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ABSTRACT in KOREAN

MicroRNA-203의 *Yes-1* 표적작용을 통한 구강암세포 성장 억제

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MicroRNA(miRNA)는 21-25개의 염기서열로 구성된 noncoding RNA 분자로, 주로 표적 유전자의 3'-UTR 부위에 상보적인 염기서열을 가지면서 그 표적유전자의 단백 합성을 억제시키거나 촉진시킨다. Oncogenic miRNA는 암과 관련된 miRNA로 최근 여러 종양에서 oncogenic miRNA 발현 분석을 통해 다양한 암의 조기진단, 예후측정에 응용가능성을 시사하고 있다. 종양에서 miRNA의 비정상적인 발현은 염색체 결손, 증폭, point mutation과 같은 유전적 원인 또는 비정상적인 DNA 메틸화 등과 같은 후생유전에 의한 것으로 알려져 있으며, 그 작용기전과 기능에 대한 연구가 최근 들어 많이 진행되고 있다. 본 연구에서는 구강암세포에서 miRNA들의 발현을 비교·분석하여 구강암 특이 miRNA를 확립하고, miRNA-203 제어에 의한 종양유전자 *Yes-1* 표적작용을 통한 구강암세포 성장억제의 분자적 기전을 밝히고자 하였다.

구강암세포에서 miRNA-203 제어에 의한 구강암세포 성장억제 작용기전을 규명하기 위해 구강암세포주 KB에 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 KB 구강암세포에서 miRNA-203의 발현이 현저히 감소하였다.
2. KB 세포에서 miRNA-203은 세포의 성장을 뚜렷이 억제시켰다.
3. KB 세포에서 miRNA-203은 세포 apoptosis를 유도하였다.
4. KB 세포에서 miRNA-203은 종양유전자 *Yes-1*의 발현을 억제하였다.
5. KB 세포에서 miRNA-203은 종양유전자 *Yes-1*을 직접 억제함으로써 luciferase 활성을 감소시켰다.

본 연구의 결과로서 구강암세포주 KB에서 miRNA-203은 종양억제 miRNA로써 기능을 하여 종양유전자 *Yes-1*의 발현을 감소시켜 구강암세포 증식억제 및 세포 apoptosis를 유도하는 것으로 사료된다.

중심어: miR-203, tumor suppressor, oral cancer cells, *Yes-1*, apoptosis

I. INTRODUCTION

Oral cancer, also known as mouth cancer, is a cancer that can develop in any part of the mouth, including the tongue, the gums, the skin lining the mouth or the lips. Close to 40,000 cases of oral cancer will be newly diagnosed annually, and the in 5 years survival rate after diagnosis and treatment only slightly more than 50% (Parkin et al., 1999). Pathophysiological studies have shown that the development of oral cancer is linked to modifiable behaviors such as tobacco use and excessive alcohol consumption (Elwood et al., 1984). 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. Despite oral cancer research has made progress to date, the molecular mechanism underlying oral cancer is not understood yet. Therefore, multi-clinical studies, including surgical excision, radiation therapy and chemotherapy, have been designed and performed to help find novel method for treating oral cancer.

MicroRNAs (miRNAs) are endogenous 19-25 nucleotide non-coding sequences (Bonnet et al., 2004), which are important post-transcriptional regulators on gene expression via sequence-specific to 3'-untranslated regions (UTR) of mRNA target genes, causing inhibition of translation and/or mRNA degradation (Valencia-Sanchez et al., 2006). miRNAs have been profiled according to their typical mechanism of transcriptional regulation in a variety of cells and cellular systems. They may regulate >90% of human gene (Miranda et al., 2006), and control a various cellular biological processes such as cell development, proliferation and differentiation, and apoptosis (Inui et al., 2010; Chitwood and Timmermans, 2010). The special sequence component in miRNAs constituting the RNA-induced silencing complex (RISC) hybridizes to the target mRNA, thereby inhibiting the transcription and reducing the stability of the target mRNA, resulting in mRNA degradation and translation inhibition (Tan et al., 2011; Hebert et al., 2012). The interaction between the miRNA-RISC complex and its target mRNA requires only

partial base-pairing (usually 6-8 nucleotides) of the miRNA; this is the so-called seed sequence. Therefore, miRNA may target hundreds or thousands of mRNAs, which allows each small RNA to substantially or weakly suppress the expression of a whole range of genes depending on the characteristics of the target sites within the 3'-untranslated region (UTR) of the target mRNA (Ui-Tei et al., 2012; Yang et al., 2012). These regulatory small RNA molecules have been implicated in cancer biology (Wang et al., 2012). A great number of studies have demonstrated that alterations of miRNA function as oncogenes or suppressive genes and that their aberrant expression contributes to human diseases such as cancer (Calin et al., 2006). Therefore, current studies have focused on the utility of miRNA as diagnostic and prognostic tools as well as potential therapeutic targets. However, their biological functions and cellular mechanisms remain largely unknown.

Many studies have examined the biological functions of miR-203 as a tumor suppressor in carcinogenesis. Saini et al. (2011) reported that miR-203 suppresses prostate cancer cell progression and metastasis by targeting a cohort of pro-metastatic genes. Wang et al. (2013) and Jin et al. (2012) reported that miR-203 inhibits cell proliferation and migration by targeting *PKCa* or *survivin* expression in lung cancer cells. In a separate study, Bueno et al. (2008) demonstrated that the genetic and epigenetic silencing of miR-203 enhanced the expression of the oncogenes *ABL1* and *BCR-ABL1* in specific hematopoietic malignancies. On the other hand, the anti-tumorigenesis function and signaling pathway of miR-203 in oral cancer cells are still unclear.

Src family kinases (also SFKs or Src), a multifunctional non-receptor tyrosine kinase family, are one of the most studied protein families in cancer biology (Ninio-Many et al., 2013). Since the identified and characterization of eight proteins, (*c-Src*, *c-Yes*, *Fyn*, *Lck*, *Lyn*, *Hck*, *Fgr*, and *Blk)*, which have a homologous structure. Each member is characterized by a unique region that specifies its respective binding partners and, its functions (Kefalas et al., 1995; Thomas and Brugge, 1997). Of the 8 SFK members, *c-Src*, *Fyn*, and *c-Yes* (also *Yes-1*) are most widely expressed, whereas the expression of others is restricted, mainly to

cells of hematopoietic origin (Thomas et al., 1997). In general, SFKs play crucial roles in regulation of cell processes, including proliferation, differentiation, adhesion, survival and migration (Kopetz et al., 2007), by mediating extracellular interactions driven by various molecules, epidermal growth factor receptor (EGFR) and integrin (Kim et al., 2009). In addition, SFKs regulate these pro-tumorigenic functions via activation of downstream signaling pathways, including mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), phosphoinositide-3-kinase (PI3K), Stat3, p130Cas and focal adhesion kinase (FAK) (Chan et al., 2012). SFKs are over-expressed and/or activated in many tumor types, and making the SFKs attractive targets for anti-cancer treatment in several solid tumors and leukemia (Kim et al., 2009; Edwards, 2010).

The SFK member *Yes-1* was initially identified as homolog of *v-Yes*, the oncogene of Yamaguchi avian sarcoma virus (Roche et al., 1997). *Yes-1* exhibits the highest homology with *c-Src*, with 70% identity outside the N-terminus. Although *c-Src* and *Yes-1* share significant sequence and structural homology, they serve not only redundant but also distinct functions, and consequences of their activation may differ in diverse cellular backgrounds (Summy et al., 2003). For example, activation of *Yes-1* is known to be associated with the formation of tight junctions in canine kidney epithelial cells, whereas activation of *c-Src* is believed to be involved in the dissociation of these structures (Clump et al., 2005). In addition, abnormal levels and/or catalytic activities of *c-Src* and *Yes-1* have been found in a number of human tumors, including lung, breast, ovarian, gastric, and colorectal tumors (Summy et al., 2003; Frame et al., 2002). Especially, in colorectal cancer, *c-Src* up-regulated in >70%, whereas *Yes-1* up-regulated in about 50%. Importantly, however, in the case of liver metastases, patients with increased *Yes-1* activity had a shorter survival time when compared with patients without *Yes-1* activation (Han et al., 1996). However, as yet, the mechanism underlying *Yes-1* regulation in cancer cells is largely unknown.

Here, we examined to determine the biological function of miR-203 as a tumor suppressor in KB oral cancer cells. Furthermore, the molecular mechanism

underlying the miR-203-mediated effect on *Yes-1* expression in KB oral cancer cells was investigated.

II. MATERIALS AND METHODS

1. Cell culture

Normal human oral keratinocytes (NHOKs) were purchased from ScienCell Research Laboratories (Carlsbad, CA) and were maintained according to the manufacturer's instructions. The human oral cancer cell line KB was obtained from the American Type Culture Collection (ATCC) and cultured as according to the cell culture instructions provided by the ATCC. Briefly, KB cells were grown in minimal essential (MEM) medium (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37℃ in an atmosphere containing 5% CO_2.

2. Affymetirx miRNA array and data pre-processing

Total RNAs from both KB cells and NHOKs were isolated with a miRNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The concentration, purity, and amount of total RNA were quantified using the Nano-Drop® ND-1000 ultraviolet spectrophotometer (Thermo Scientific, USA). The miRNA array was scanned using an Affymetrix GeneChip Platform (Affymetrix, Santa Clara, CA). Briefly, for each sample, total RNA was subjected to a tailing reaction (2.5 mM $MnCl_2$, ATP and Poly A Polymerase; incubation for 15 min at 37℃) 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℃ and 60 rpm for 16 h, washed, stained on a

Fluidics Station 450, and scanned on a GeneChip® Scanneer3000 7G (Affymetrix, Santa Clara, CA). The image data were analyzed with the miRNA QC Tool software for quality control.

3. Identification of miR-203 target genes

DNA samples isolated from KB cells over-expressing miR-203 were run on the DMET microarray (Affymetrix Inc., Santa Clara, CA) 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 the Affymetrix fluidic stations and scanned with the Affymetrix GeneChip® Scanner 3000 7G. Data were generated with Affymetrix GeneChip® Command console software and analyzed with the DMET Console software.

4. Construction of miRNA expression vector

miR-203 and scrambled miR-203 were purchased from Ambion (Austin, TX). The miR-203, mimic miR-203, and psiCHECK-2-promoter-*Yes-1*-3'-UTR were transfected into cultured KB cells using Lipofectamine™2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The experimental group of cells was treated with 1 ng/ml miRNAs for 2 days.

5. Transfections and Cell viability assay

MTT assay was applied to estimate the effect of miR-203 on KB 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 well overnight. After incubation, the cultured

cells were transfected pSuper-miR-203 at a defined concentration (5, 20, 200 ng/ml) for 24 h or 48 h at 37℃ as the dose-dependent test using Lipofectamine™2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.. In addition, 200 ng/ml of pSuper-miR-203 was transfected into the KB cells and incubated for 24 h and 48 h as a time-dependence test. Then, the cells were incubated for a further 4 h in 20 μl of 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). To dissolve the formazan crystals transformed from MTT, the cells were resuspended in 150 μl dimethyl sulfoxide (DMSO) and the optical density (OD) of the solution was determined using a spectrometer at an incident wavelength of 495 nm. The experiments were repeated three times at least.

6. Apoptosis evaluation

The apoptotic cells were quantified by assessing the characteristic nuclear changes of apoptosis using fluorescence microscopy (Eclipse TE200; Nikon Instruments, Melville, NY). The KB cells were seeded at a density 2 x 10^3 cells/well in a 4-well chamber slide and incubated overnight. pSuper-miR-203 (200 ng/ml) was transfected into the cultured KB cells using LipotectamineTM 2000 for 48 h. The cells were fixed with 1% paraformaldehyde and washed 3 times with phosphate buffered saline (PBS). For nuclear staining, KB cells were incubated with 4', 6'-diamidino-2-phenlyindole dihydrochloride (DAPI, Roche Diagnostics, Madison, WI) containing with at a concentration of 10 μg/mL for 15 min.

7. Quantization of apoptosis

For apoptosis analysis, cultured cells were harvested and washed by PBS. The cells were resuspended in a binding buffer (BD Biosciences, San Diego, CA).

Annexin V-fluorsecein isothiocyanate (FITC) and 7-amino-actinomycin D (7-AAD) were added to the cells, which were then incubated in the dark for 15 min, and then added and resuspended in 300 μl of the binding buffer. The cells were analyzed using a flow cytometry with a fluorescence-activated cell sorting Calibur system (Becton-Dickinson, San Jose, CA). Data analysis was performed using standard Cell Quest software (Becton-Dickinson, San Jose, CA).

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

To perform qPCR, total RNA was isolated from KB cells using TRIzol reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's protocol. Reverse transcription was carried out with 1 μg of total RNA and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega, Madison, WI). The expression levels of *Yes-1* (*Yes-1*-F, 5'- ATCCCGAGCAATTATGTAGCGCCT-3'; *Yes-1*-R, 5'-TCTCGAGGGATTTCCCAAGCATCT-3') and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*-F, 5'-TCAACAGCAACTCCCACTCTTCCA -3'; *GAPDH*-R, 5'-ACCCTGTTGCTGTAGCCGTATTCA-3') were measured by qPCR and visualized by DNA agarose gel electrophoresis. The differences in expression are presented as a histogram after densitometric analysis using a VersaDoc^{TM} imaging system (Biorad, Hercules, CA). GAPDH was used as an internal standard for data calibration. For calculation of differential gene expression, the $2^{-\triangle\triangle ct}$ formula was used.

9. Immunoblotting

To determine the level of activated *Yes-1* in the KB cells transfected with pSuper-miR-203, the proteins were extracted as previously described (Jiang et al., 2012). Briefly, the KB oral cancer cells were cultured in 60-cm dishes. After the

cells reached confluence, they were transfected with 200 ng/ml of pSuper-miR-203 for 48h. After incubation, the cells were washed twice with ice-cold PBS and lysed using a RIPA buffer for protein extraction according to the manufacturer's instructions. The total protein concentrations were determined using the Bradford Assay (BioRad, Hercules, CA). An equal amount of protein was resolved by 10% SDS-PAGE and transferred to PVDF membrane for immunoblotting. Membranes were blocked for 2 h in 5% non-fat dry milk in TBST. Membrane was probed with primary antibodies against *Yes-1* (cell signaling, Danvers, MA), β-actin(Santa Cruz Biotechnology, Dallas, TX) overnight at 4℃ and then incubated with secondary antibody for 2 h at room temperature. The immunoactivity was visualized using an enhanced chemiluminescent (ECL) system (Amersham Biosciences, Piscataway, NJ) and a single visual enhancer system (Pierce, Rockford, IL) to magnify the signal.

10. Luciferase assays

The 3'-UTR of *Yes-1* containing the predicted target sites of miR-203 were amplified from human genomic DNA by proof reading the Phusion High-Fidelity PCR master mix with the following primers: *Yes-1*-3'-UTR-F, 5'-CCGCTCGAGTTCAAGTAGCCTATTTTATATG-3';*Yes-1*-3'-UTR-R, 5'-ATAGTTTAGCGGCCGCTGAGTTCAATAATGTTCTGAAT-3'.ThePCRproductwasclonedinbetweentheNot1 and *Xho*1 sites of the psiCHECK™-2 Luciferase Reporter Vector (Promega, Madison, WI). The transformants were validated by restriction digestion and direct sequencing. PsiCHECK™-2-*Yes-1*-3'-UTR vector was co-transfected with miR-203 into KB cells, with psiCHECK™-2-vector as their control. Then, the cells were harvested and lysed for luciferase assays 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's protocol. The Renilla/firefly luciferase activity was calculated for each reaction.

11. Statistical analysis

The results are expressed as the mean ± SEM from at least three separate experiments. All statistical analyses were performed using Excel and SPSS software. The differences between the groups were determined statistically using a Student's test. Values of $p < 0.05$ were considered significant.

Ⅲ. RESULTS

1. The miR-203 expression was down-regulated significantly in human KB oral cancer cells

To identify KB-related miRNAs, we performed a miRNA microarray on the KB cells and NHOKs samples (n = 3 per cells) using an Affymetrix Genechip and the analysis method described previously (Unver et al., 2010). In this analysis, up- or down-regulated miRNA differentially expressed in the KB cells and NHOKs were identified (Fig. 1A). We focused on miR-203 because its expression was down-regulated significantly up to approximately 8 fold in KB cells, compared with NHOKs. The microRNA array result was verified by examining the expression pattern of miR-203 by miRNA qRT-PCR using the miRNA isolated from both KB cells and NHOKs (Fig. 1B). Analysis of qRT-PCR showed that miR-203 expression was significantly down-regulated in the KB cells by up to 90% compared to the NHOKs, as shown in Fig. 1C. These data indicate that down-regulation of miR-203 is clearly involved in human oral cancer cell development.

2. Over-expressed miR-203 inhibits proliferation and induces apoptosis of KB cell.

To determine whether over-expression of miR-203 affects cell proliferation of KB cells, the pSuper-miR-203 construct was generated to induce the over-expression of miR-203 in KB cells by transfecting with Lipofectamine™2000, left for 48 h. Cell viability was assessed using a MTT assay. As shown in Fig. 2A, the viability of

KB cells transfected with 200 ng/ml pSuper-miR-203 gradually decreased in a time-dependent manner, and was decreased by approximately 25% compared to cells treated with either the non-transfected or empty vector only. Moreover the cell viability of KB cells decreased with increasing concentration of pSuper-miR-203, as shown in Fig. 2B. By contrast, the up-regulation of miR-203 did not alter the viability of NHOKs (data not shown). These results indicate that over-expression of miR-203 reduces cell viability of KB cells, as shown in Fig. 2A, B. Cell morphology was also assessed by DAPI staining, which detects the nucleus condensation. The number of cells with typical morphology of apoptosis was increased under the over-expressing of miR-203 in KB cells at least 9 fold higher than either the un-transfected or empty vector cells, as shown in Fig. 2C. Otherwise, cells remained intact with no chromatin condensation in either the untransfected control or KB cells transfected with empty vector only. Furthermore, similar results were obtained by FACS analysis. FACS analysis for apoptosis was conducted using Annexin V-FITC-7-AAD dye, apoptotic markers. As shown in Fig. 2D, the proportion of total apoptotic cells was increased significantly by 31% in response to miR-203 transfection compared with those transfected with empty vector only. These data suggested that over-expression of miR-203 induced the apoptosis of KB oral cancer cells.

3. Over-expressed miR-203 regulates the expression of *Yes-1* in KB oral cancer cells.

Since miR-203 is down-regulated in KB oral cancer cells, a microarray was performed after transfecting the pSuper-miR-203 construct into KB cells to determine which specific genes are affected by miR-203 over-expression. As shown in Fig. 3, various potential suppressor genes, including Yippee-like 4 (Drosophila),

transcription factor 4, and interleukin 15, were significantly up-regulated by over-expression of miR-203 in KB cells. On the other hand, miR-203 over-expression down-regulates the expression of other potential oncogenic genes, such as ets homologous factor, deiodinase iodothyronine type Ⅱ, and *Yes-1*. As shown in Fig. 3, *Yes-1* decreased strongly by miR-203 in KB cells. *Yes-1* is part of the Src kinase family (Thomas et al., 1997), proto-tumorigenic function (Chan et al., 2012). Therefore, we hypothesized that over-expression of miR-203 might induced apoptosis via the down-regulation of *Yes-1* in KB cells. To further confirm that miR-203 inhibits *Yes-1* expression in KB cells, the pSuper-miR-203 constructs were transfected into KB cells and analyzed the mRNA and protein levels of *Yes-1*. As shown in Fig. 4A. B, both qPCR and qRT-PCR results showed that *Yes-1* was significantly decreased by approximately 50% in KB cells transfected with 200 ng/ml pSuper-miR-203 compared to untransfected control and empty vector only cells. In addition, the expression of *Yes-1* protein was notably decreased by miR-203, as shown in Fig. 4C. A histogram after densitometric analysis normalized to β-actin showed that the level of *Yes-1* protein expression was decreased compared to non-transfected control. Together, these results suggested that miR-203 decreased the expression of *Yes-1* indicating that it is one of its down-stream target genes in KB cells.

4. *Yes-1* is direct target gene of miR-203.

To determine the mechanisms of the miR-203-mediated down-regulation of *Yes-1* in KB cells, we performed a target gene scan using TargetScan (http://www.targetscan.org/) and miRBase (http://www.mirbase.org/) and found that *Yes-1* was one of predicted target gene. Furthermore, *Yes-1* has a potential complementary binding sequence, which is located from nucleotide 200 to 208 in its 3'-UTR, and it indicates that miR-203 would directly target this site. Therefore, we cloned the 2,791bp (full length) of *Yes-1* 3'-UTR into the firefly luciferase

based-psiCHECK-2 vector. In addition, a psiCHECK-2-empty vector was transfected into KB cells to assess the basal level of luciferase activity (Fig. 5A). As shown in Fig. 5B, the relative luciferase activity of the reporter which contained 3'-UTR of *Yes-1* was notably decreased in miR-203 by approximately 1.5 fold compared to control in KB cells. In contrast, psiCHECK-2-empty vector plus pSuper-miR-203, and psiCHECK-2-*Yes-1* plus pSuper-empty vector only were all similar with the basal level of luciferase activity. These results suggested that *Yes-1* is a novel and specific target gene of miR-203, and miR-203 could be one of its mechanisms for regulating *Yes-1* expression in KB cells.

Ⅳ. DISCUSSION

miRNAs play important roles as gene regulators and intensive research of their functions. Recently, miRNA studies have become increasingly important in human cancer research owing to their roles as oncogenes or tumor suppressors. However, the precise cellular mechanisms associated with the tumorigenesis or anti-oncogenesis functions of miRNAs remain largely shade. Recent studies have shown that the dys-regulation of miRNAs is closely associated with oncogenesis in various cancer cells. In particular, the down-regulation of tumor suppressor miRNAs such as miR-34a (Chim et al., 2010), miR-145 and miR-133a/b (Kano et al., 2010) enhanced the oncogenic effects in various cancer cells. Aberrant miR-203, acts as tumor suppressor, silencing has been reported in various tissues, such as oral cancer (Kozaki et al., 2008), hepatocellular carcinoma (Furuta et al., 2010). Moreover, Mathe et al. (2009) demonstrated that miR-203 expression was reduced in cancerous compared with noncancerous tissues. In addition to the previously reported study associated with the pathophysiological role of miR-203 in cancer cells, the expression of miR-203 was most significantly down-regulated in KB cells compared with NHOKs (normal tissues) in the present study (Fig. 1). Therefore, these data suggest that miR-203 is potentially clinically useful for developing prognostic biomarkers. Furthermore, these data also suggest that down-regulated expression of miR-203 might be closely associated with oral cancer progression.

Recently, miRNA research associated with cancer therapies has suggested that the over-expression of tumor suppressor miRNAs such as miR-124 (in gastric cancer) (Xie et al., 2014), miR-494-3p (in prostate cancer) (Shen et al., 2014), miR-23a/b (in lymphoma) (Li et al., 2013), and miR-205 (in oral cancer) (Kim et al., 2014) can inhibit proliferation and induce apoptosis of cancer cells. More recently, Tian et al. (2014) reported that over-expression of miR-203 could suppress proliferation and induce apoptosis of laryngeal squamous cell carcinoma. Therefore, based on our results and those of previous studies, we hypothesized that

the over-expression of miR-203 could induce apoptosis of KB cells through the alteration of cellular responses. As shown in the Fig. 2, cell viability was gradually decreased in KB cells transfected with miR-203 in both time- and dose-dependent manners. Furthermore, over-expressed miR-203 increased the extent of nuclear condensation, a representative typical apoptotic pattern, and increased the apoptotic population of KB cells, as shown by DAPI staining and FACS analysis, respectively. Likewise, previous studies (Kozaki et al., 2008; Yuan et al., 2011; Bo et al., 2011) also suggested that miR-203 might act as a tumor suppressor miRNA in oral cancer, esophageal squamous cell carcinoma, and bladder cancer, we further confirmed that miR-203 is a reasonable potent tumor suppressor miRNA in oral cancer cells. Although our studies would be needed to elucidate the detailed molecular biological mechanism associated with the miR-203-induced apoptosis of KB cells, Hemida et al. (2012) reported that anti-apoptotic factors, such as *Bcl-2* and *Bcl-xL*, were significantly expressed in HeLa cancer cells with over-expressed miR-203. In addition, Bo et al. (2011) reported that up-regulated miR-203 expression was inversely correlated with *Bcl-w* expression, which is a well-known anti-apoptotic gene, in bladder cancer cells.

miRNAs are believed to elicit their effects by base-pairing complement between the seed sequence of miRNAs and the 3'UTR of their target mRNAs, is the key determinant of miRNA-target recognition (Nahvi et al., 2009). Therefore, we performed the miR-203 target gene array to explore the potential molecular biological mechanism of miR-203-induced apoptosis in KB cells. The results of the miR-203 target gene array in KB cells revealed that over-expression of miR-203 altered the expression patterns of oncogene. Even though miR-203 induced the expression of some tumor suppressor genes, we focused on the down-regulated oncogenes in KB cells transfected with miR-203 because miRNAs usually suppress protein expression by affecting mRNA translation and/or destabilization through interaction with the 3'-UTR of their target mRNAs. As shown in Fig. 3, potential oncogenes, such as *Yes-1*, ets homologous factor *(EHF)*, and deiodinase iodothyronine type II (*DIO2*), were significantly down-regulated in KB cells

transfected with miR-203. *EHF* is a member of the ETS gene family, one of the largest families of transcriptional regulators and includes 30 distinct members (Oikawa and Yamada, 2003; Seth and Watson, 2005). Translocations, amplification and over-expression of ETS genes are found in leukemia, lymphomas and solid tumors (Seth and Watson, 2005). The oncogenic activity of ETS factors is attributed to their ability to induce expression of genes that promote cell proliferation and survival (Seth and Watson, 2005). As a result of its involvement in cellular processes, uncoordinated expression of EHF has the potential to contribute to tumor growth and consequently disease progression (Brenne et al., 2011). However, the biological function of *EHF* is largely unknown in cancer. DIO2 and DIO1 catalyze the conversion of thyroxine (T4) to the biologically active triiodothyronine (T3) (Gereben et al., 1999). It is well know that gradual or partial loss of the thyroid-specific differentiation markers are observed with increasing dedifferentiation from normal to well-differentiated thyroid carcinoma and undifferentiated thyroid carcinoma (Schmutzler et al., 2004; Schreck et al., 1994). However, its oncogenic activities remain largely unknown in various cancer cells. Although we are still conducting functional studies for both *EHF* and *DIO2* in KB cell, the expression of *Yes-1* was most significantly down-regulated by miR-203 in KB cells (Fig. 3). We focused on the miR-203-*Yes-1* suppression-apoptosis axis in the present study.

Yes-1 is a proto-oncogene tyrosine-protein kinase, and member of the Src kinase family (SFKs). A number of studies have linked increased expression of *Yes-1* in cancer with increased cell motility and tumor invasion (Barraclough et al., 2007; Kleber et al., 2008). Furthermore *Yes-1* activity was found to correlate with the predicted cancer risk based on size, histology, and degree of dysplasia (Pena et al., 1995). Several recent studies have uncovered evidence for miRNA-mediated regulation of SFKs. Gregersen et al., (2010) reported that miR-145 inhibits cell proliferation by directly targeting *Yes* and *Stat1*, which regulates tumorigenesis in colon cancer cells. miR-34a and miR-203 contributes to gastric cancer development by down-regulating of *Src*, and miR-205 inhibits *Src*-mediated oncogenic pathways

in renal cancer (Hao et al., 2013). In addition, Bo et al. (2011) reported that the up-regulated miR-203 expression was negatively correlated with *Bcl-w* expression, which is a well-known anti-apoptotic gene and a potential target gene of miR-203, in bladder cancer cells. Based on these results, we hypothesized that the miR-203 induced the apoptosis of KB cells would be related with the suppression of *Yes-1*. As shown in Fig. 4, both mRNA induction and protein expression of *Yes-1* were significantly suppressed by miR-203 in KB oral cancer cells. These present data suggest that *Yes-1* may be a target oncogene of the miR-203-induced apoptosis in KB oral cancer cells.

However, miRNAs are believed to elicit their effect by silencing the expression of the target genes via a direct interaction with their own regulatory region, such as the promoter (Fujita and Iba, 2008) and/or 3'-UTR (Didiano and Hobert, 2008; Nahvi et al., 2009). Therefore, to determine the potential miR-203 binding site in the *Yes-1* gene, we performed a target gene scan and identified a potential complementary binding sequences located from position 200 to 208 in its 3'-UTR as shown in Fig 5. Next, to verify whether miR-203 can suppress the expression of *Yes-1* in KB cells, we generated the psiCHECK-2-*Yes-1* construct, which contained the potential complementary binding sequences for miR-203 in the 3'-UTR of *Yes-1*. MiR-203 directly targets the 3'-UTR of *Yes-1*, as its over-expression was associated with suppression of luciferase activity (Fig. 5). In contrast, the over-expressed mimic miR-203 did not have any effect on *Yes-1* expression. These results suggest that miR-203 regulates the expression of *Yes-1* in KB oral cancer cells by down-regulating *Yes-1* directly via targeting its 3'-UTR.

In conclusion, the present study elucidated the function of miRNA-203 as a tumor suppressor associated with the suppression of *Yes-1* in KB oral cancer cells. Further analyses of the mechanism underlying regulation of cancer progression by miR-203 will contribute to the development of new cancer treatments and provide a new marker for the diagnosis of cancer in the future.

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Ⅵ. FIGURE LEGENDS

Fig. 1. MicroRNA array and qRT-PCR in KB cells compared with NHOK cells.

(A) Total RNA from both KB 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 three independent experiments. (B) The relative content of miR-203 in both NHOKs and KB cells were assessed by miRNA array using Affymetrix Genechip and Affymetrix software. (C) The relative expression of miR-203 was assessed by qRT-PCR, as described in the "Materials and Methods".

Fig. 2. Over-expressed miR-203 increased cell cytotoxicity via apoptotic cell death in KB oral cancer cells.

The measurement of KB cells after miR-203 transfection (A) over time and (B) across different concentrations. Cell cytotoxicity was assessed by MTT assay after transfection into KB cells following defined treatment condition. (C) The observation of apoptotic cell death was observed by DAPI staining after transfection of 200 ng/ml pSuper-miR-203. The numbers of apoptotic dying cells were counted and are presented as a

histogram. (D) The population of apoptotic cells was increased in KB oral cancer cells after miR-203 transfection.

Fig. 3. miR-203 target gene array in KB cells.

DNA samples isolated from KB cells with overexpressed 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 three independent experiments with varying SEM less than ± 15.9 %. EHF; ets homologous factor, DIO2; deiodinase iodothyronine type Ⅱ, YPEL4; yippee-like 4, TCF4; transcription factor 4, IL15; interleukin 15.

Fig. 4. Over-expressed miR-203 decreased expression of the oncogenic gene *Yes-1* in KB cells.

(A) The expression level of *Yes-1* was measured by qPCR after miR-203 transfection. The amplified PCR products of *Yes-1* were electophoresed on agarose gel and then presented as a histogram after densitometric analysis. (B) The expression level of *Yes-1* was assessed by qRT-PCR after miR-203 transfection into KB cells. (C) *Yes-1* protein expression was quantified by western blotting using *Yes-1* specific antibody.

Fig. 5. *Yes-1* is a direct target of miR-203 in KB oral cancer cells.

(A) The sequence of the miR-203 target sites on the *Yes-1*-3'UTR. (B) The luciferase activity of *Yes-1* promoter against miR-203. psiCHECK-2-*Yes-1* was cloned as previously described in "MATERIALS AND METHODS", Luciferase activity was normalized to total protein.

Ⅶ. FIGURES

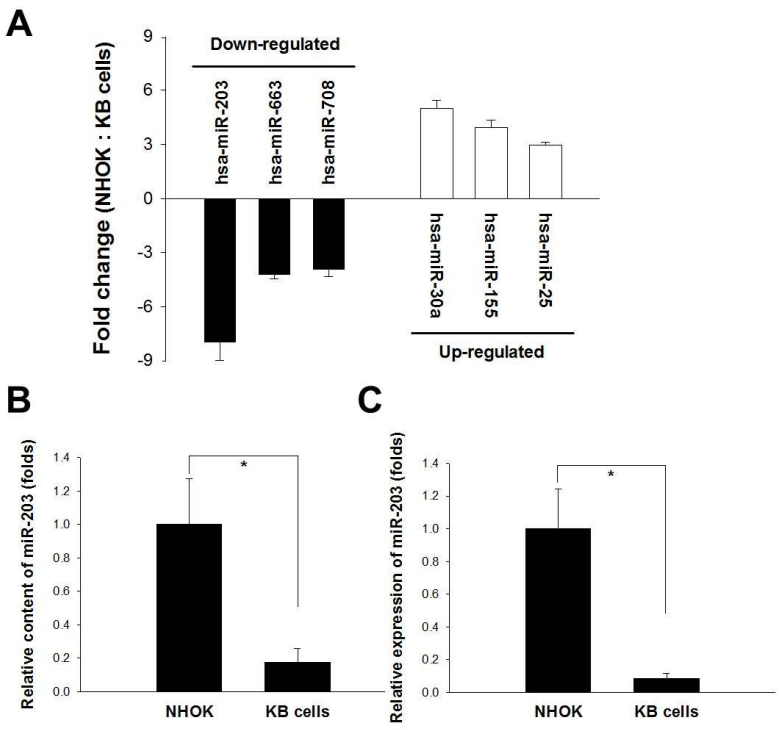

Fig. 1. MicroRNA array and qRT-PCR in KB cells compared with NHOK cells.

Ⅶ. FIGURES

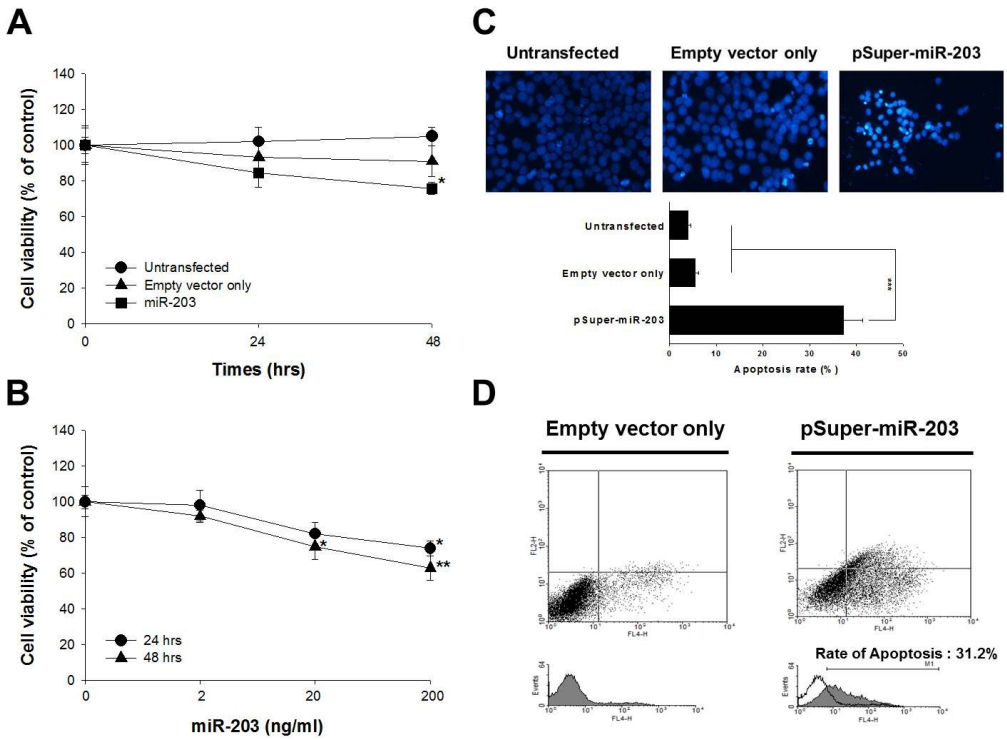

Fig. 2. Over-expressed miR-203 increased cell cytotoxicity via apoptotic cell death in KB oral cancer cells.

Ⅶ. FIGURES

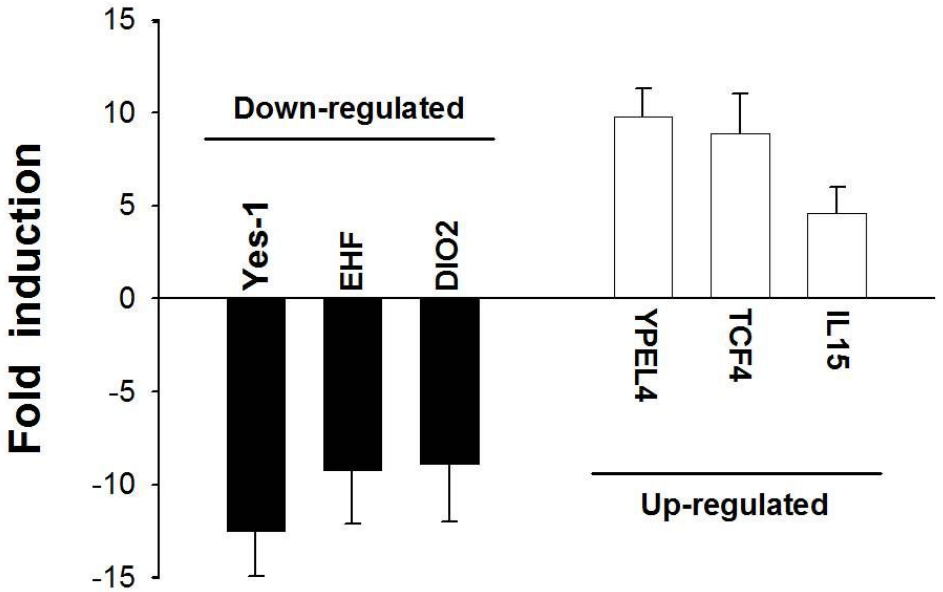

Fig. 3. miR-203 target gene array in KB cells.

Ⅶ. FIGURES

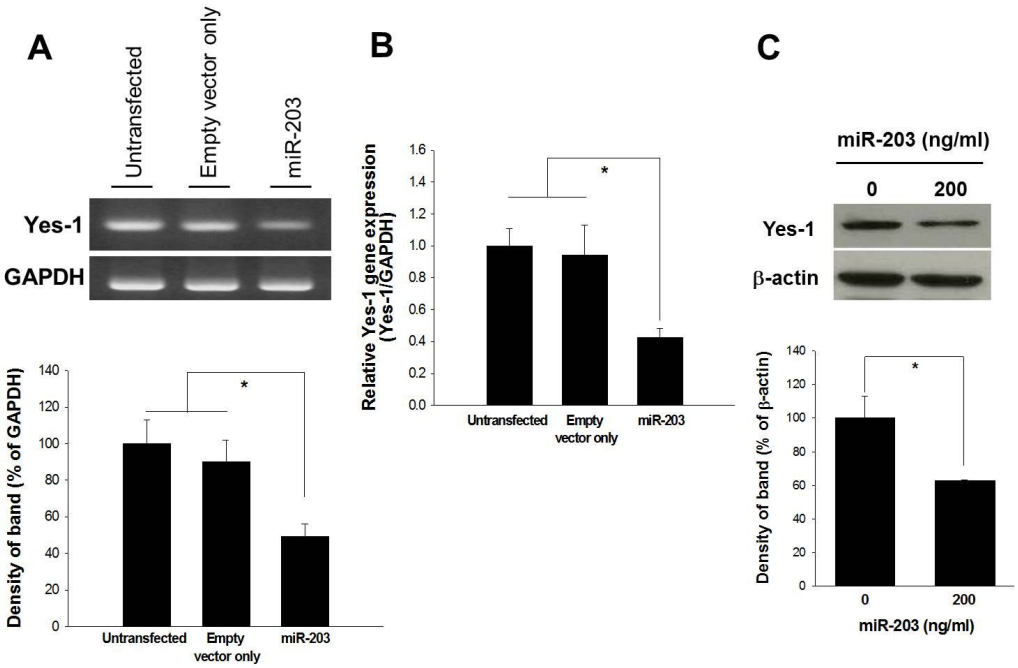

Fig. 4. Over-expressed miR-203 decreased expression of the oncogenic gene *Yes-1* in KB cells.

Ⅶ. FIGURES

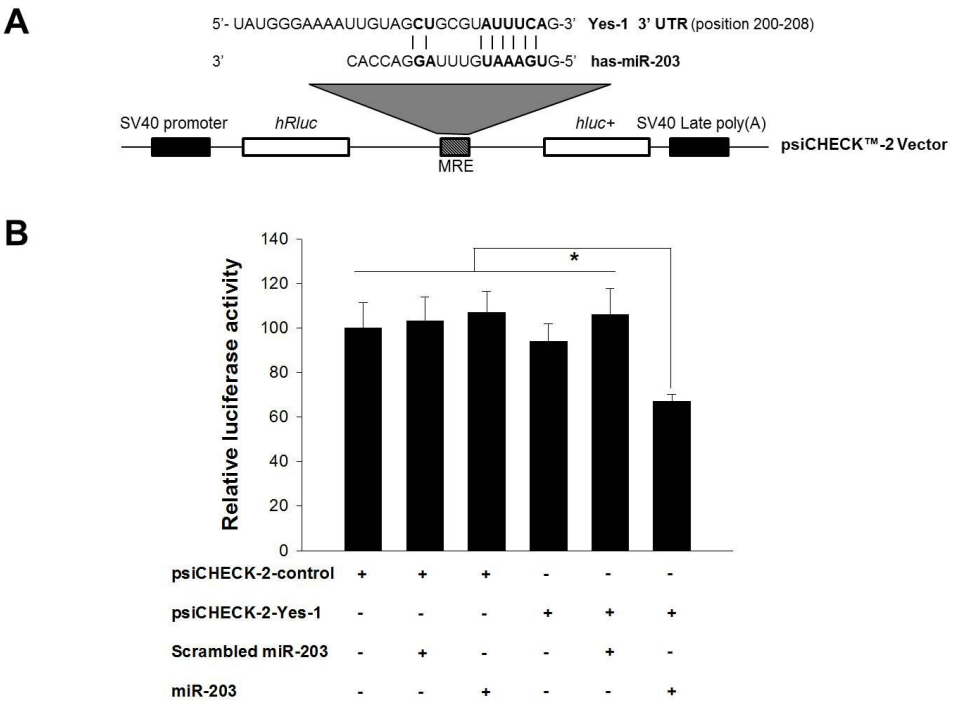

Fig. 5. *Yes-1* is a direct target of miR-203 in KB oral cancer cells.

ABSTRACT

MicroRNA-203 suppresses the oral carcinoma oncogenic acitivity via downregulation of *Yes-1*

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The purpose of this study was to elucidate the molecular mechanisms of microRNA-203 (miR-203) as a tumor suppressor in KB human oral cancer cells. MicroRNA microarray results showed that the expression of miR-203 was significantly down-regulated in KB cells compared with normal human oral keratinocytes. The viability of KB cells was decreased by miR-203 in the time- and dose-dependent manners. In addition, over-expressed miR-203 not only increased the nuclear condensation but also significantly increased the apoptotic population of KB cells. These results indicated that the over-expression of miR-203 induced apoptosis of KB cells. Furthermore, the target gene array analyses revealed that the expression of *Yes-1*, a member of the *Src* family kinases (SFKs), was significantly down-regulated by miR-203 in KB cells. Moreover, both the mRNA and protein levels of *Yes-1* were strongly reduced in KB cells transfected with miR-203. Therefore, these results indicated that *Yes-1* is predicted to be a potential

target gene of miR-203. Through a luciferase activity assay, miR-203 was confirmed to directly targets the *Yes-1* 3' untranslated region (UTR) to suppress gene expression. Therefore, our findings indicate that miR-203 induces the apoptosis of KB cells by directly targeting *Yes-1*, suggesting its application in anti-cancer therapeutics

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Keywords: miR-203, tumor suppressor, oral cancer cells, *Yes-1*, apoptosis