MicroRNA-205 Directly Regulates the Tumor Suppressor, Interleukin-24, in Human KB Oral Cancer Cells

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MicroRNA (miRNA) is a form of small noncoding RNA that regulates the expression of genes either by inhibiting mRNA translation or by inducing its degradation. Small microRNA play important roles in regulating a large number of cellular processes, including development, proliferation and apoptosis. This study examined the biological functions of miR-205 as a tumor suppressor in KB oral cancer cells. The results showed that miR-205 expression was significantly lower in KB oral cancer cells than in human normal oral keratinocytes. Furthermore, the miR-205 over-expressed in KB oral cancer cells increased the cell cytotoxicity and induced apoptosis through the activation of caspase-3/-7. The transfection of miR-205 into KB oral cancer cells strongly induced IL-24, a well known cytokine that acts as a tumor suppressor in a range of tumor tissues. In addition, miR-205 targeted the IL-24 promoter directly to induce gene expression. Overall, miR-205 has significant therapeutic potential to turn on silenced tumor suppressor genes by targeting them with miRNA.

INTRODUCTION

Oral cancer is the most frequently diagnosed malignant tumor with approximately 500,000 people being newly diagnosed annually worldwide (Chen et al., 2008; Warnakulasuriya, 2009). Pathophysiological studies have shown that the development of oral cancer is closely associated with environmental carcinogens, particularly smoking, alcohol and betel quid etc. (Elwood et al., 1984) Over the last 10 years, despite the improved treatments for oral cancer, only 50% of patients diagnosed with oral cancer survive more than 5 years. In addition, although oral cancer patients can be cured completely by effective clinical treatment, some patients still suffer from side effects and cosmetic problems that decrease their quality of life. Therefore, multi-clinical studies, including surgical excision, radiation ther-

apy and chemotherapy, have been designated and performed to treat oral cancer.

MicroRNA (miRNA) is an endogenous, nonprotein-coding sequence (Bonnet et al., 2004) that regulates the expression of genes, and affects approximately 60% of all human genes, either by inhibiting or inducing mRNA transcription (Majid et al., 2010). miRNA have been profiled according to their typical mechanism of transcriptional regulation in a variety of cells and cellular systems. Moreover, numerous cellular process of miRNA itself have been examined, including organism development (Carrington and Ambros, 2003), cellular differentiation (Bentwich, 2005), proliferation (Brennecke et al., 2003; Jiang et al., 2012) and apoptosis (Cho et al., 2009; Lynam-Lennon et al., 2009; Wang and Lee, 2009). In addition, many studies, including O'Hara et al. (2008), reported that alterations of miRNA have been associated with specific clinical phenotypes, such as disease progression or recurrence, development of metastases and postoperative survival. Therefore, current studies have focused on the utility of miRNA as diagnostic and prognostic tools as well as potential therapeutic targets. Although many studies related to miRNA are currently underway, their biological functions or cellular mechanisms are still largely unknown.

Recently, miscellaneous studies have examined the biological functions of microRNA-205 (miR-205) as a tumor suppressor in carcinogenesis. Yue et al. (2012) reported that miR-205 is a potential tumor suppressor in human glioblastoma cells by down-regulating the expression of VEGF-A via direct interactions with the putative miRNA-205 binding site at the 3'UTR site. As another mechanism of tumor suppression by miR-205, Dar et al. (2011) reported that miR-205 suppressed cell proliferation by regulating the E2F1 protein in melanoma cells. In addition, Matsushima et al. (2011) examined the role of miR-205 in inhibiting cellular invasion and migration in esophageal squamous carcinoma. Interestingly, Majid et al. (2010) showed that a new functional mechanism of miR-205 specifically activates tumor suppressor genes, such as interleukin-24 (IL-24) and interleukin-

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32 (IL-32), by targeting specific sites in their promoters.

IL-24 is a well known tumor suppressor gene in a variety of cancers, and the expression of IL-24 is decreased during carcinogenesis (Chada et al., 2004; Gopalan et al., 2008). Furthermore, IL-24 induces apoptosis directly, and has divergent anticancer properties, such as anti-angiogenic (Lebedeva et al., 2007), immunostimulatory (Caudell et al., 2002) and apoptosis-inducing properties (Gopalkrishnan et al., 2004; Gupta et al., 2006; Sauane et al., 2003). One study suggested that the over-expression of IL-24 in various carcinoma cells would arrest carcinogenesis and considered it to be an ideal candidate for anticancer therapy (Sauane et al., 2008).

The present study examined whether miR-205 is down-regulated significantly in oral cancer cells. The molecular mechanism of miR-205 mediated IL-24 up-regulation was also analyzed.

MATERIALS AND METHODS

Cell culture and transfection

Normal human oral keratinocytes (NHOKs) were purchased from ScienCell Research Laboratories (USA). The NHOKs were maintained in KGM and a supplementary growth factor bullet kit (Clonetics Corp., USA). The human oral squamous cell carcinoma cell line, KB, was obtained from the American Type Culture Collection (ATCC) and cultured according to the cell culture instructions provided. Briefly, KB cells were grown in MEM medium (Gibco, USA) containing 10% fetal bovine serum (Invitrogen, USA) at 37°C in an atmosphere containing 5% CO₂. The cells were harvested for RNA isolation, and whole cell extracts were subjected to Western blot analysis.

Affymetrix miRNA array and data pre-processing

Total RNA from both KB cells and NHOK were isolated with miRNeasy mini kit (Qiagen, USA) 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 (DNA link, Korea). Briefly, for each sample, total RNA were subjected to a tailing reaction (2.5 mM MnCl₂, ATP, Poly A Polymerase - incubation for 15 min at 37°C) followed by ligation of the biotinylated signal molecule to the target RNA sample (16Flash Tag ligation mix biotin, T4 DNA ligase - incubation for 30 min at RT) and adding of stop solution. Each sample was hybridized to a GeneChip® miRNA Array at 48°C and 60 rpm for 16 h then washed and stained on Fluidics Station 450 and scanned on a GeneChip® Scanner 3000 7G (Affymetrix). The image data were analyzed with the miRNA QC Tool software for quality control.

miRNA isolation, quantitative real-time PCR (qRT-PCR) and quantitative PCR (qPCR)

The total RNA, including mature miRNA, were isolated using a miRNeasy Mini kit (Qiagen, USA), and the RNA was quantified by spectrophotometry (Nanodro® ND-1000) according to the manufacturer's instructions. Reverse transcription of the miRNA was performed using a miScript Reverse Transcription kit (Qiagen, USA) starting from 1 µg of total RNA. TaqMan miRNA assays kits (ABI, USA) were used to examine the specific miRNA expression by qRT-PCR according to the manufacturer's protocol. The qRT-PCR results, which were recorded as threshold cycle numbers (Ct), were normalized against an internal control (U6 RNA), and the comparative threshold cycle method (^{ΔΔ}Ct) was used to determine the levels of miRNA expression. The

level of IL-24 (IL-24F, 5'-AAGCCTTCTGGGCTGTGAAAGACA-3'; IL-24R, 5'-TGGACAAGGTAACAG CTCTCA GCA-3') induction was measured by qRT-PCR. The deviations in the samples represent 3 separate experiments.

To perform qPCR, the total RNA was isolated using TRIzol reagent (Gibco BRL, USA) according to the manufacturer's instructions. Reverse transcription was carried out with 1 µg total RNA using the TernoScript RT-PCR system (Invitrogen, USA). The levels of IL-24 (IL-24F, 5'-CAAATGCAGATGGTT GTGCTCCCT-3'; IL-24R, 5'-TGGACAAGGTAACAGCTCTCA GCA-3') and GAPDH (GAPDH-F, 5'-TCAACAGCAACTCCCA CTCTTCCA-3'; GAPDH-R, 5'-ACCCTGTTGCTGTAGCCGTA TTCA-3') induction were measured by qPCR, and visualized by DNA agarose gel electrophoresis. The differences in expression are presented as a histogram after densitometry using a VersaDoc™ imaging system (Biorad, USA).

Identification of miR-205 target genes

DNA samples isolated from KB cells with overexpressed miR-205 were run on the DNET microarray (DNA Link, Korea) 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 was generated with Affymetrix GeneChip® Command console software and analyzed with the DMET Console software.

Construction of miRNA expression vector

The sequence of miR-205 was retrieved from the Ensemble database and mirbase. miR-205 was amplified from human genomic DNA. The primers used for miR-205 amplification were 5'-AGCAGATCTAAAGATCCTCAGACAATCCATGTGC TTCTTTGTCTTCATTCCACCGGAGTCTGTG-3' (forward) and 5'-GACCTCGAGTGTCTCAGCTCCATGCCTCCTGAACTTC ACTCC ACTGAAATCTGGTTGGGTATGAGACAGACTCCGG TGG (reverse). The PCR products were digested with *Bgl*II and *Xho*I. The digested PCR products after phenol extraction were inserted into the restriction enzyme site of pSuper vector to generate pSuper-miR205. The pSuper-miR-205 mutation, in which the binding site of miR-205 is mutated from 5'-UCCU UCAUCCACCGGAGUCUG-3' to 5'-UGUAGUCGACCACCG GAGUCUG-3' was generated using miR-205 mutation. The Forward primer, 5'-AGCAGATCTAAAGATCCTCAGACAATC CATGTGCTTCTCTTGTGTAGTC GACCACCGGAGTCTGTG-3' and Reverse primer, 5'-GACCTCGAGTGTCTCAGCTCCATGC CTCCTGAACTTCACTCCACTGAAATCTGGTTGGGTATGAG ACAGACTCCGGTGG-3' were used as the control-miR. The clones were confirmed by sequencing.

Cell viability assay

The cells were seeded at a density of 5 × 10³ cells/well in 96-well plates and allowed to attach to the well overnight. After incubation, the cultured cells were transfected using Lipofectamine™ 2000 (Invitrogen) as according to the manufacturer's instructions. The cells were incubated with pSuper-miR-205 at a defined concentration (2, 20, and 200 ng/ml) for 24 h at 37°C as a dose-dependent test. Furthermore, 200 ng/ml of pSuper-miR-205 was transfected into the KB cells and incubated for 24, 48 and 72 h, as a time-dependent test. After incubation under the defined conditions, the cells were incubated for a further 4 h in 20 µl of 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT). To dissolve the formazan transformed from MTT, the cells were resuspended in 150 µl di-

methyl 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, independently.

Quantization of apoptosis

The cells were plated in 12-well plates at a density of 1×10^5 cells per well and incubated overnight. pSuper-miR-205 mutation and pSuper-miR-205 were then transfected into the cultured KB cells using Lipofectamin 2000. Twenty-four hours later, apoptosis was quantified by assessing the characteristic nuclear changes in apoptosis (i.e., chromatin condensation and nuclear fragmentation) using fluorescence microscopy (Eclipse TE200; Nikon Instruments, USA) after DAPI (4',6'-diamidino-2-phenylindole dihydrochloride, Roche Diagnostics) staining at a concentration of 10 μ g/ml for 15 min.

Immunoblotting

To determine the level of activated caspase-3 and poly (ADP-ribose) polymerase (PARP) in the KB oral cancer cells transfected with pSuper-miR-205, the proteins were extracted, as described previously. Briefly, the KB oral cancer cells were cultured in 6-cm dishes. After the cells reached confluence, they were transfected with 200 ng/ml of pSuper-miR-205 or the same concentration of pSuper-scram-ble for 72 h. 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 a bicinchoninic acid (BCA) protein assay (Pierce, USA). An equal amount of protein was resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting, as described previously. The immunoreactivity was visualized using an ECL system (Amersham Biosciences, USA) and a Signal Visual Enhancer system (Pierce) was used to magnify the signal.

Caspase-3/7 activity assay

The apoptosis executioner caspase-3/7 activity was determined using the cell-permeable fluorogenic substrate, PhiPhiLux-G₁D₂ (OncoImmunin Inc., USA), according to the manufacturer's instructions.

Luciferase assays

A 555-bp promoter region of IL-24 (from -531 to +24) was obtained by PCR using the following primers: IL-24/555F, 5'-TCAGGTACCACTCCTCAACTTCTGGCCC-3'; IL-24/55R, 5'-AAGTCT AGACAGAAGTAAAGGTTTGCA-3'. The PCR product was cloned to the pGL3-basic Luciferase Reporter Vector (Promega, USA) by *KpnI* and *BglII* digestion. The forward and reversed clones were confirmed by sequencing. The luciferase assay was performed according to the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using StatView version 5.0 for Windows. A Student's *t*-test was used to compare the different groups. *P* values < 0.05 were considered significant.

RESULTS

miR-205 is significantly down-regulated in human oral cancer

To determine the expression pattern of miRNA in KB cells, a miRNA microarray was performed on the KB cells and NHOKs samples (*n* = 3 per each cells) using an Affymetrix Genechip

Fig. 1. MicroRNA array in KB cells compared with NHOK cells. Total RNA from both KB cells and NHOK were isolated with miR-Neasy mini kit 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 Plateform (DNA Link, Korea) and stained on Fluidics Station 450 and scanned on a GeneChip® Scanner3000 7G (Affymetrix). The image data were analyzed with the miRNA QC Tool software for quality control.

and the analysis method described previously (Unver et al., 2010). In this analysis, up-regulated and down-regulated miRNA differentially expressed in the KB cells and NHOKs were identified (Fig. 1). The miR-205 in KB cells was down-regulated significantly, up to approximately 7 fold, compared to the NHOKs. qRT-PCR and qPCR were performed as described previously to validate the miRNA microarray results. The level of miR-205 expression in the KB cells was reduced by up to 80% compared to the NHOKs, as shown as Fig. 2A. The microRNA array data was verified by examining the expression pattern of miR-205 by miRNA qRT-PCR using the miRNA isolated from both KB cells and NHOKs. The expression of miR-205 in KB cells was down-regulated by up to approximately 95% compared to the NHOKs (Fig. 2B). This shows that miR-205 was down-regulated significantly in the KB oral cancer cells as well as in other cancer cells, such as melanoma (Dar et al., 2011; Xu et al., 2012), bladder cancer (Wang et al., 2012), prostate carcinoma (Boll et al., 2012), endometrial cancer (Karaayvaz et al., 2012) and breast cancer (Adachi et al., 2011). These results suggest that over-expressed miR-205 would increase the level of apoptotic cell death in KB cells.

miR-205 induces cell apoptosis through the activation of caspase-3/-7 in human KB oral cancer cells

To test the hypothesis, the pSuper-miR-205 construct was generated to over-express the miR-205 and transfected into the cultured KB cells using Lipofectamin 2000 (Invitrogen, USA) according to the manufacturer's instructions. The level of cell viability at 200 ng/ml pSuper-miR-205 was monitored for 72 h. As shown in Fig. 3A (Upper panel), the transfected KB cells with 200 ng/ml pSuper-miR-205 showed approximately lower cell viability 40% (*p* < 0.05) compared to the untransfected and pSuper-miR-205 mutation. The up-regulated miR-205 did not alter the cell viability in the NHOKs (data not shown). Therefore, the viability of the oral KB oral cancer cells was monitored for 72 h after transfection with pSuper-miR-205 according to the dose. As shown in Fig. 3A (Lower panel), up-regulated miR-205 decreased the cell viability in a dose-dependent manner.

Fig. 2. miR-205 notably decreased in KB oral cancer cells compared with normal human oral keratinocyte. (A) The relative content of miR-205 in both NHOKs and KB cells were accessed by miRNA array using affymetrix Genechip and Affymetrix software. (B) The relative expression of miR-205 was accessed by qRT-PCR according to methods described in "Materials and Methods".

Fig. 3. Over-expressed miR-205 increased the cell cytotoxicity via apoptotic cell death in KB oral cancer cells. (A) The measurement of cell cytotoxicity by up-regulated miR-205 as time-dependent (upper panel) and dose-dependent manner (low panel). Cell cytotoxicity was performed by MTT assay after transfection into KB cells as following defined treatment condition. (B) The observation of apoptotic cell death was performed by DAPI staining after transfection of 200 ng/ml pSuper-miR-205 and 200 ng/ml pSuper-miR-205mutation. The number of apoptotic dying cells were counted and presented as histogram. (C) Apoptosis executioner caspase-3/7 activity was determined using the cell-permeable fluorogenic substrate PhiPhiLux-G1D2 (Oncolmmunin Inc., USA) according to the manufacturer's instructions.

The KB cells transfected with 200 ng/ml of pSuper-miR-205 exhibited 40% lower cell viability ($p < 0.05$), as shown in the upper panel data in Fig. 3A. The cell death induced by the up-regulated miR-205 in KB cells was examined further by DAPI staining, which detects the nuclear morphology. As shown in Fig. 3B, the number of cells with the typical morphology of cell death, such as condensed and/or fragmented nuclei, increased under the over-expressed miR-205. Cell death was not detected in either the untransfected or mock cells. The percentage of cell death is presented as a histogram. As shown in Fig. 3B (lower panel), and previous data, the level of cell death was at least 10 fold higher than either the untransfected or pSuper-miR-205mutation. To determine if miR-205 has proapoptotic properties, the level of caspase-3/7 activation was examined as a possible cell death mechanism in KB cells with up-regulated miR-205. As shown in Fig. 3C, the KB cells containing up-regulated miR-205 showed a marked increase in the number of caspase-3/7-positive apoptotic cells. Moreover, the typical

morphology of KB cells was lost and the number of KB cells was decreased significantly by the up-regulated miR-205 compared to the untransfected and pSuper-miR-205mutation. In contrast, neither untransfected nor pSuper-miR-205 mutation activated caspase-3/7 or altered the cell morphology compared to the KB cells with up-regulated miR-205. Therefore, up-regulated miR-205 induced oral cancer KB cell death through the activation of caspase-3/7. An immunoblotting assay for the expression of the apoptotic factors, such as PARP and cleaved caspase-3, was performed to confirm the miR-205-induced apoptosis in KB cells. As shown in Fig. 2C, overexpressed miR-205 significantly cleaved PARP more than the pSuper-miR-205 mutation. In addition, in the pSuper-miR-205 transfected cells, cleaved caspase-3 was expressed significantly more than the pSuper-miR-205 mutation. Therefore, overexpressed miR-205 induces the apoptosis of KB oral cancer cells.

Fig. 4. miR-205 downstream target gene array in KB cells. DNA samples isolated from KB cells with overexpressed miR-205 were run on the DNET microarray (DNA Link, Korea). Hybridized DMET arrays were washed and stained in the Affymetrix fluidic stations and scanned with the Affymetrix GeneChip® Scanner 3000 7G. Data was generated with Affymetrix GeneChip® Command console software and analyzed with the DMET Console software.

Overexpression of miR-250 promotes the expression of IL-24 directly in the KB oral cancer cells

A microarray was performed after transfecting the pSuper-miR-205 construct into the KB cells to determine which genes are affected by up-regulated miR-205. The over-expressed miR-205 induced IL-24 the strongest, which is a well known cytokine, an anti-cancer molecule and a strong inducer of cancer cell apoptosis (Fig. 4). Even though, overexpressed miR-205 induced the various potential apoptotic factor, such as Lipocalin 2, Interleukin-8, IL-24 is most consistently increased in KB cells. Furthermore, IL-24 is a well known apoptotic factor as tumor suppressor in various cancer cells (Hanna et al., 2012; Wu and Mo, 2009; Wu et al., 2009). Therefore, we hypothesized that the overexpression of miR-205 induces the apoptosis *via* the induction of IL-24 in KB cells. pSuper-miR-205 constructs were transfected into KB cells determine if miR-205 enhances IL-24 expression in KB cells. The level of IL-24 gene induction was assessed by qPCR and is presented as a histogram after den-

sitometric analysis of the IL-24 PCR product bands on the DNA gel image from electrophoresis. As shown in Fig. 5A, IL-24 expression increased approximately 2 fold ($p < 0.05$) and 3 fold ($p < 0.01$) with 20 ng/ml and 200 ng/ml of the pSuper-miR-205 constructs, respectively, compared to the non-transfected control. In addition, these qRT-PCR results showed that 200 ng/ml of the pSuper-miR-205 construct transfected into KB cells increased the induction of the IL-24 genes by at least 20 fold ($p < 0.01$) compared to the non-transfected control and pSuper-miR-205 mutation, which is similar to the qPCR results (Fig. 5B). Western blotting was performed with the specific IL-24 antibody after transfecting 200 ng/ml the pSuper-miR-205 constructs into KB cells to confirm the over-expressed miR-205-mediated expression of the IL-24 gene. The β -actin was used as an internal control. As shown in Fig. 3D, the protein expression of IL-24 was increased significantly by over-expressed miR-205 in the KB cells. In addition, the histogram after densitometric analysis normalized to β -actin showed that the level of IL-24 expression was approximately 9 fold ($p < 0.01$) compared to non-transfected control. Therefore, these results show that the over-expression of miR-205 promotes the expression of IL-24 in KB cells.

miR-205 interacts directly with the promoter target sequence of IL-24

To determine the mechanisms of miR-205 over-expression-mediated IL-24 expression, the 2-kb promoter of the IL-24 gene was scanned for sequences complementary to miR-205 using gene runner software. As shown in Fig. 6A, IL-24 promoter scanning analysis revealed a potential binding sequence located at position -151 in the IL-24 promoter. Therefore, a 555-bp fragment (from +24 to -531) of the IL-24 promoter containing the miR-205-binding site was cloned into the firefly luciferase-based pGL3-Basic vector (Promega, USA). pGL3-Basic-IL-24 was co-transfected with either a pSuper-miR-205 or pSuper-miR-205 mutation into the KB cells, and the luciferase activity was then measured. In the KB cells co-transfected with pGL3-Basic-IL-24 and pSuper-miR-205mutation, the luciferase activity was similar to the pGL3-control co-transfected with pSuper-miR-205mutation as a control (Fig. 6B). In contrast, the luciferase activity was approximately 5.5 times higher ($p < 0.01$) in the KB cells transfected with pGL3-Basic-IL-24 and co-pSuper-miR-205. This suggests that miR-205 interacts directly with the promoter target sequence of IL-24, and would be one of mechanism for regulating directly IL-24 expression in KB cells.

Fig. 5. Over-expressed miR-205 increased tumor suppressor gene, IL-24, KB oral cancer cells. (A) The expression level of IL-24 was measured by qPCR after pSuper-miR-205. The amplified PCR productions of IL-24 were electrophorized on agarose gel and then presented as histogram after densitometric analysis. (B) The expression level of IL-24 was accessed by qRT-PCR after transfection of pSuper-miR-205mutation and

pSuper-miR-205 into KB cells. And then data analyzed by calculating method described in "Materials and Methods". (C) The expressed IL-24 was quantified by Western blotting using IL-24 specific antibody.

Fig. 6. IL-24 is a direct target of miR-205 in KB oral cancer cells. (A) The sequences of the miR-205 target sites located at -131 bp is shown to the transcription start sites. (B) The luciferase activity of IL-24 promoter against miR-205. pGL3-basic-IL-24-555 bp was cloned as previously described in "Materials and Methods". Luciferase activity was normalized to total protein.

DISCUSSION

In recent years, the role of MicroRNA has been emphasized in cellular regulation, including development, proliferation and apoptosis. On the other hand, this study clearly identified the important role of miR-205 as a potential tumor suppressor in KB cells. Several research groups performing microRNA profiling reported that miR-205 was down-regulated or up-regulated in tumor tissues compared to normal tissues. Although Majid et al. (2010) reported a reduction of miR-205 in prostate cancer cells, there is still controversy regarding the expression of miR-205 in various tumor tissues. For example, Sempere et al. (2007) and Feber et al. (2008), reported that miR-205 was down-regulated significantly in breast and esophageal cancer, respectively. In contrast, Gottardo et al. (2007) and Iorio et al. (2009) reported that miR-205 was significantly up-regulated in kidney, bladder and ovarian cancers. This indicates that the observed changes in miR-205 expression are tissue-specific. On the other hand, in the present study, the expression of miR-205 was significantly lower in the KB cells than NHOKs (normal tissues). This indicates that the down-regulation of miR-205 might be used as a prognostic marker for oral cancer. Moreover, reduced miR-205 expression in the KB cells might be important for oral cancer progression. Therefore, it was hypothesized that the over-expression of miR-205 in KB cells would alter the tumor cell specific cellular responses. To test this hypothesis, miR-205 over-expression was induced in both KB cells and NHOKs. These results clearly show that the cell cytotoxicity was increased significantly by the over-expression of miR-205 in the KB cells only, not normal cells. This suggests that miR-205 is a reasonable potent tumor suppressor miRNA. Although, previous studies (Majid et al., 2010), (Song and Bu, 2009) and (Liu et al., 2012) also suggested that miR-205 might act as a tumor suppressor miRNA in the prostate cancer tissues, this is the

first report showing that miR-205 plays important roles as the chemotherapeutic effect in oral cancer cells. The DAPI staining data showed that chromatin condensation, a significant apoptotic signal, was detected in the KB oral cancer cells transfected with the miR-205 gene containing the over-expression recombinant construct. Nevertheless, further studies will be needed to elucidate the apoptosis induced by the over-expression of miR-205 in the KB cells, including terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) and the detection of the apoptotic factors. Majid et al. (2010) also performed DAPI staining to confirm the up-regulated miR-205 mediated apoptosis in a range of prostate cancer cell lines and observed the chromatin condensation within the dying prostate cancer cells by the over-expressed miR-205. Moreover, they reported that pro-apoptotic factors, such as Bcl2-antagonist (BAK), Bcl2-associated X protein (Bax) and BH3-interacting domain death agonist (Bid), were expressed significantly by the over-expressed miR-205 in the prostate cancer cells. In addition, KB oral cancer cells transfected with miR-205 over-expression vector expressed the caspase-3/-7 compared to both the untransfected control and pSuper-miR-205 mutation transfected. According to Majid et al. (2011), the miR-205-mediated induction of apoptosis in melanoma cancer cells was associated sequentially with the cleavage of caspase-3 and PARP and the release of cytochrome c from the mitochondria. Furthermore, the over-expression of miR-205 induced senescence in melanoma cells via the increase in SA-β-gal activity, which is a representative marker of senescence. On the other hand, the apoptosis signaling by miR-205 in KB oral cancer cells is still largely unknown. Therefore, further studies will be needed to determine the mechanism of apoptosis by the over-expressed miRNA-205.

miRNA 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). Some papers stated that miRNA can up-regulate the expression of their own target gene through an interaction with the regulatory region. For example, Majid et al. (2011) reported that miR-205 directly up-regulates the expression of IL-24 and IL-32, which are well known pro-apoptotic cytokines in various cancer cells. In addition, Bhatnagar et al. (2010) reported that the down-regulation of miR-205 conferred resistance to chemotherapy-induced apoptosis via the down-regulation of BCL2L2 encoded by Bcl-2, which is a well known anti-apoptotic gene and a potential target gene of miR-205, in prostate cancer cells. These reports suggest that miRNA might regulate the gene expression of their own target gene through either down-regulation or up-regulation. Therefore, a microRNA array was performed to profile the target genes of miR-205 in KB cells. According to the results of this microRNA array (data is not shown), the IL-24 gene in KB cells was up-regulated by most significantly by over-expressed miRNA-205. Although Majid et al. (2010) reported that both IL-24 and IL-32 were up-regulated significantly by over-expressed miR-205 in prostate cancer cells, such up-regulation of IL-32 was not observed in the KB cells in the present study. This was attributed to the tissue specific differences between prostate and oral cancer. On the other hand, these results suggest that IL-24 is the target gene of miR-205 in KB cells. IL-24, an IL-10-related cytokine, induced cancer-specific apoptosis through the generation of reactive oxygen species (ROS) and ceramide, which both cause endoplasmic reticulum stress and suppress protein translation (Gupta et al., 2006). Tian et al. (2012) reported that IL-24 induces Bcl-2 denitroxilation and ubiquitin-degradation in cancer specific apoptosis. Therefore, it was hypothesized that the miR-205 mediated apoptosis in KB cells would be mediated through the over-expression of IL-24. Interestingly, the IL-24 promoter was a direct target of miR-205. Several lines of evidence suggest that the gene induction was specific to miR-205 and the targeted promoter sites. Figure 5 shows the induction of IL-24 required for overexpressing miR-205. Furthermore, scrambled miR-205 had no impact on the expression of the IL-24 gene. Furthermore, the luciferase activity assay showed that overexpressed miR-205 activated the promoter of the IL-24 gene. In contrast, the overexpressed miR-205 mutation did not have any effect on IL-24 promoter activation. Therefore, the expression of IL-24 requires miR-205 by interacting directly with the promoter of IL-24. Similarly, Majid et al. (2010) reported that miR-205 targets the IL-24 promoter directly to induce gene expression. These results suggest that over-expressed miR-205 in KB cells induces tumor suppression by upregulating IL-24 directly by targeting its promoter binding site. Nevertheless, to understand the mechanism, further study will be needed to confirm the anticipated molecular components associated with miRNA-activated gene expression. In conclusion, this study elucidated the function of miRNA-205 as a tumor suppressor in up-regulating IL-24 by targeting specific sites in its promoter. miRNA-205 has significant therapeutic potential as a molecular medicine for the treatment of oral cancer by turning on silenced tumor suppressor genes by targeting with miRNA.

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