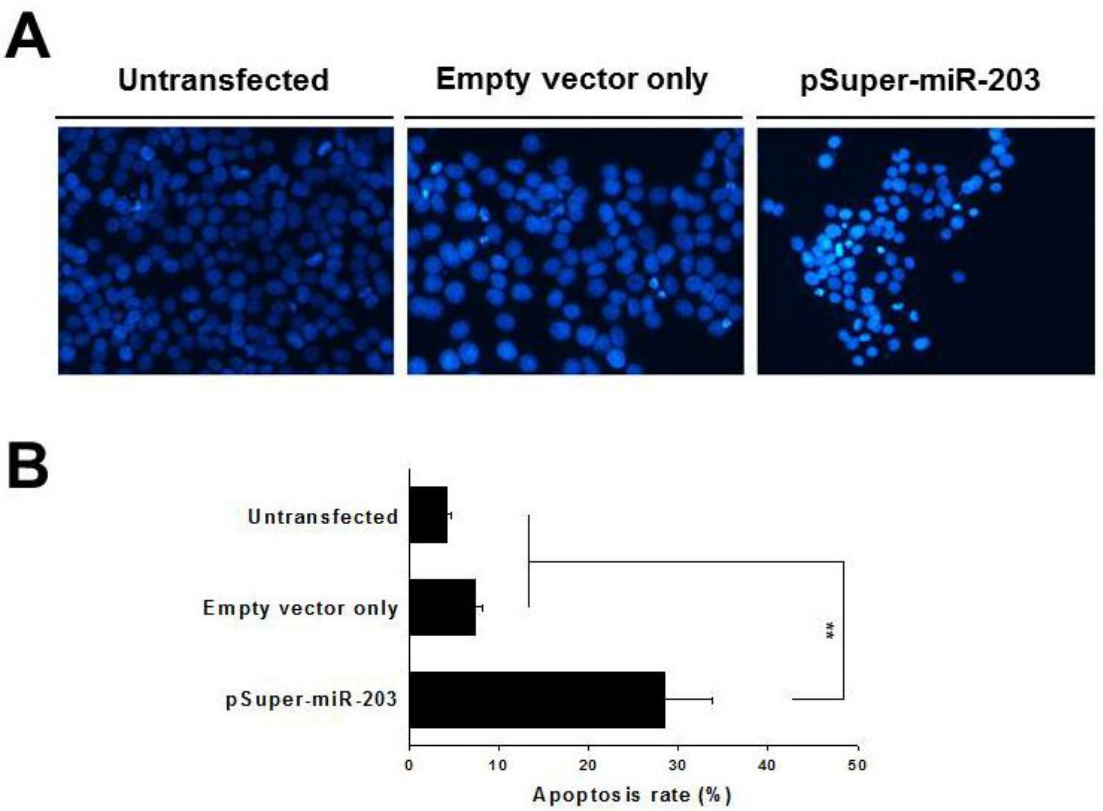

Fig. 3. Over-expressed miR-203 changed the nuclear morphology of cells in YD-38 cells.

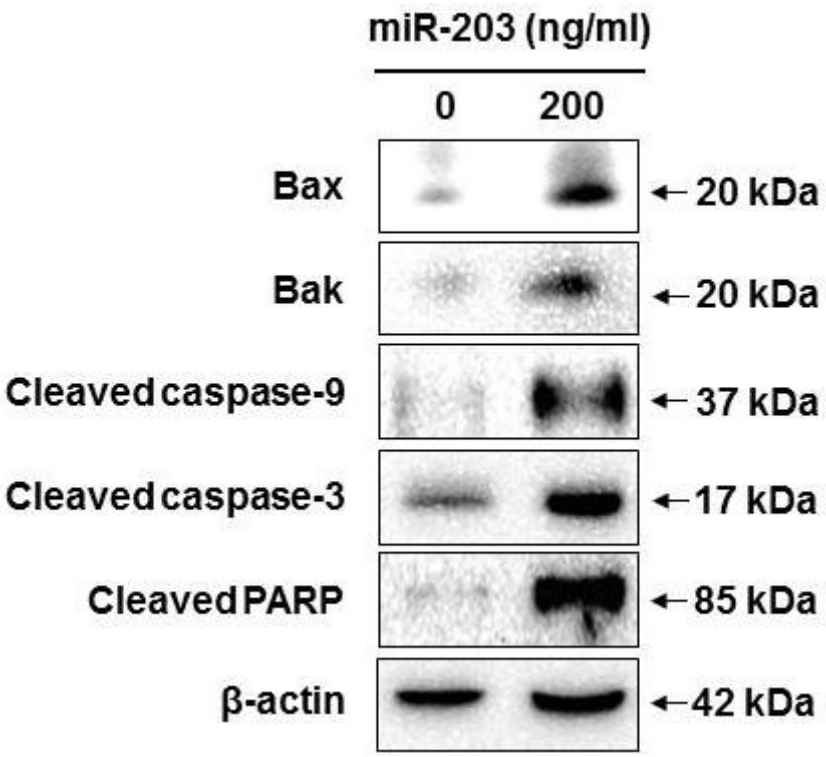

Fig. 4. Over-expressed miR-203 induced cell apoptosis in YD-38 cells.

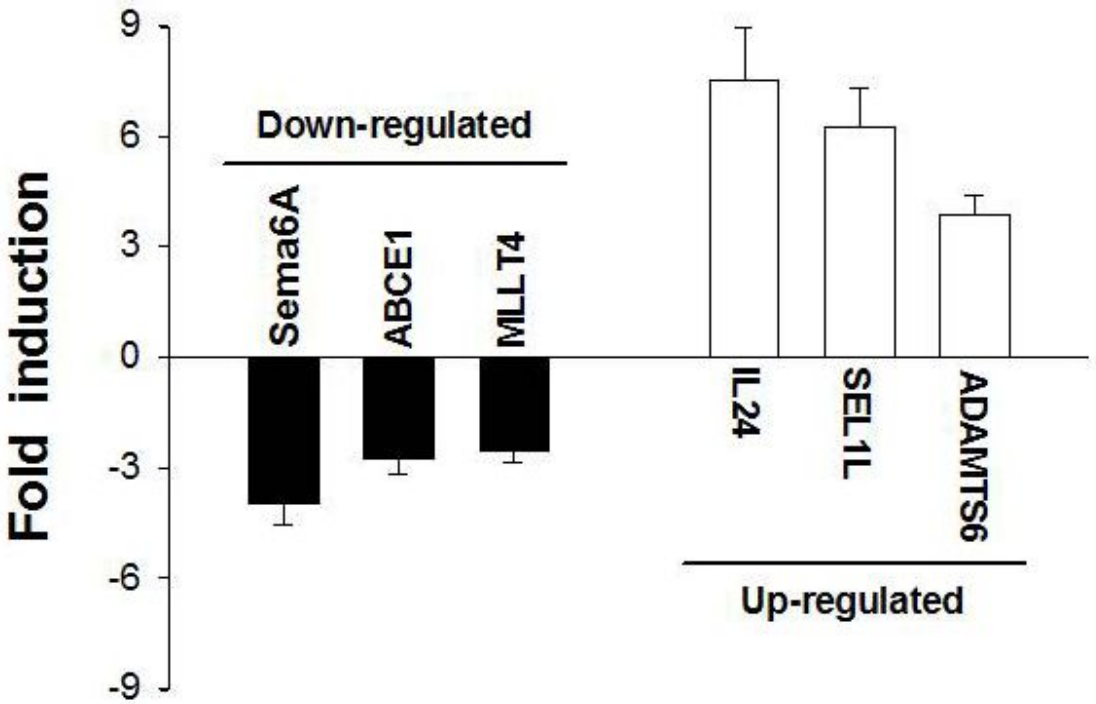

Fig. 5. *Sema6A* is a target gene of miR-203 in YD-38 cells.

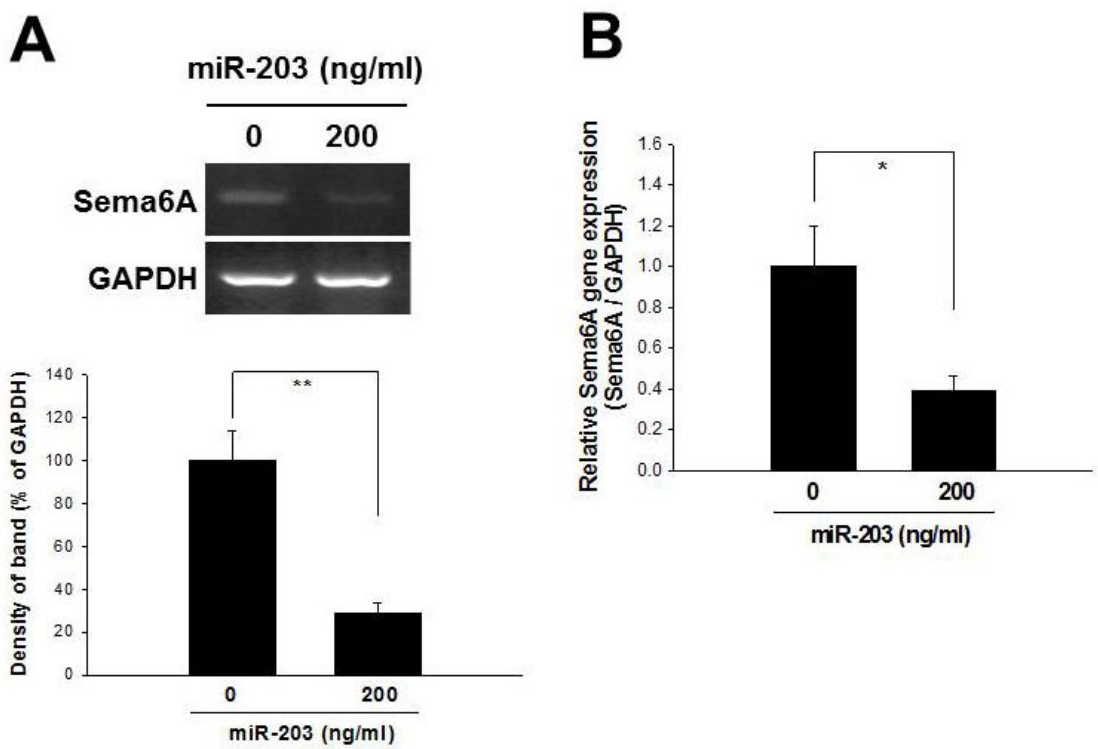

Fig. 6. Over-expressed miR-203 decreased expression of the oncogenic gene *Sema6A* in YD-38 cells.

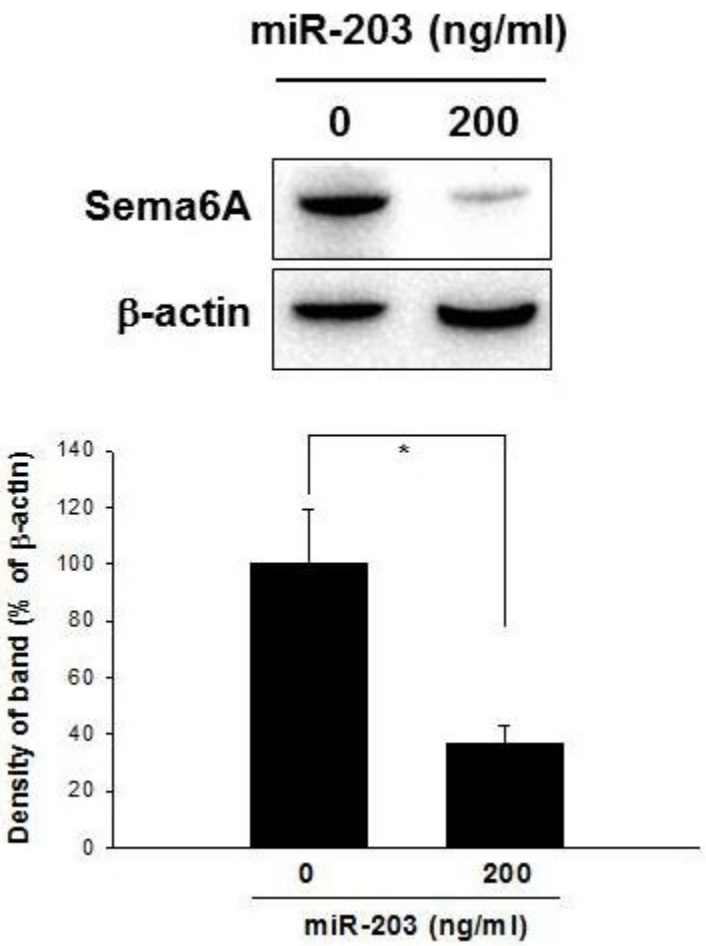

Fig. 7. Over-expressed miR-203 decreased expression of Sema6A protein in YD-38 cells.

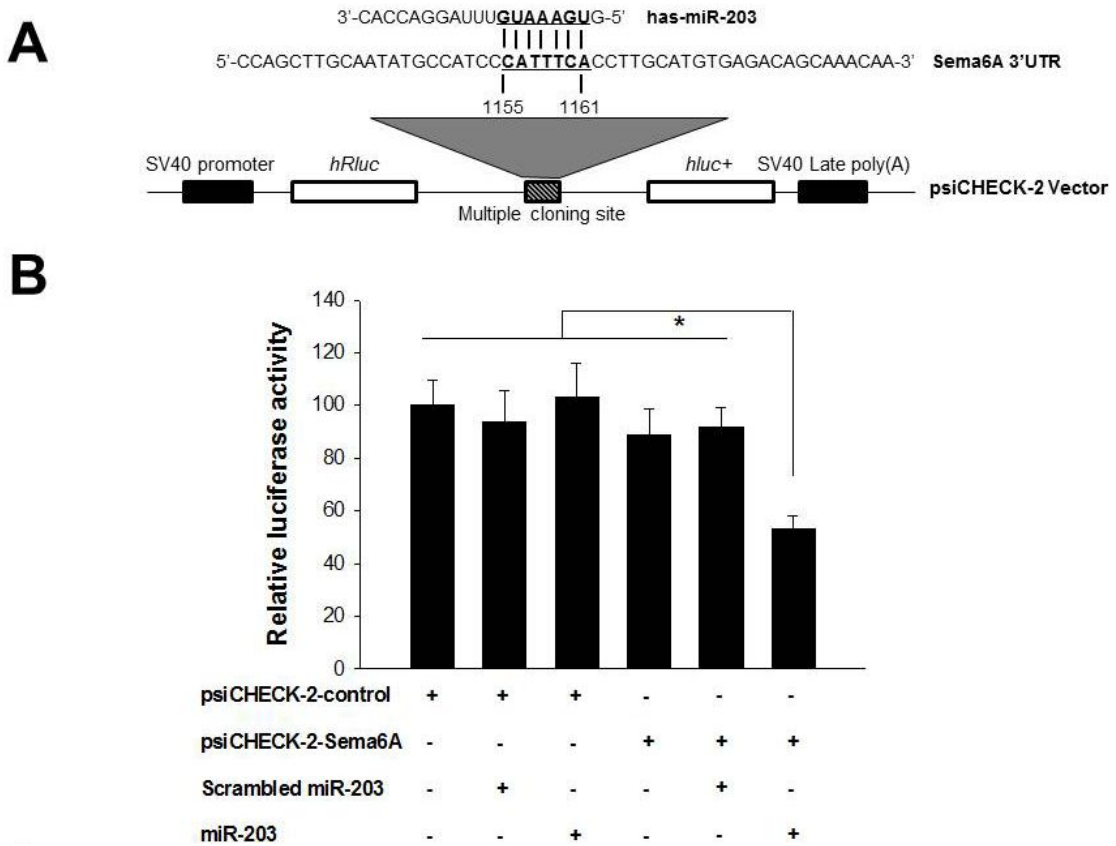

Fig. 8. *Sema6A* is a direct target of miR-203 in YD-38 cells.