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Full Length Research Paper

miR-181a regulates multiple pathways in hypopharyngeal squamous cell carcinoma

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MicroRNAs (miRNAs) constitute a class of small non-coding RNAs that play essential roles in a variety of biological processes including apoptosis, proliferation and differentiation. Altered expression of miRNAs in head and neck cancer has been reported, but the consequences of aberrant expression of specific miRNAs on cancer pathways remain vague. Therefore, it is important to identify and elucidate pathways that may be regulated by specific miRNAs in this disease. miR-181a was up-regulated in head and neck versus normal tissues, thus pathway analysis was performed to profile the changes in the activities of ten signaling pathways relevant to cancer influenced by down-regulation of miR-181a. Expression of four pathway reporters were significantly increased (p53/DNA damage, TGFβ, MAPK/ERK and MAPK/JNK), while expression of two pathway reporters were decreased (Wnt and NFkB) upon miR-181a down-regulation. Notch, Myc/Max, hypoxia and cell cycle/pRB-E2F pathways were not significantly affected by miR-181a down-regulation. This study provides insight into the understanding of miRNA regulation on major cancer pathways.

Key words: MicroRNA, head and neck cancer, miR-181a, pathway analysis, luciferase assay, FaDu cell line, transfection, qRT-PCR.

INTRODUCTION

Head and neck cancer is the sixth most common cancer affecting men globally (Parkin et al., 2005) and it includes cancers that originated from nasopharynx, oropharynx, hypopharynx, larynx, oral cavity, lips, nose and paranasal sinus, thyroid, as well as salivary glands. A total of 2884 cases of head and neck cancer were reported in Peninsula Malaysia in the year 2006, with 34% of overall cases contributed by nasopharyngeal cancer. The world-wide five-year relative survival rate is less than 50%, which is much lower than other cancers, such as cancer of colorectal, cervix and breast origin and this has remained unchanged for more than three decades (Jemal et al., 2011). Poor prognosis regardless of advances in treat-ment can be attributed to late presentation, failure of advanced disease stage to respond to treatment, as well as lack of proper markers for screening. The understanding of the pathways underlying this cancer is expanding (Hunter et al., 2005) although there is still much to learn about the accurate mechanisms (Tran et al., 2010). Recently, non-coding genes such as microRNAs (miRNAs) are regarded as regulators in many molecular pathways underlying various cancers (Tran et al., 2010).

miRNAs are endogenous, small, non-coding RNAs of 17 to 25 nucleotides that regulate almost 30% of human genes by inducing mRNA cleavage or translational

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suppression at the post-transcriptional level (Bartel, 2009). Firstly discovered in *Caenorhabditis elegans*, its crucial involvement in various cancer-related cellular processes such as cell differentiation, cell division, apoptosis and cell cycle regulation (Miska, 2005) made this molecule worth studying in head and neck cancer. miRNAs paired with their target messenger RNA (mRNA) through imperfect pairing with the 3′ untranslated region (UTR) resulted in their destabilization or ribosomal blockade (Bushati and Cohen, 2007). At the moment, miRNAs deregulation is considered a hallmark of cancer (Calin and Croce, 2006), in which they can function as oncogenes or tumor suppressors (Croce, 2009). Current miRNAs database showed that 1,424 human miRNAs have been deposited in miRBase release 17.0 (http://www.mirbase.org).

Although, altered expression of miRNAs in head and neck cancer has been reported (Ramdas et al., 2009; Hui et al., 2010, Nurul-Syakima et al., 2011), their biological function remains largely unknown and the consequences of aberrant expression of specific miRNAs on cancer pathways remain unclear. Thus, one of the vital priorities is the need to identify and elucidate pathways that may be regulated by specific miRNAs in this disease. Previous global miRNA expression study showed that expression level of miR-181a is up-regulated in head and neck cancer tissue versus normal tissue (Nurul-Syakima et al., 2011). In addition, other miRNA studies in head and neck cancers also revealed the up-regulation of the miR-181 family (Kozaki et al., 2008; Wong et al., 2008; Cervigne et al., 2009). Furthermore, miR-181a up-regulation is also observed in carcinoma of breast (Calin and Croce, 2006) and ovary (Iorio et al., 2007). This up-regulation suggests that miR-181a is an oncogenic miRNA (oncomiR). On the contrary, miR-181a expression is down-regulated in glioma, suggesting its role as a tumor suppressor (Shi et al., 2008). Hence, this study aims to investigate and validate the effect of miR-181a down-regulation on major cancer pathways in head and neck cancer.

MATERIALS AND METHODS

Cell line and culture condition

The human hypopharyngeal FaDu (HTB-43) cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and was maintained with Minimal Essential Medium (MEM) with Earle's salt (Nacalai Tesque, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Nacalai Tesque, Tokyo, Japan) and 100 mg/L penicillin/streptomycin (Invitrogen, Carlsbad, California) in a 37°C incubator with humidified 5% CO_2 (Galaxy R+ CO_2 Incubator; RS Biotech Laboratory Equip-ment Ltd., Irvine, Scotland, UK).

miR-181a hairpin inhibitor transfection

The biological effects of miR-181a down-regulation was assessed by transfecting FaDu with human miRIDIAN miR-181a hairpin inhibitor and inhibitor negative controls (Dharmacon, Lafayette, CO) using DharmaFECT Transfection Reagent 1 (Dharmacon, Lafayette, CO) according to the manufacturer's instructions. A total of 7×10^4 cells were plated in 96 well plates a day prior to transfection and were transfected with 25 nM of miR-181a hairpin inhibitor or 25 nM negative control using 0.2 µL transfection reagent per well. The cells were incubated at 37°C with 5% CO_2.

Validation of miR-181a down-regulation using qRT-PCR

Real time reverse transcription polymerase chain reaction (qRT-PCR) was utilized to assess expression level of miR-181a after its down-regulation to validate its inhibition. After 72 h of transfection, cells were harvested and total RNA including miRNA was extracted using innuPREP microRNA Isolation Kit (Analytik Jena, Jena, Germany). The total RNAs were quantified using BioPhotometer (Eppendorf, Hamburg, Germany) and further analyzed by electrophoresis in a denaturing gel to assess the RNA integrity. qRT-PCR was performed using the NCode™ EXPRESS SYBR® GreenER™ miRNA qRT-PCR Kit Universal (Invitrogen, Carlsbad, CA, USA) with 10 pmol primers. RNU-48 was used as normalization control and reference gene. The cDNA was synthesized from 1 µg total RNA according to the manufacturer's protocol.

miR-181a-specific primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The qPCR reaction was run in triplicates using Rotorgene 6000 (Qiagen, Valencia, CA, USA) in a 20 µL total volume according to manufacturer's protocol with adjustment of annealing time to 15 s. The qRT-PCR amplification products were subsequently analyzed by melting curve analysis and confirmed by 1.8% agarose gel electrophoresis. A negative control without cDNA template was included to assess the specificity of the qPCR reaction. Fold change of miR-181a expression was analyzed using $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). This method was validated by using protocol described by Livak and Schmittgen (2001). Primer sequences for qRT-PCR reaction are summarized in Table 1.

Dual luciferase pathway reporter transfection

Pathway analysis was performed using the Cignal Finder 10 Pathway Reporter Arrays (SA Biosciences, Fredrick, MD) according to the manufacturer's instructions. Reverse transfection protocol was implemented. Briefly, a day before transfection, cells were passed 1:4 in a 25 cm^3 flasks and were incubated at 37°C with 5% CO_2 overnight. Then, 100 ng of each dual luciferase Cignal transcription factor-responsive reporter, negative and positive control constructs were diluted in serum-free MEM along with miR-181a hairpin inhibitor and inhibitor negative control. The diluted nucleic acids were mixed with the diluted 0.3 µL DharmaFECT 1 transfection reagent, delivered to 2×10^5 cells resuspended in Opti-MEM® (Invitrogen, Carlsbad, CA, USA) containing 5% FBS and 1% non-essential amino acids (NEAA; Invitrogen, Carlsbad, CA, USA) in a 96 well plate and was incubated in a 37°C incubator with 5% CO_2. After transfection for 24 h, culture medium was changed to the complete growth medium (MEM supplemented with 10% FBS, 0.1 mM NEAA, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin) and was further incubated for another 48 h. Each transfection condition was carried in triplicate.

Luciferase assay

After 72 h, cells were harvested and luciferase activity was measured using the Dual-Glo Luciferase Assay system (Promega, Madison, WI) on a Panomics luminometer (Panomics, Inc., Fremont, CA). The firefly/*Renilla* activity ratio generated from the transcription factor-responsive reporter transfections was divided by

Table 1. Sequence of qRT-PCR primers used in this study.

miRNA	Primer sequence (5'-3')	Size (bp)	Reference
RNU48	Forward: AGTGATGATGACCCCAGGTAACTC Reverse: CTGCGGTGATGGCATCAG	106	Goodarzi et al., 2010
miR-181a	Forward: AGGGCTATCAGGCCAGCCTTCA Reverse: ACCCCAAGGTACAGTCAACGGTCA	102	Primer-BLAST

the firefly/*Renilla* activity ratio generated from the negative control transfections to obtain the relative luciferase units. Subsequently, fold change of reporters' expression between miR-181a hairpin inhibitor transfectant and their negative control was calculated from the relative luciferase units.

Statistical analysis

The results are expressed as mean ± standard error (SE). Statistical analyses were performed with SPSS version 16 (SPSS, Chicago, Illinois, USA) and statistical significances were determined using the Student's t-test. A p-value <0.05 was considered statistically significant.

RESULTS

Validation of $2^{-\Delta\Delta Ct}$ method

The $2^{-\Delta\Delta Ct}$ method that was employed in this study demands for validation before being used in qRT-PCR analysis (Livak and Schmittgen, 2001). In validation of $2^{-\Delta\Delta Ct}$ method, primers' efficiencies were determined and a plot of the log cDNA dilution versus ΔCt was made for each primer set. qRT-PCR was performed on 10-log dilution series of cDNA and a standard curve was generated in Rotor-Gene 6000 Series software for each primer pairs (Figure 1A and B). Data were then exported to Excel 2007 (Microsoft, Seattle, WA) and ΔCt was calculated ($Ct_{miR-181a} - Ct_{RNU-48}$) for each cDNA dilution. Graph log cDNA dilution versus ΔCt was plotted and the slope of the line was determined. As shown in Figure 1C, the absolute value of the slope (R^2) was close to zero, concluding that the efficiencies of the target and reference gene are equal. Thus, the ΔΔCt calculation for the relative quantification of miR-181a can be used to analyze the qRT-PCR data.

qRT-PCR validation of miR-181a down-regulation

Expression level of miR-181a after transfection with its inhibitor was assessed by qRT-PCR and was compared to the negative control. After calculating fold change of expression using $2^{-\Delta\Delta Ct}$, there was ~22 fold reduction of miR-181a expression in cells transfected with 25 nM miR-181a hairpin inhibitor compared to negative control (Figure 2).

miR-181a down-regulation resulted in various cancer pathways alteration

To determine the signaling pathways induced upon miR-181a down-regulation, a transcription factors array consisting of 10 dual-luciferase reporters assays were used. Each of the reporters encodes for an inducible transcription factor responsive firefly luciferase reporter and constitutively expressing *Renilla* construct in 40:1 ratio. Experimental reporter was represented by firefly luciferase, while normalization reporter was represented by *Renilla* luciferase. Student t-test showed that there were six pathways significantly affected by miR-181a down-regulation (Table 2). As summarized in Figure 3, expression of four pathway reporters (p53/DNA damage, TGFβ, MAPK/ERK and MAPK/JNK) were increased, while two pathway reporters (Wnt and NFkB) were decreased upon transfection. Notch, Myc/Max, hypoxia and cell cycle/pRB-E2F pathways were not significantly affected by miR-181a down-regulation.

DISCUSSION

Being a master regulator of gene expression and cellular pathways, miRNAs are considered to possess great therapeutic potential (Nelson and Weiss, 2008). miRNAs deregulation was revealed in different disease conditions including head and neck cancer. Inhibition of a single miRNA can result in an enormous effect within a cell and can significantly modify the cellular phenotype due to its ability to target several genes directly and indirectly (Visone and Croce, 2009). Hence, miRNA down- or up-regulation can be a key factor in the treatment of human diseases (Liu et al., 2008). However, many aspect need to be clarified before a miRNA can serve as a therapeutic agent, including understanding of the molecular pathway underlying disease progression. Thus, this study aimed to investigate the effect of miR-181a down-regulation on major cancer pathways in head and neck cancer. To identify the major pathways that are affected by this miRNA, a reporter assay was performed and p53/DNA damage, TGFβ, MAPK/ERK, MAPK/JNK, Wnt and NFκB pathways were found to be significantly influenced by miR-181a down-regulation. These pathways play important roles in head and neck carcinogenesis (Blons and Laurent-Puig, 2003; Uraguchi et al., 2004; Levy and Hill,

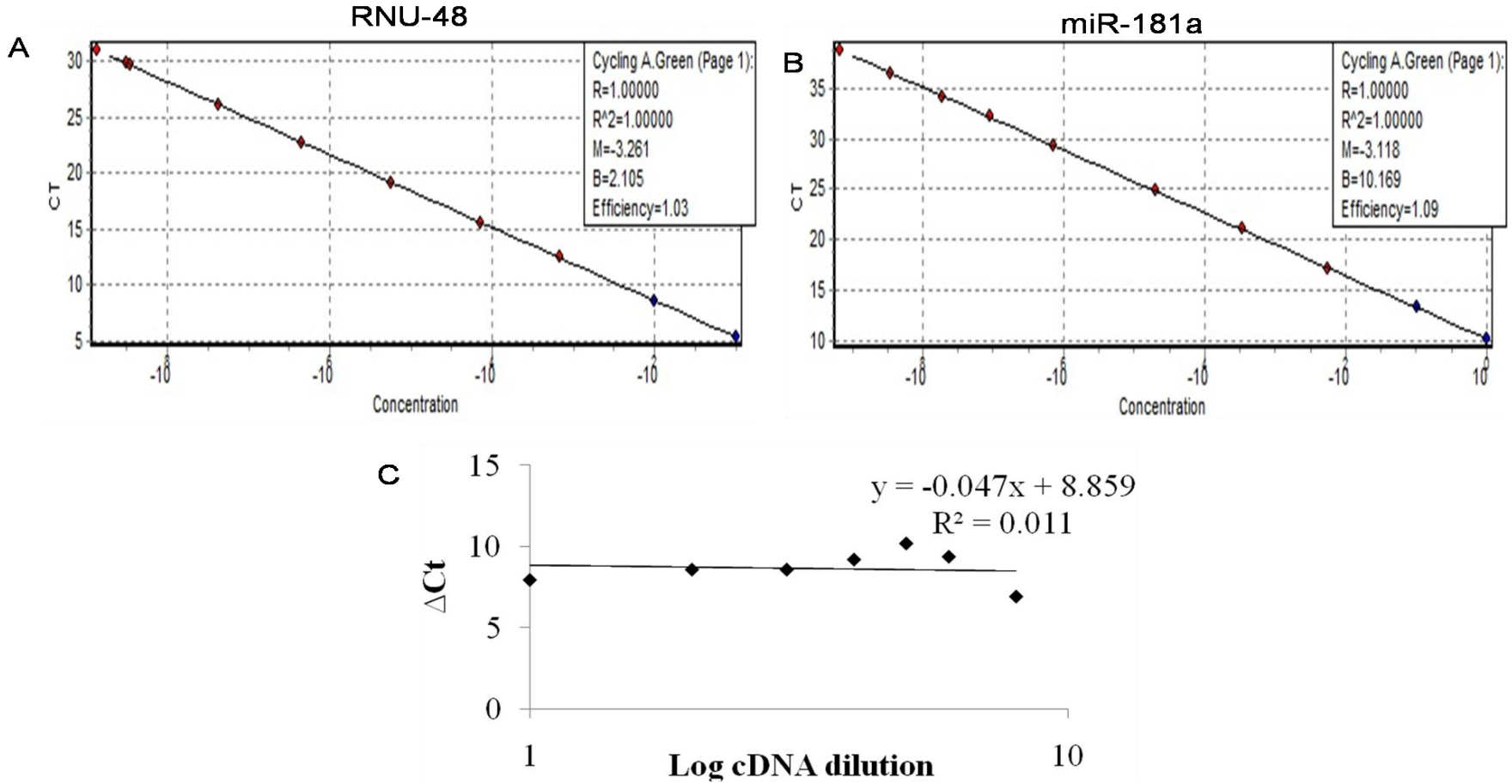

Figure 1. $2^{-\Delta\Delta Ct}$ validation. (A) and (B), Efficiency graph of each primer pairs used in qRT-PCR. Serial dilutions of cDNA were amplified by qRT-PCR using miRNA specific primers. Good efficiency is denoted by value of 90 to 110% (0.9 to 1.1) with slope of the standard curve (M) approximately -3.3 (± 0.1) and $R^2 > 0.99$. R^2 is a statistical term that shows how good one value is at predicting another and its value of >0.99 provides good confidence in correlating two values. (C) cDNA was synthesized from 1 µg total. 10-log dilutions were performed and were amplified by qRT-PCR. ΔCt was determined and plotted against log cDNA dilutions. These data were fitted using least-squares linear regression analysis and observed for R^2 value almost equal to zero.

2006; Squarize et al., 2006; Stadler et al., 2008). One of the most frequent alterations in head and neck cancer is the disruption of the p53 pathway through mutations, losses of heterozygosity (LOH) or interaction with viral proteins such as human papilloma virus (HPV) (Blons and Laurent-Puig, 2003). The p53 protein is involved in the repairs of the cellular integrity after DNA damage and when activated, the p53 protein can induce growth arrest and cell death (Nylander et al., 2000). miR-181a is highly expressed in head and neck cancer versus normal tissues (Nurul-Syakima et al., 2011), thus, this miRNA is an oncomiR candidate. Inhibition or down-regulation of miR-181a expression might have therapeutic potential as this study showed that

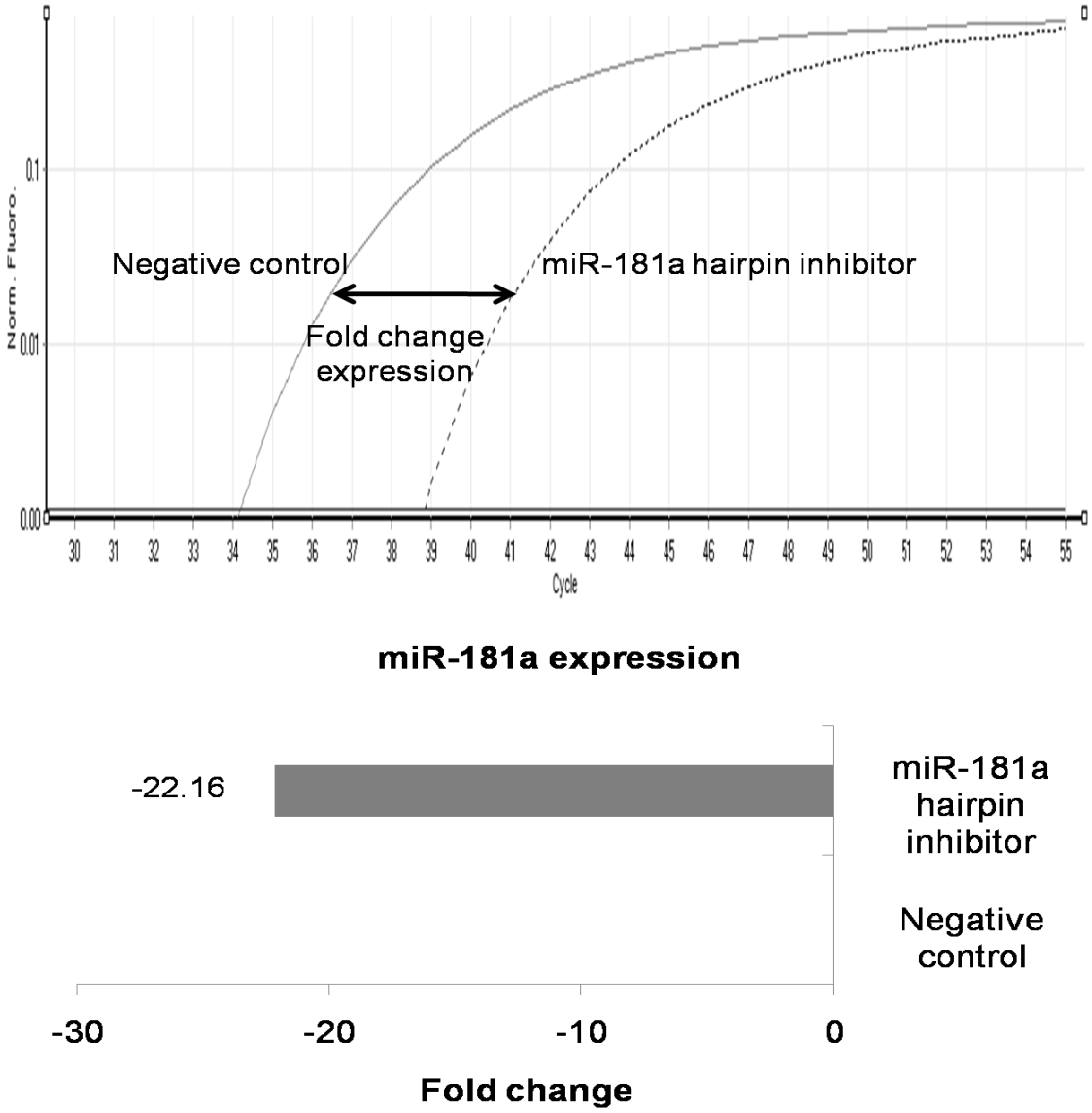

Figure 2. Expression level of miR-181a in cells treated with miR-181a hairpin inhibitor and negative control. miR-181a expression was reduced by 22.16-fold compared to negative control.

Table 2. Ten cancer pathways involved in this study.

Pathway	Transcription factor	Expression	Fold change	p-value
Wnt	TCF/LEF	↓	0.262	0.012
Notch	RBP-Jκ	-	1.276	0.48
p53/DNA damage	p53	↑	3.727	0.023
TGFβ	SMAD2/3/4	↑	3.057	0.034
Cell cycle/pRB-E2F	E2F/DP1	-	0.509	0.13
NFκB	NFκB	↓	0.551	0.041
Myc/Max	Myc/Max	-	0.54	0.345
Hypoxia	HIF1A	-	0.607	0.472
MAPK/ERK	Elk-1/SRF	↑	3.441	0.001
MAPK/JNK	AP-1	↑	1.742	0.006

Significance value (p) was determined by Student's t-test. $p<0.05$.

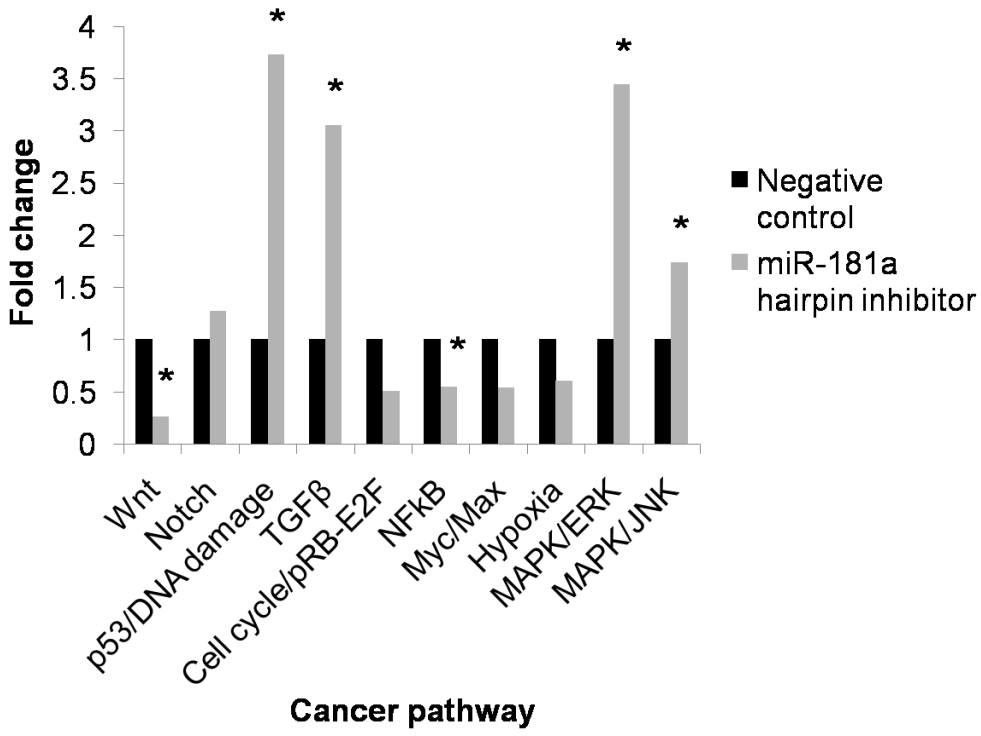

Figure 3. Pathway reporter assay of key response elements in 10 major cancer-related pathways. The ratio of reporter signals between cells treated with miR-181a hairpin inhibitor and negative control is shown as increased or decreased fold change. * indicates $p<0.05$.

miR-181a down-regulation led to increased expression of p53 transcription factor. In the past few years, solid connections between the miR-34 family and p53 tumor suppressor network in the regulation of apoptosis have been demonstrated (He et al., 2007; Hermeking, 2007). More recent in-depth study showed that the down-regulation of miR-34a expression in the livers of methyl-deficient rats could contribute to a carcinogenic process by inhibition of the p53 pathway, thus compromising the cellular apoptotic program (Tryndyak et al., 2009). In combination with the current study, these findings provided evidence of miRNA involvement in p53 pathway.

Generally, transforming growth factor beta (TGFβ) signaling pathway regulates tumor development via a tumor cell-autonomous mechanism or through tumor–stroma interaction, and has the ability to suppress or promote tumor depend on cellular context (Ikushima and Miyazono, 2010). Alteration in TGFβ signaling is central to tumorigenesis and tumor progression by affecting the cellular process including cell proliferation and cell invasion (Blobe et al., 2000). Absence or decreased SMAD4 expression and the signal transducers of the TGFβ family signaling cascade (Hata and Davis, 2009) has been found in various cancers, including pancreatic, colorectal and head and neck cancer (Bornstein et al., 2009). Interestingly, the SMADs also play a regulatory role in the processing of miRNA in the nucleus. miRNA involvement in TGFβ signaling pathway was demonstrated by Kong et al. (2008). TGFβ was found to induce miR-155 expression and promoter activity through SMAD4, while knockdown of miR-155 suppressed TGFβ-induced epithelial-mesenchymal transition (EMT), tight junction dissolution, cell migration and invasion in breast cancer (Kong et al., 2008). In addition, miR-106b and miR-93 impair TGFβ-induced cell cycle arrest while miR-25 cooperates with miR-106b and miR-93 in preventing the onset of TGFβ-induced apoptosis in gastric cancer (Petrocca et al., 2008). In this study, miR-181 down-regulation resulted in increased expression of TGFβ transcription factors (SMAD2/3/4). Since SMADs expression was reduced in cancer, their increased expression upon miR-181a down-regulation in this study was probably due to inhibition of cancer cell growth.

Mitogen-activated protein kinase (MAPK) signaling pathways are involved in the regulation of gene expression, mitosis, proliferation, motility, metabolism and apoptosis (Werlen et al., 2003). Conventional MAPKs comprised of three family members: the extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38-MAPK (Wada and Penninger, 2004). The MAPK/ERK pathway is activated in response to many mitogens and generally regulates cell proliferation and cell cycle (Schmidt et al., 2000). Due to its ability to promote proliferation and increase cell survival, Balmanno and Cook (2009) have proposed an oncogenic property for MAPK/ERK. Conversely, other study proved that

activation of MAPK/ERK pathway resulted in the promotion of apoptosis, suggestive of an anti-proliferative role (Martin and Pognonec, 2010). JNKs (also known as stress-activated protein kinases; SAPKs) are ubiquitously expressed and the MAPK/JNK pathway participates in many diverse intracellular signaling pathways that control various cellular processes including cell growth, differentiation, transformation or apoptosis (Johnson and Lapadat, 2001). MAPK/JNK activation generally leads to apoptosis promotion; however, like MAPK/ERK, in certain conditions it can also support cell survival or tumor progression (Katz et al., 2007). In this study, the signals of MAPK/ERK and MAPK/JNK reporters were both significantly increased after miR-181a down-regulation, thus suggesting that they were activated upon inhibition of miR-181a in head and neck cancer cell line. These findings support tumor suppressive ability of the two pathways. However, due to their dual potential in cancer progression, further investigation is required. Among other miRNAs that are found to be involved in MAPK signaling pathways are miR-199* (Kim et al., 2008), miR-21 (Thum et al., 2008), miR-221/miR-222 (Terasawa et al., 2009) and miR-34a (Ichimura et al., 2010).

Wnt signaling commences proliferation, dedifferentiation and EMTs in various types of carcinoma cells (Pannone et al., 2010). Increased Wnt expression and signaling hasten the progression of carcinomas via activating EMTs and local invasiveness (Uraguchi et al., 2004). Therefore, therapeutic inhibition of Wnt and its signaling pathway serve as a valuable approach to stop the progress of head and neck progression (Molinolo et al., 2009). Decreased expression of Wnt pathway reporter was observed in this study after down-regulation of miR-181a, suggesting its potential usage in head and neck cancer therapy. Other miRNAs that appear to regulate Wnt signaling pathway includes miR-8 and miR-27. The miR-8 family members function as a negative regulator of Wnt signaling through their evolutionarily conserved role in regulating the pathway (Kennell et al., 2008). Meanwhile, Wang and Xu (2010) found out that miR-27 promotes osteoblast differentiation by modulating Wnt signaling, thus offering a novel target for the development of preventive or therapeutic agents against osteogenic disorders.

Nuclear factor κB (NFκB) pathway represents a cascade responsible for cell growth and maintenance of cellular integrity (Yamamoto and Gaynor, 2001) by protecting the cells against xenobiotic injury, as well as ensuring ample nutrition and oxygenation via angiogenesis (Kato et al., 2000; Xiong et al., 2004). Many evidences implicate the NFκB pathway in the pathology of head and neck cancer (Tamatani et al., 2001; Zhang et al., 2005). In a study done by Lun and colleagues, constitutively activated NFκB pathway promotes tumor cell growth (Lun et al., 2005). Their findings imply that therapies that incorporate inhibitors of the NFκB pathway are worth considering. In this study, down-regulation of miR-181a in head and neck cancer cell lines showed inhibitory effect against NFκB pathway, further strengthening the therapeutic value of this miRNA. NFκB pathway is also found to be regulated by miR-146a (Taganov et al., 2006), miR-221/222 (Galardi et al., 2011) and miR-27b* (Thulasingam et al., 2011).

As disease-specific miRNAs are identified, the validation of targets within a disease pathway of interest may lead to novel therapeutic strategies. This study provided insights of the influence of miR-181a on major cancer pathways in a head and neck cancer cell line model. However, more researches are warranted in order to gain clear understanding on its regulation in this cancer. Due to its short size (17 to 25 nucleotides), a miRNA can have several binding site on the target gene. More also, different target binding sites may have different effect on its regulation, thus it is important to validate miRNA/mRNA target pairs. Finally, utilization of animal model to study the effect of miRNA down-regulation is essential in order to elucidate its therapeutic potential *in vivo*.

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