Differential expression of microRNAs in development of GH-secreting pituitary adenoma

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Differential expression of microRNAs in development of GH-secreting pituitary adenoma

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The Doctoral Dissertation
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

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JUNE 2017

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JUNE 2017

ACKNOWLEDGEMENTS

I am deeply indebted to my supervisor Professor Eun Jig Lee for his kind help, guidance, support and encouragement throughout my study. This study would not have been possible without his assistance and cooperation. I would like to express my sincere gratitude to my reviewers, Professor Young Suk Jo, Cheol Ryong Ku, Sang Kil Lee, and Sahng Wook Park who had the patience and fortitude to read my thesis and provided constructive criticism to help me defend it. Their guidance not only improved my thesis but also will benefit my future work. I am especially grateful to my laboratory colleagues for their assorted efforts and supports.

I sincerely appreciate my parents providing their endless support and unwavering love. Their patience and supports have been constant during work on this thesis. They always play for my side and strengthen my will to achieve my ambition.

Finally, I give all glory to God who is the reason that I live.

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ABSTRACT

Differential expression of microRNAs in development of GH-secreting pituitary adenoma

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Recent studies have suggested that aberrant microRNA (miRNA) expression profiles are associated with tumor formation, migration, and invasion. However, the role of miRNA in the development of pituitary adenoma is limited. Herein, we analyzed the different miRNA expression profiles during the cascade of pituitary tumorigenesis, using of somatotroph-specific aryl hydrocarbon receptor interacting protein (AIP) knock-out (sAIPKO) mouse model.

To explore possible oncogenic factors in sAIPKO, we used a miRNA microarray to profile changes in the expression of miRNAs. Top/significantly deregulated miRNAs were analyzed from 4 to 50 wks of pituitary adenoma progression (hyperplasia to tumor) by quantitative

realtime-polymerase chain reaction (qRT-PCR) analyses. Candidate miRNAs were further validated in human tissue samples which had no *AIP* gene mutation. Clinical indicators for growth hormone (GH)-secreting pituitary adenoma were analyzed according to the different expression profile of each miRNAs, including basal and glucose-suppressed nadir GH, insulin-like Growth Factor 1 (IGF-1), tumor size, invasiveness, and Ki-67 labeling index. Target mRNAs of candidate miRNAs were also verified in GH3 cells and sAIPKO model.

Fourteen miRNAs were significantly changed during GH-secreting pituitary tumorigenesis in sAIPKO mice. Through analysis of qRT-PCR using sAIPKO model during the mouse pituitary adenoma progression and their contemporary littermate animals, seven miRNAs that showed similar trends to miRNA microarray. miR-216a-5p and miR-652-3p were selected from those candidate miRNAs using human GH-secreting pituitary adenoma tissues. Direct target genes of candidate miRNAs were predicted and their expression regulation by binding miRNA was also verified in GH3 cells and sAIPKO mouse model. Finally, the effect of miR-216a-5p and miR-652-3p on proliferation and invasion in GH3 cells was observed. In conclusion, it is strongly suggested that miR-21a-5p and miR-652-3p may regulate GH-secreting pituitary adenoma tumorigenesis. Future studies will focus on their utilizing possibility as therapeutic targets in a larger number of human GH-secreting pituitary adenoma tissues.

Key words: GH-secreting pituitary adenoma, microRNA microarray, miR-216a-5p, miR-652-3p

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I. INTRODUCTION

Pituitary adenomas (PAs) are common benign tumors making up approximately 15% of all intracranial tumors[1] and two-thirds of all PAs are functioning PAs. Functioning PAs cause dysregulated secretion of anterior pituitary hormones. Especially, GH-secreting PAs correspond to 20% of all PAs causing acromegaly, which is a chronic endocrine disorder results from hypersecretion of Growth Hormone (GH). Tumorigenesis of PAs are generally considered a model of multicausal process of carcinogenesis including genetic alterations, endocrine factors, and somatic mutations.[2]

Recently, epigenetic events such as hypermethylation or microRNA (miRNA)-dependent dysregulation of protein, are likely to be related to the impairment of gene or protein expression related to pituitary tumorigenesis.

MiRNAs are small noncoding RNAs composed of 20 to 30 nucleotides found in the genomes of animals, plants, and protozoa.[3] They bind to 3' untranslated regions of target mRNAs, resulting in the block of translation of mRNA or degradation.[4] Due to these features, they play roles in cell differentiation, cell proliferation, and cell death.[5] Many studies have demonstrated that abnormal expression of certain miRNAs in different types of human cancers suggesting that they play an important role in tumorigenesis.[6] However, most of the miRNA step-wise variations in the onset and progression of pituitary adenomas are unknown, because of the lack of human tissue samples in the early stages of pituitary adenoma. We established an *in vivo* system that covers alteration of miRNA expression profiles in serial cascade for tumorigenesis (hyperplasia to tumor).

In this study, we established the miRNAs involving GH-secreting pituitary tumorigenesis by comparing the miRNAs of the pituitary gland in control, hyperplasia and tumorous condition in mice models we previously developed,[7] somatotroph-specific aryl hydrocarbon receptor interacting protein (AIP) knock-out (sAIPKO) mouse model. Using this model, we investigated the expression profile of miRNAs at various stages of pituitary adenoma progression. Revealing stage-specific expression of miRNAs in sAIPKO model will help to develop not only early diagnosis markers, but also therapeutic strategies for GH-secreting pituitary adenomas.

II. MATERIALS AND METHODS

1. Ethical Statement

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University Health System (approval number: 2013-0214) and were carried out strictly in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were housed as described previously.[7]

2. Human tissue collection

All patients were initially part of a cohort at Severance Hospital Pituitary Tumor Center. From 2011 to 2012, 17 acromegalic patients have received TSA in Severance Hospital Pituitary Tumor Center. All TSAs were performed by a senior neurosurgeon (Sun Ho Kim). a highly sensitive and specific chemiluminescent immunoassay was used for GH measurement with World Health Organization international standard (WHO IS 98/574). Because recent guideline suggested ultrasensitive method for GH measurement using WHO IS 98/574, the patients of whom GH levels were measured by this kit before and after TSA were included in this study.

Correlation between expression of miRNAs and GH-secreting pituitary adenoma was analyzed with clinical indicators such as including basal and glucose-suppressed nadir GH, IGF-1, tumor size, invasiveness, and Ki-67 labeling index.

Normal pituitary glands were offered by Korea National Forensic Service, and obtained from autopsies of three females and three males, aged from 52 to 75, and devoid of endocrine diseases.

This study was conducted in accordance with the Declaration of Helsinki and was approved by Institutional Review Board (IRB) of Yonsei University Health System (IRB No: 4-2012-0417).

3. Experimental animals

sAIPKO mouse model was previously developed with mice in which express Cre-recombinase in pituitary somatotrophs[8] and *Aip*$^{lox/lox}$ mice generated as previously described[7].

4. Isolation of total RNA and miRNAs

Total RNA and miRNA were performed from pituitary glands of the control mice, sAIPKO mice, rat pituitary adenoma cell lines (GH3 cells) (ATCC, VA, USA), and human tissues using mirVana miRNA Isolation Kit (Applied Biosystems®, Carlsbad, CA, USA) or NucleoZol (Machery-Nagel, Düren, Germany). The RNA concentration was measured by NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

5. Affymetrix miRNA Array

The Affymetrix Genechip miRNA 4.0 array process was executed according to the manufacturer's protocol. Three hundred nanogram RNA samples were labeled with the FlashTag™ Biotin RNA Labeling Kit (Genisphere, Hatfield, PA, USA). The labeled RNA was quantified, fractionated and hybridized to the miRNA microarray according to the standard procedures provided by the manufacturer. The labeled RNA was heated to 99°C for 5 min and then to 45°C for 5 min. RNA-array hybridization was performed with agitation at 60 rotations per minute for 16 hrs at 48°C on an Affymetrix® 450 Fluidics Station. The chips

were washed and stained using a Genechip Fluidics Station 450 (Affymetrix, Santa Clara, California, United States). The chips were then scanned with an Affymetrix GCS 3000 canner (Affymetrix, Santa Clara, California, United States). Signal values were computed using the Affymetrix® GeneChip™ Command Console software. Raw data were extracted automatically in Affymetrix data extraction protocol using the software provided by Affymetrix GeneChip® Command Console® Software (AGCC). The CEL files import, miRNA level RMA+DABG-All analysis and result export using Affymetrix® Expression Console™ Software. Array data were filtered by probes annotated species. The comparative analysis between the test sample and the control sample was carried out using fold-change and independent T-test in which the null hypothesis was that no difference exists among 2 groups. False discovery rate (FDR) was controlled by adjusting *p* value using Benjamini-Hochberg algorithm. All Statistical test and visualization of differentially expressed genes was conducted using R statistical language v. 3.1.2.

6. Cell culture

GH3 cells were grown in DMEM/high glucose supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, and penicillin-streptomycin solution (Hyclone Co., Logan, UT, USA). Cells were maintained at 37°C in a humidified incubator containing 5% CO2. Lipofectamin2000 (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instructions for transfection of miRNA mimics or inhibitors (Ambion, Austin, TX, USA).

7. qRT-PCR analyses

200ng of the small RNAs and 500ng of the total RNAs were used for the RT reaction with TaqMan® MicroRNA Reverse Transcription Kit (Applied

Biosystems®) and RealHelix™ qPCR Kit (NanoHelix, Korea). The qRT-PCR analyses were performed using StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Carlsbad, CA, USA) according to the manufacturer's protocols.

TaqMan RT primer sets used for experiments were U6 snRNA (ID:001973), rno-miR-216a-5p (ID: 002220), and miR-652-3p (ID: 002352). The primers used for this study will be offered upon request.

8. Cell proliferation assay

For MTS assay, GH3 cells were seeded in 96-well plates and cultured in 10% FBS contained DMEM/High glucose media. Mimic and Inhibitor of miR-216a-5p and miR-652-3p were transiently transfected after 24hr. MTS reagent (Promega, Madison, WI, USA) concentration was measured as absorbance at 24hr, 48hr, 72hr, 96hr. Experiments were repeated on at least three different occasions and time-response curves were performed.

9. Matrigel invasion assay

Cell migration and invasion assays were performed using a Matrigel Invasion Chamber. Transfected GH3 cell suspension (2.5×10^4cells) in 500 μL DMEM/high glucose were seeded in chamber tops. For invasion assays, cells were plated in Transwells pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA). Chamber inserts were deposited on 24-well plates containing 400μL DMEM/high glucose supplemented with 10% FBS in the lower chamber. After 22hr of incubation for migration and invasion, non-migrating cells were removed from the upper chambers. Invaded cells were stained with 0.05% crystal violet. Stained cells were quantified with ImageJ.

10. Statistical analyses

Statistical analysis was performed using Microsoft excel software and SPSS software package for Windows (Version 18.0; IBM Corp., Armonk, NY, USA). Tumor incidence data were evaluated with two-tailed Student's t test. Results are expressed as mean ± SEM. Differences were assessed by 1-way ANOVA. A *p*-value of <0.05 was considered statistically significant.

III. RESULTS

1. A GH-secreting pituitary adenoma progression model

Our previous study using rGHp-Cre$^{tg/+}$; Aip$^{lox/+}$ GH-secreting pituitary adenoma model showed the presence of pituitary gland hyperplasia as early as 18 wks of age; this hyperplasia progressed to GH-secreting pituitary adenoma by 80 wks of age[7]. We observed pituitary hyperplasia at 18 wks of age that progressed to macroscopically visible tumors between 24 and 30 wks of age. In reticular staining of pituitary gland of 40 wks of age or older sAIPKO, destruction of pituitary acinar nest area was observed.

2. Differential miRNA expression in sAIPKO mouse model

miRNA expression profiles in the pituitary of 20-wk old hyperplasic sAIPKO mice (rGHp-Cre$^{tg/+}$; Aip$^{lox/+}$), 50-wk old turmeric sAIPKO mice (rGHp-Cre$^{tg/+}$; Aip$^{lox/+}$), and its age-matched littermate control mice (Aip$^{lox/+}$) were analyzed using affymetrix miRNA microarray to identify the dysregulated miRNAs during GH-secreting pituitary adenoma progression (Figure 1). We chose 20-wk old mice as the hyperplasic model because, at this age, the mice had developed pituitary gland hyperplasia.

The significance of the differentially expressed miRNAs was clarified by QC tool software. The miRNA microarray using pituitary tissues of sAIPKO animals compared to controls revealed that miR-330-3p.1, miR-185-5p. miR-339-5p, and miR-216a-5p (p-value<0.05) were significantly downregulated in hyperplasic sAIPKO mice and miR-330-3p.1, miR-185-5p, miR-181d-5p, miR-342-3p, and miR-652-3p (p-value<0.05) were significantly downregulated in turmeric sAIPKO mice, whereas, miR-323-5p, miR-674-3p, miR-376c-3p, miR-139-5p, and miR-485-3p (p-value<0.05) were significantly upregulated in hyperplasic sAIPKO mice and miR-183-3p and miR-539-5p (p-value<0.05) were significantly upregulated in turmeric sAIPKO mice (Table. 1, 2).

The top differentially dysregulated miRNAs were analyzed at 4, 10, 30, and 50 wks of pituitary adenoma progression (Figure 2). At 4 wks of age, the expression of and miR-183-3p and miR-539-5p were upregulated, but their upregulated expression was not statistically significant. However, miR-185-5p (p-value=0.018), miR-339-5p (p-value=0.038), miR-216a-5p (p-value=0.023), and miR-652-3p (p-value=0.009) were downregulated in sAIPKO mice compared to control littermates (Figure 2A). At 10 wks of age, the expression of miR-376c-3p (p-value=0.016) and miR-539-5p (p-value=0.029) were upregulated, whereas the expression of miR-339-5p (p-value=0.033), miR-216a-5p (p-value=0.010), miR-181d-5p (p-value=0.023), and miR-342-3p (p-

value=0.032) were downregulated in sAIPKO mice compared to control littermates (Figure 2B). At 30 wks of age, the expression of miR-485-3p (*p*-value=0.008), miR-183-3p (*p*-value=0.038), and miR-539-5p (*p*-value=0.001) were upregulated, whereas the expression of miR-216a-5p (*p*-value=0.0006), miR-181d-5p (*p*-value=0.002), miR-342-3p (*p*-value=0.026), and miR-652-3p (*p*-value=0.009) were downregulated in sAIPKO mice compared to control littermates (Figure 2C). Further, at 50 wks of age, the expression of miR-183-3p (*p*-value=0.001) and miR-539-5p (*p*-value=0.020) were upregulated, whereas the expression of miR-185-5p (*p*-value=0.049), miR-339-5p (*p*-value=0.003), miR-216a-5p (*p*-value=0.048), miR-181d-5p (*p*-value=0.023), miR-342-3p (*p*-value=0.007), and miR-652-3p (*p*-value=0.005) were downregulated in sAIPKO mice compared to control littermates (Figure 2D).

The overall expression trend of miRNAs are shown in Figure 2E~H.

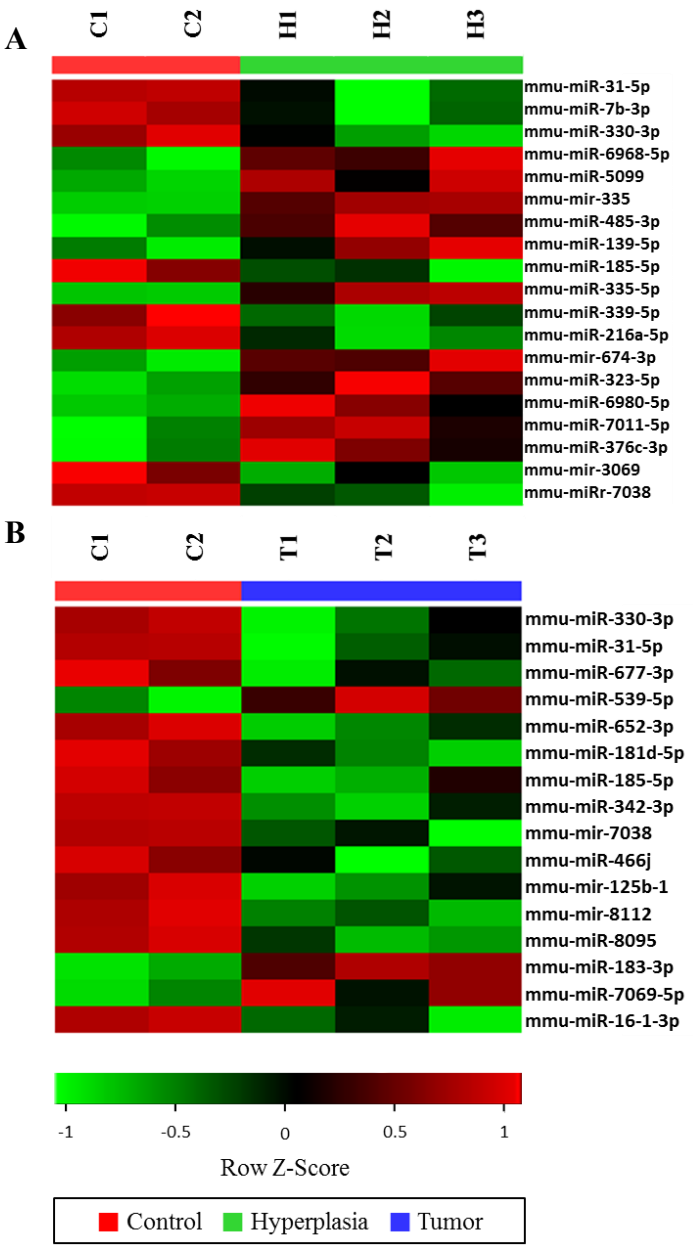

Figure 1. Heatmap and unsupervised hierarchical clustering of the miRNA expression in pituitary gland of a s*AIP*KO model. Differentially expressed miRNAs between hyperplasia versus control **(A)** and tumor versus control **(B)**. (Hyperplasia = 20-wk old sAIPKO, tumor = 50-wk old sAIPKO)

Table 1. Top/Significantly[1] downregulated miRNAs in sAIPKO mice

miRNA ID	Fold change	Predicted Targets[2]
miR-330-3p.1	**-4.41**	Hipk1, Insig2, Pcdh9, Tmem215, Cyclin D3, Cyclin D1
miR-185-5p	**-2.44**	Gpr165, Pet2, Saal1, Crem, Rit2, Top1, Magi2
miR-339-5p	**-2.51**	Fbxo30, Map3k7, Slc15a1, Smtnl2
miR-216a-5p	**-5.64**	Bcl2l11, Pcdhb13, Dazap1, Taf7l, Trub2, Dzip1l, Tmtc1
miR-181d-5p	**-1.84**	BC018101, Gm13242, Zfp600, Rex2, Zfp820
miR-342-3p	**-2.28**	Fam160b2, Socs6, Nip7, Dxd3x, Pus7
miR-652-3p	**-3.48**	Tox, Isl1, Nptn, Plag1, Krtap4-16

Table 2. Top/Significantly[1] upregulated miRNAs in sAIPKO mice

miRNA ID	Fold change	Predicted Targets[2]
miR-323-5p	**2.58**	Col5a2, Kif16b, Tet2, Pfkfb4
miR-674-3p	**2.36**	Neu3, Mbtps2, Alg9, Tbc1d23, St3gal4, Bcl6
miR-376c-3p	**2.31**	Bc031353, Kcnmb2, Arfgef1
miR-139-5p	**2.27**	Ltbp3, Rreb1, Mtmr6, Ube2d1
miR-485-3p	**1.77**	Trdmt1, Cnot2, Ptgdr, Csn3, Pdhx
miR-183-3p	**2.91**	p18, MEN1, p27, Zfp600, Gm13212, Rex2, Nfs1
miR-539-5p	**2.17**	p18, p21, Men1, p27, Mettl14, Slc39a9, Sfrs18

[1] |FC|>=1.5 and p-value<0.05

[2] Bioinformatic tool (TargetScan: http://www.targetscan.org/index.html) was used to detect the putative miRNA-gene targets.

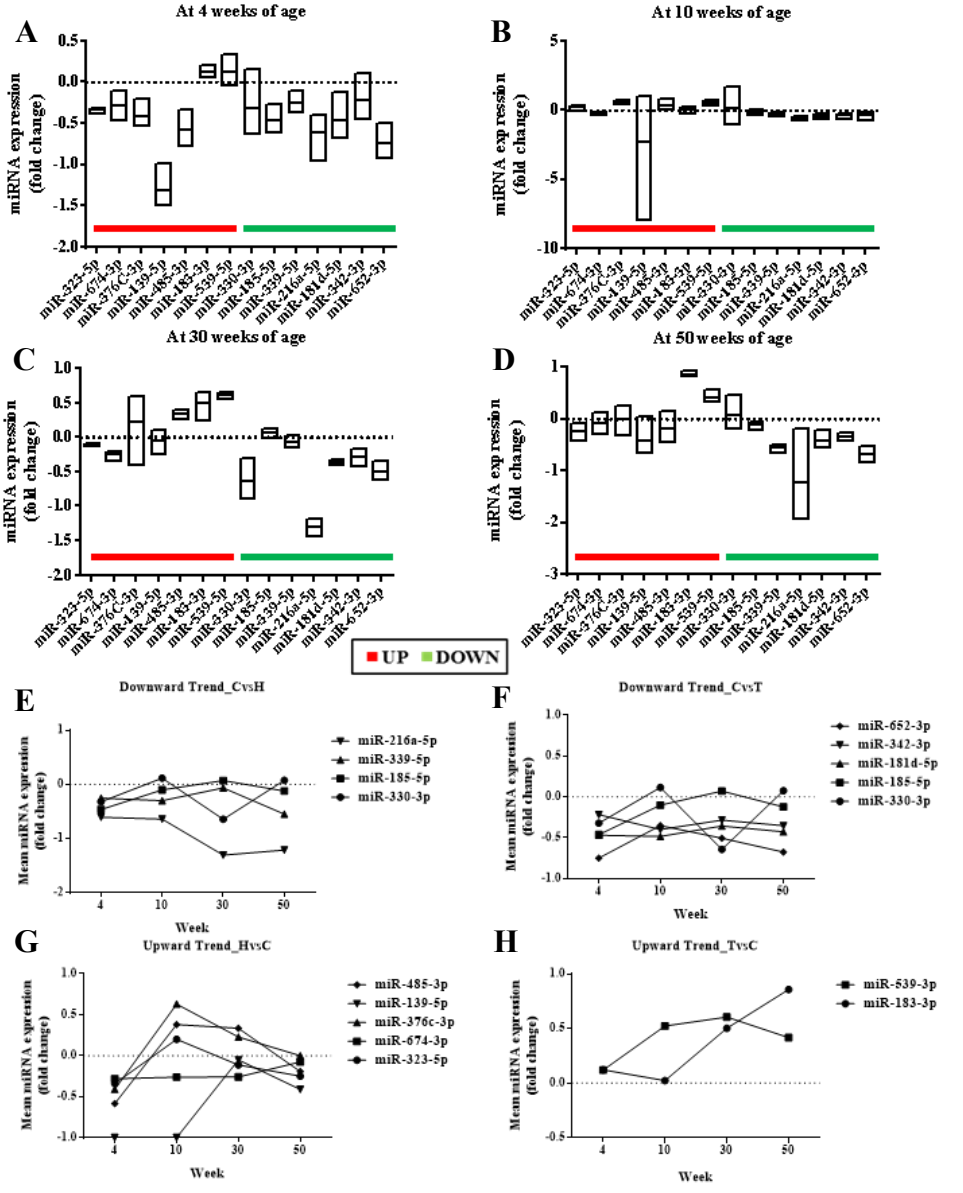

Figure 2. Expression of miRNAs during the pituitary adenoma progression in sAIPKO model. (A)-(D). Expression profiles of miRNA at 4, 10, 30, 50 wks of PA progression was analyzed by qRT-PCR. Red colored miRNAs indicate upregulated ones, whereas green colored miRNAs indicate downregulated in hyperplasia and tumor. Four mice pituitary samples were used for each wks of

age. **(E)-(H).** Expression trends of miRNAs between hyperplasia vs. control and tumor vs. control. U6 snRNA was used to normalize the results. The fold change was calculated by ΔΔCt method.

3. Expression patterns of miRNAs in human GH-secreting pituitary adenoma tissues

Differentially expressed miRNAs identified from mouse miRNA microarray data were further analyzed in 17 human GH-secreting pituitary adenomas to ratiocinate the mouse miRNA microarray data to human GH-secreting pituitary adenoma (Figure 3). We checked the expressions of a few differentially expressed miRNAs (miR-339-5p, miR-216a-5p, miR-181d-5p, miR-342-3p, miR-652-3p, miR-183-3p, and miR-539-5p) of which expression level were significantly dysregulated in 4, 10, 30, and 50 wks of age sAIPKO mice. Seventeen human GH- secreting pituitary adenoma tissues were analyzed with two clinical indicators; ki67 labeling index and basal GH secretion. miR-216a-5p was inversely correlated with ki67 labeling index (decreased by 65%, *p*-value=0.007, Figure 3A), and miR-652-3p was inversely correlated with ki67 labeling index and basal GH secretion (decreased by 70%, *p*-value=0.03, Figure 3A and 3B). Hence, we next focused on these two miRNAs (miR-216a-5p and miR-652-3p).

We also used six human normal anterior pituitary samples to compare the expression of miR-216a-5p and miR-652-3p. Both miR-216a-5p (*p*-value=0.004, Figure 3C) and miR-652-3p (*p*-value=0.0005, Figure 3D) were downregulated in GH-secreting pituitary adenoma samples compared to normal tissues.

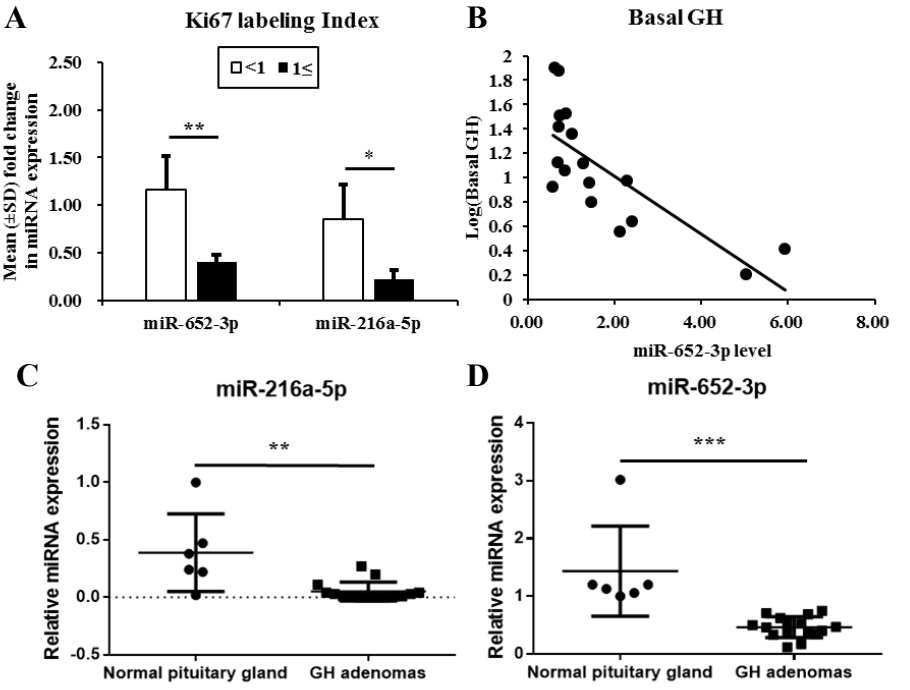

Figure 3. Expression patterns of miR-216a-5p and miR-652-3p in human GH-secreting pituitary adenoma tissue samples. (A) miRNA expression patterns were measured in human GH-secreting pituitary adenoma tissue samples. Two of miRNAs (miR-652-3p, -216a-5p) showing downward trend in animal models were also downregulated in human tissues of which ki67 labeling index is higher than 1. (**B)** Basal GH expression is inversely correlated with miR-652-3p expression. An inverse relationship between Basal GH level and miR-652-3p expression was evident. (**C) and (D)** Expression level of miR-216a-5p and miR-652-3p were downregulated in GH-secreting pituitary adenoma tissues compared to normal anterior pituitary. Six normal pituitary tissue samples and 16 GH adenoma tissue samples were used. The fold change was calculated by ΔΔCt method. Data represented as mean ± SE from triplicated experiments. ***, **, and * indicate $p<0.001$, $p<0.01$ and $p<0.05$, respectively.

4. Identification of target genes for miR-216a-3p and miR-652-3p

In order to investigate the possible role of dysregulated expression of miR-216a-5p and miR-652-3p in GH-secreting pituitary adenoma, we employed bioinformatic tools (TargetScan v7.1) to search for potential targets of the identified miRNAs. The selection criterion applied to select target genes were as follows: (i) target genes that play roles in cell proliferation and/or in cell signaling pathway were selected.; (ii) target genes that are targeted identified miRNAs in mouse, rat, and human simultaneously were selected. As predicted by TargetScan v7.1, JAK2 was identified as a potential target of miR-216a-5p (Figure 4A) and PRRX1 was identified as a potential target of miR-652-3p (Figure 4B). Additionally, the expression of miRNAs (miR-216a-5p and miR-652-3p) and their target mRNAs (JAK2 and PRRX1) were analyzed in sAIPKO compared to control mouse. miR-216a-5p (decreased by 55%, *p*-value=0.0007, Figure 5A) and miR-652-3p (decreased by 35%, *p*-value=0.0006, Figure 5B) were downregulated, whereas Jak2 (increased up to 2.2-fold, *p*-value=0.012, Figure 5C) and Prrx1 (increased up to 1.5-fold, *p*-value=0.037, Figure 5D) were upregulated in sAIPKO. These results indicate that each JAK2 and PRRX1 could be the functioning target genes of miR-216a-5p and miR-652-3p.

To validate the influence of identified miRNAs on the expression of their predicted target genes, we have performed *in vitro* experiments. Firstly, we checked the basal expression level of miR-216a-5p and miR-652-3p in GH3 cells, as well as in the normal rat anterior pituitary tissues (Figure 6A and 6B). Results showed the downregulation of miR-216a-5p (decreased to 1.3%, *p*-value=0.014, Figure 6A) and miR-652-3p (decreased to 1.5%, *p*-value=0.017, Figure 6B) in GH3 cells compared to the normal rat anterior pituitary tissues. Next, we performed transfection with miRNA mimic, inhibitor, and their negative controls (NC) using GH3 cells. As shown in Figure 6C and 6D, transfection of mimic significantly increased the expression of miR-216-5p (increased up to 45-fold, *p*-

value=0.028, Figure 6C) and miR-652-3p (increased up to 55-fold, *p*-value=0.016, Figure 6D), whereas, transfection of inhibitor significantly decreased them (decreased by 60%, *p*-value=0.034, and decreased by 90%, *p*-value=0.029, respectively).

The effect of miR-216a-5p and miR-652-3p on the expression of their target gene was next assessed. QRT-PCR analysis revealed that mimics of miR-216a-5p and miR-652-3p downregulated the expression of JAK2 (decreased by 30%, *p*-value=0.025, and decreased by 65%, *p*-value=0.002, respectively) and PRRX1, whereas inhibitor upregulated them (increased up to 1.4-fold, *p*-value=0.048, and increased up to 1.7-fold, *p*-value=0.045, Figure 7A and 7B, respectively).

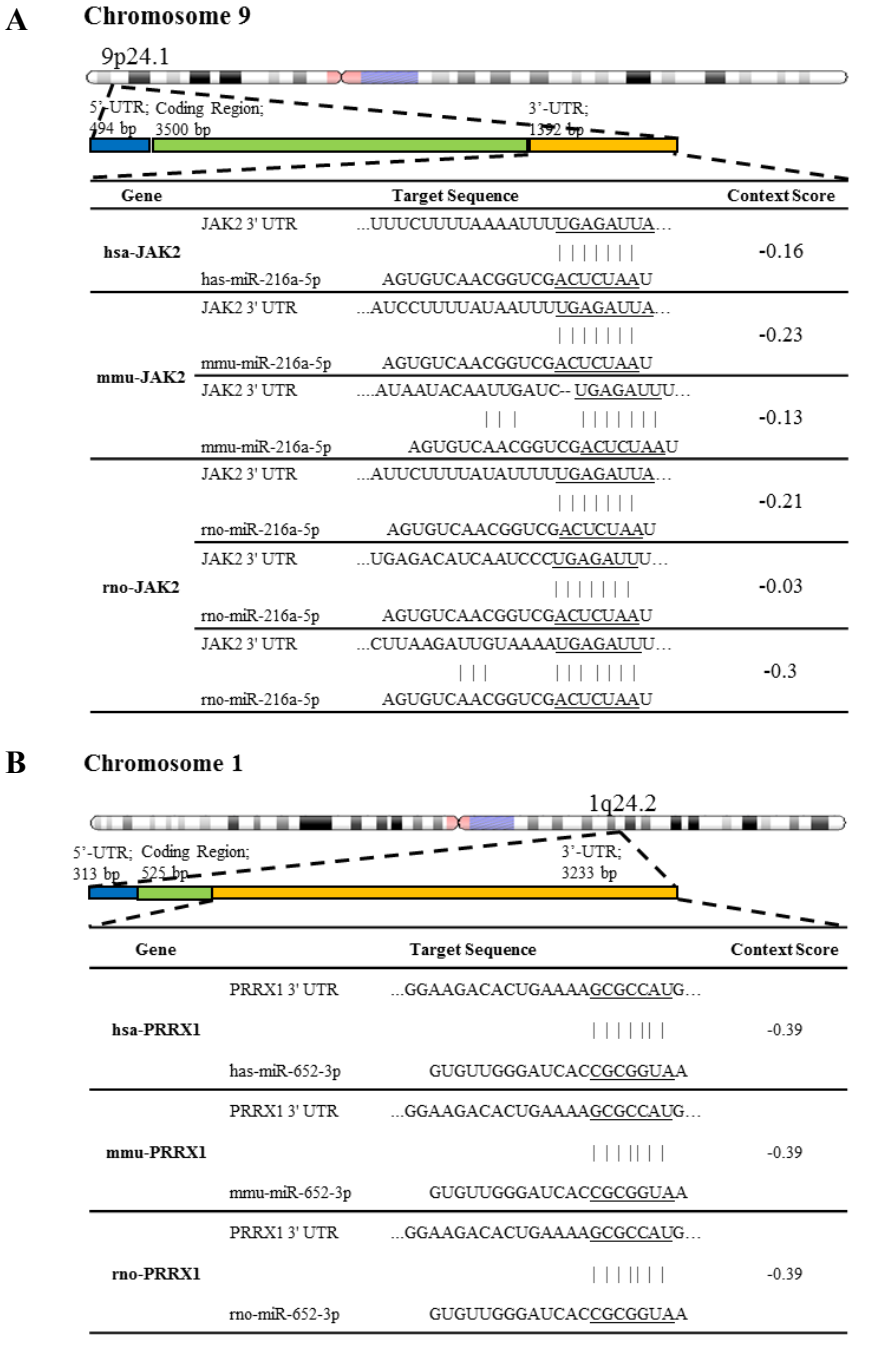

Figure 4. Identification of target gene for miR-216a-5p and miR-652-3p in various species. (A) JAK2 is a direct target gene of miR-216a-5p, detected by

TargetScan. Alignments of sequence in the JAK2 3'-UTR (underlined) with miR-216a-5p in human (has-), mouse (mmu-), and rat (rno-). (**B)** PRRX1 is a direct target gene of miR-652-3p, detected by TargetScan. Alignments of sequence in the PRRX1 3'-UTR (underlined) with miR-652-3p in human (has-), mouse (mmu-), and rat (rno-).

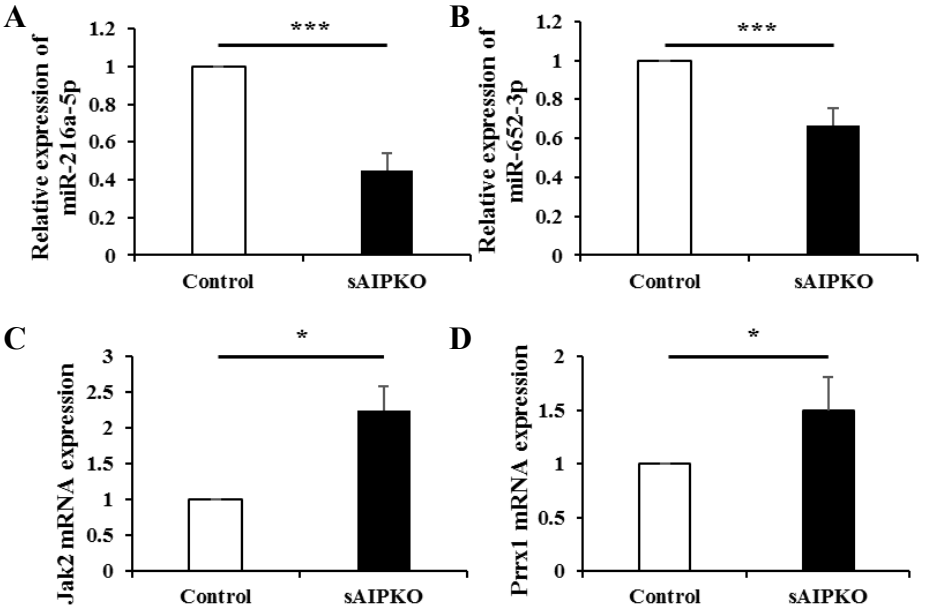

Figure 5. Expression of miRNAs and their target mRNAs in GH-secreting PA animal models. Expression of miRNAs (**A and B**) and target gene (**C and D**) in 30-wk-old sAIPKO mice compared to control. The fold change was calculated by ΔΔCt method. Data represented as mean ± SE from triplicated experiments. *** and * indicate $p<0.001$ and $p<0.05$, respectively.

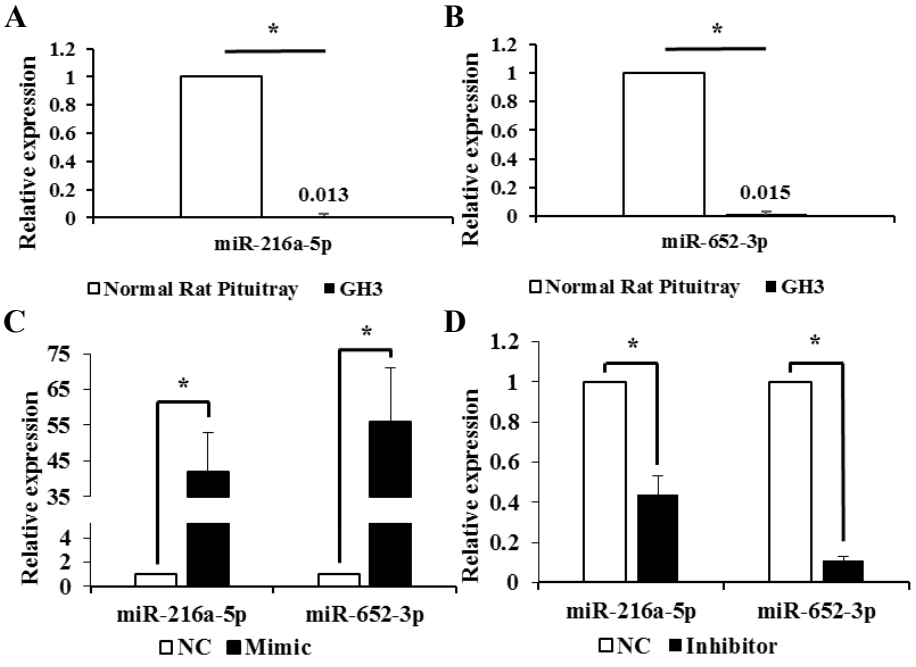

Figure 6. Expression levels of miR-216a-5p and miR-652-3p in normal rat pituitary and GH3 cells. (A and B) Expression of miRNAs in normal rat anterior pituitary and GH3 cells. (**C and D)** qRT-PCR of miR-216a-5p and miR-652-3p levels in mimic and inhibitor transfected GH3 cells. The fold change was calculated by ΔΔCt method. Data represented as mean ± SE from triplicated experiments. * indicates $p<0.05$.

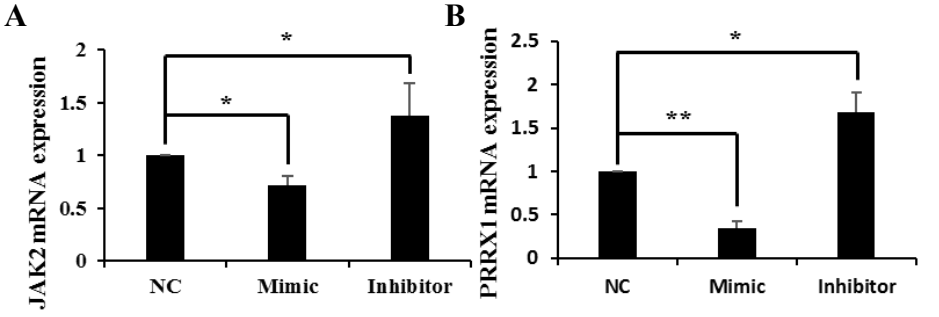

Figure 7. Effect of miRNAs on their target gene expression in GH3 cells. Expression of Jak2 (**A**) and Prrx1 (**B**) was regulated by mimic and inhibitor of miR-216a-5p and miR-652-3p in GH3 cells. The fold change was calculated by ΔΔCt method. Data represented as mean ± SE from triplicated experiments. ** and * indicate $p<0.01$ and $p<0.05$, respectively.

5. miR-216a-5p and miR-652-3p regulate the cell proliferation and invasion in GH3 cells

To understand the role of miR-216a-5p and miR-652-3p, we next investigated their effect on cell proliferation (Figure 8) and invasion (Figure 9). The effect of miR-216a-5p and miR-652-3p expression on cell proliferation was studied on GH3 cells. As shown in Figure 8, a significant reduction of absorbance was observed in 96h after transient transfection with mimic of miR-216a-5p (decreased by 20%, *p*-value=0.043, Figure 8A) and miR-652-3p (decreased by 25%, *p*-value=0.045, Figure 8B, respectively) compared to NC-transfected cells. Conversely, a significant increase was observed in inhibitor-transfected cells (increased by 30%, *p*-value=0.037, Figure 8C, and increased by 20%, *p*-value=0.044, Figure 8D, respectively).

To characterize the effects of the analyzed miRNAs on invasion, inhibitors and NC were transfected in GH3 cells, and analyzed by matrigel invasion assay (Figure 9A and 9B). Interestingly, mimics of miR-216a-5p and miR-652-3p decreased invasion of GH3 cells (decreased by 60%, *p*-value=0.002, and decreased by 50%, *p*-value=0.018, Figure 9A, respectively) whereas inhibitors increased (increased up to 2-fold, *p*-value=0.026, and increased up to 1.8-fold, *p*-value=0.0017, Figure 9B, respectively).

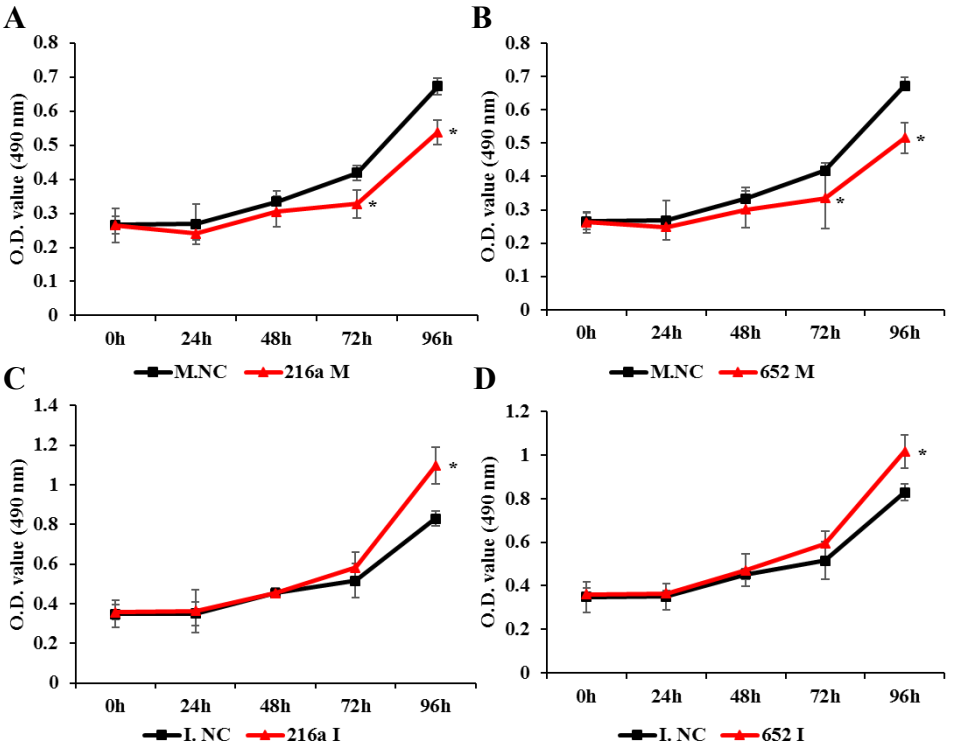

Figure 8. Effect of miR-216a-5p and miR-652-3p on proliferation in GH3 cells. (A)-(D). Effect of identified miRNAs on GH3 cell proliferation. Data represented as mean ± SE from triplicated experiments. * indicates $p<0.05$.

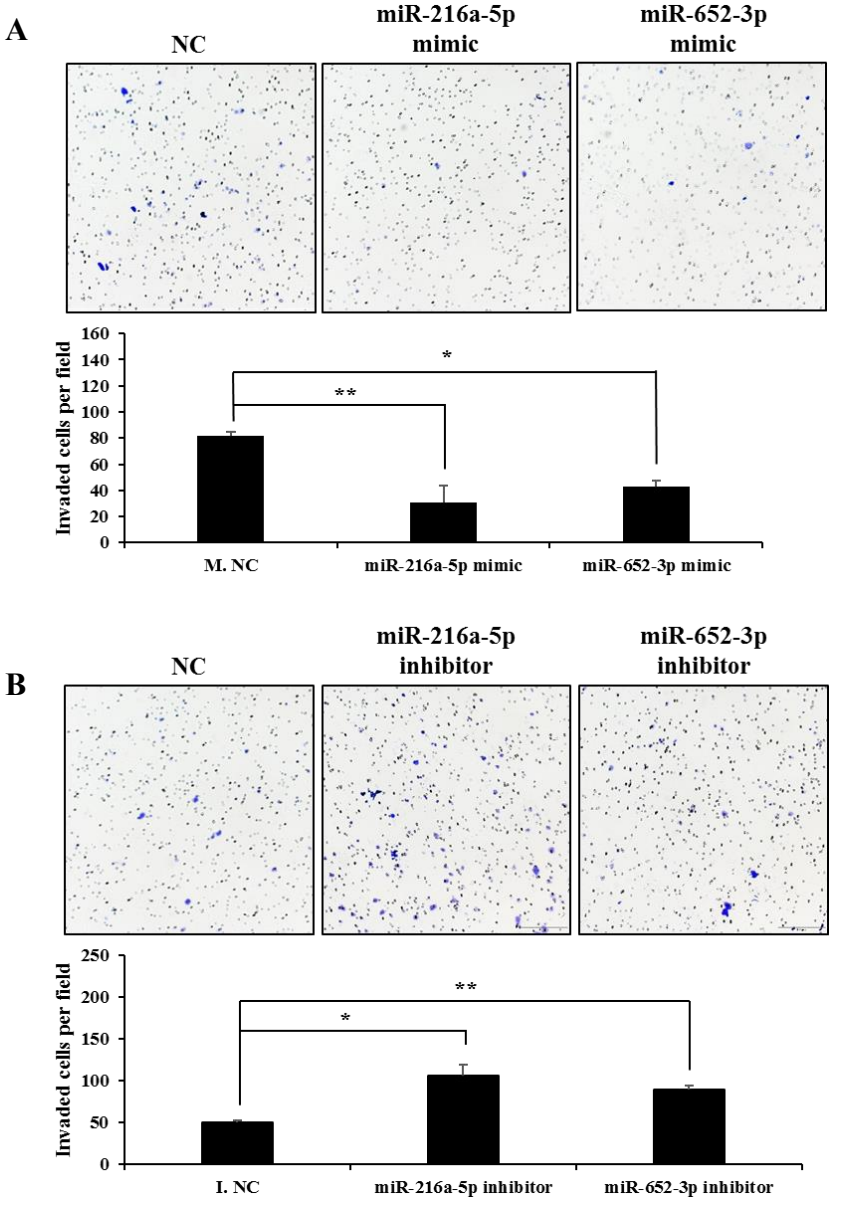

Figure 9. Effect of miR-216a-5p and miR-652-3p on invasion in GH3 cells. (A), (B), (C), and (D). The ability of invasion of GH3 cells after transfection with miRNA mimics or inhibitors were detected by Matrigel invasion assay. Data represented as mean ± SE from triplicated experiments. ** and * indicate $p<0.01$ and $p<0.05$, respectively.

IV. DISCUSSION

miRNAs are expressed differently depending on the tissues, maintaining accurate time trends.[9] Recently, the involvement of miRNAs in pituitary development has been demonstrated. Indeed, miR-26b appears to regulate two major transcription factors that allow differentiation of the pituitary gland.[10] In addition, several studies have demonstrated the participation of miRNAs in anterior pituitary hormone secretion such as pro-opiomelanocortin (POMC) and ACTH secretion by miR-375,[11] FSH secretion by mir-361-3p,[12]and LH secretion by miR-325-3p.[13] Moreover, the fundamental roles of miRNAs in tumorigenesis of pituitary adenoma has been defined.[14]

Especially, there are several reports studied the role of miRNAs in GH-secreting pituitary adenomas. miR-326, miR-432, miR-570, miR-34b, miR-548c-3p, miR-326 and miR-603 are related to pathogenesis of pituitary adenoma, respectively targeting HMGA1, HMGA2, and E2F1.[15] High expression levels of HMGA1, HMGA2, and E2F1 proteins in GH-secreting pituitary adenomas may be clarified by the reduced levels of these miRNAs.

The role of miRNAs in the drug resistance to pharmacological treatment of GH-secreting pituitary adenomas was also studied. Long-acting somatostatin analogs are used as medical therapy for GH-secreting pituitary adenomas. However, some patients show drug resistance to this treatment, suggesting relation to reduction in the expression of somatostatin receptor subtype 2 (SSTR2). miR-185 is known to be related to expression reduction, targeting SSTR2 directly. Overexpression of miR-185 inhibits GH3 cell proliferation and activate late apoptosis.[16] Sporadic GH-secreting pituitary adenomas is also known to display low levels of aryl hydrocarbon receptor-interacting protein (AIP), suggesting that both AIP and miR-34a may be linked to pituitary adenoma

tumorigenesis.[17] It is also found that simultaneous treatment of miR-26 inhibitor and miR-128 mimic blocks both tumorigenicity and invasiveness of GH3 cells and MtT/S cell line. Palumbo T. and colleagues identified PTEN as a direct target of miR-26b and BMI1 as a direct target of miR-128. These miRNAs not only control cell tumorigenicity and invasiveness *in vitro*, but also cell proliferation *in vivo* regulating PTEN-AKT pathway.[18]

Despite the above studies, the role of miRNAs in pathogenesis leading to the control-hyperplasia-tumor of growth hormone-secreting pituitary tumors remains poorly studied.

We utilized well-characterized spontaneous mouse model (sAIPKO) of GH-secreting pituitary adenomas to analyzed the global miRNA expression profile, due to unavailability of early stage samples from human GH-secreting patients. Notably, this model histopathologically recapitulates human GH-secreting pituitary adenomas. We previously demonstrated that this model showed the presence of pituitary gland hyperplasia as early as 18 wks of age. Pituitary hyperplasia was observed at 18 wks of age that progressed to macroscopically visible tumors between 24 and 30 wks of age. Invasive pituitary adenomas were observed at 80 wks of age. Beginning at 18 wks, pituitaries of mouse models showed significantly greater volume as compared to control littermates. Pituitaries of mouse models exhibit further growth from 12 to 18 wks, and continue to grow through the lifespan, whereas pituitaries of control stabilized after 12 wks.[7]

Through miRNA microarray, we identified several differentially expressed miRNAs in GH-secreting pituitary adenoma animals compared to control mice (Table 1 and 2). We observed significant downregulation of 5 miRNAs (miR-339-5p, miR-216a-5p, miR-181d-5p, miR-342-3p, and miR-652-3p) and upregulation of 2 miRNAs (miR-183-3p and miR-539-5p) from 4-50 wks of age compared to controls. miR-339-5p is known to be downregulated and have tumor-suppressive

roles in several cancers targeting MDM2,[19] PRL-1,[20] NACC1 and BCL6.[21] miR-181d-5p is known to be related to MGMT-regulation in glioblastoma.[22] miR-342-3p is reported as tumor suppressor regulating proliferation and invasion targeting RAP2B,[23] E2F1,[24] and FOXM1[25] in several human cancers. Upregulation of miR-183-3p is a potent prognostic marker for lung adenomacarcinoma,[26] and miR-539-5p is reported to be related to human nasopharyngeal carcinoma.[27] To complement miRNA microarray data using animal model, we performed additional experiments using human pituitary tissues. 5 miRNAs, however, did not show correlation with clinical indicators using human tissues, whereas 2 miRNAs (miR-216a-5p and miR-652-3p) did (Figure 3). Ki-67 expression and basal GH level are known to be representative markers of aggressiveness in the World Health Organization Classification of Endocrine Tumors, and especially, ki-67 is an independent indicator of pituitary adenoma progression.[28] Comparing expression level of miRNAs between human normal posterior pituitary and GH-secreting pituitary adenoma tissues also compensate our miRNA microarray data using animal model, which could be a vulnerable point. Recent studies have shown that miR-216a-5p is related to several cancers such as pancreatic cancer targeting MALAT1,[29] oral squamous cell carcinoma targeting EIF4B,[30] and colorectal cancer targeting KIAA1199/CEMIP.[31] miR-652-3p has also known to be related to several cancers such as pancreatic cancer targeting ZEB1,[32] lung cancer targeting Lgl1,[33] and breast cancer.[34]

In the present study, we found potential target genes that have a role in cell proliferation and invasion, because there was no known about molecular targets of miR-216a-5p and miR-652-3p in GH-secreting pituitary adenomas. Using bioinformatic tools, we found that each JAK2 and PRRX1 are direct targets of miR-216a-5p and miR-652-3p. Previous studies have demonstrated that JAK2 and PRRX1 may play important roles not only in pituitary adenomas,[35-38] but also in other cancers.[39-42] JAK2 is known to be related to growth of pituitary adenomas by erythropoietin,[35] and GH hypersecretion is the result of STAT3 expression,

which is a transcription factor that is phosphorylated by JAK2.[36] JAK2 is also identified as the target gene of miR-216a inhibiting pancreatic cancer,[39] and JAK2/STAT3 pathway involves in the pathogenesis of several types of tumors.[40] PRRX1 participate in pituitary organogenesis,[37] and vasculogenesis during rat embryonic pituitary development.[38] PRRX1 also involved in epithelial-mesenchymal transition (EMT) in gastric cancer,[41] and associated with invasive properties of glioblastoma cells.[42]

We also considered whether there could be relations between two miRNAs (miR-216a-5p and miR-652-3p) and *AIP* gene, because we started this study using somatotroph-specific *AIP* knock out mouse model. Location of miR-216a-5p (chromosome 2), miR-652-3p (chromosome X), and AIP (chromosome 11) did not overlap and also human tissues that we used were turned out to have no mutations in *AIP* genes. According to the research above, we concluded that there is no direct mechanism between two miRNAs and *AIP* gene.

V. CONCLUSION

Analyzing miRNA microarray, differential expression of 14 miRNAs; 7 downregulated (miR-330-3p.1, miR-185-5p, miR-339-5p, miR-216a-5p, miR-181d-5p, miR-342-3p, and miR-652-3p) and 7 upregulated (miR-323-5p, miR-674-3p, miR-376c-3p, miR-139-5p, miR-485-3p, miR-183-3p, and miR-539-5p) were observed. 2 miRNAs (miR-216a-5p and miR-652-3p) were selected above these candidate miRNAs through additional *in vivo* experiments using human GH-secreting pituitary adenoma tissues. Effect of miR-216a-5p and miR-652-3p on cell proliferation and invasion were analyzed using GH3 cells. Correlation between expression of candidate miRNAs and cell proliferation and invasion was verified, indicating their possibility to function as a tumor suppressor to inhibit tumorigenesis of GH-secreting pituitary adenoma potentially by downregulating JAK2 and PRRX1.

Our findings indicate that miR-216a-5p and miR-652-3p might have a role in GH-secreting pituitary adenomas by targeting JAK2 and PRRX1. Further studies are needed to specify the mechanism of miRNAs regulating JAK2 and PRRX1 in GH secreting pituitary adenoma.

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ABSTRACT (IN KOREAN)

성장호르몬 분비 뇌하수체 종양의 miRNA 발현 및 역할 규명

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이 양 종

최근 비정상적인 마이크로 RNA (miRNA) 의 발현이 인체의 여러 종양 형성, 전이, 침습에 관련 있음이 밝혀지고 있다. 그러나 뇌하수체 선종의 발병에 miRNA가 어떤 역할을 하고 있는지에 대한 연구는 한정되어 있다. 본 연구에서는 뇌하수체 성장호르몬 분비세포 특이적으로 Aip 유전자를 적중시킨 조건적 Aip 유전자 마우스 동물모델 (somatotroph specific AIP knock out; sAIPKO) 을 이용하여 뇌하수체 종양 형성 과정 중 일어나는 miRNA 발현 프로파일을 분석하였다.

먼저 miRNA의 발현 변화를 프로파일하는 microRNA microarray 를 통하여 sAIPKO에서 종양의 발생에 관여할 가능성이 있는

miRNA를 추출하였다. 여러 후보 miRNA들 중 가장 유의성 있게 발현에 변화를 보인 miRNA를 qRT-PCR을 통해 4주~50주령 동물 모델에서 분석하였고 인간 조직 sample에서 혈중 성장호르몬 수치, 혈중 IGF-1 수치, 종양 크기, 침습성, 그리고 ki-67 발현 지수 등을 토대로 추가 검증되었다. 또한 각 miRNA의 표적 mRNA의 발현도 GH3 세포와 sAIPKO 동물 모델에서 확인하였다.

sAIPKO 마우스의 GH 분비 뇌하수체 종양 형성 과정 중에 14개의 miRNA가 유의하게 변화했으며 그 중 7개의 miRNA가 qRT-PCR을 통해서 microarray와 동일한 발현 양상을 보였다. 이들 중 인간 성장호르몬분비성 뇌하수체 종양 조직을 이용하여 miR-216a-5p와 miR-652-3p를 선별하였다. 두 개의 miRNA의 표적 mRNA의 발현이 miRNA의 발현량에 따라 조절되는 것을 확인하였으며 GH3 세포주의 세포 증식과 침습에도 영향을 주는 것을 확인하였다. 이와 같은 결과들을 볼 때에, miR-216a-5p와 miR-652-3p가 뇌하수체종양의 발생 과정 중에 해당 표적 mRNA를 통하여 관여 할 수 있음을 알 수 있다.

핵심 되는 말: 성장호르몬분비성 뇌하수체종양, 마이크로RNA 마이크로 어레이, miR-216a-5p, miR-652-3p

PUBLICATION LIST

1. Lee YJ, Cho JM, Ku CR, Kim SH, Lee EJ Increased miR-338-3p expression correlates with invasiveness of GH producing pituitary adenomas. Endocrine 2017;[Accepted].