Development of biomarkers for detecting the invasiveness of GH secreting pituitary adenoma

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Development of biomarkers for detecting the invasiveness of GH secreting pituitary adenoma

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Jin Mo Cho

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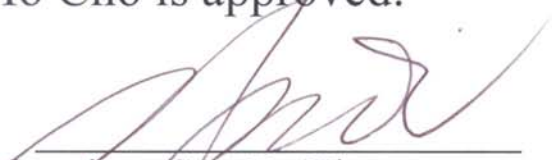
This certifies that the Doctoral Dissertation

of Jin Mo Cho is approved.



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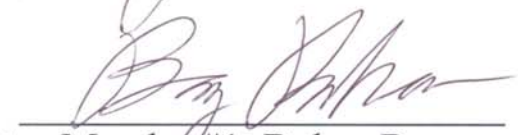

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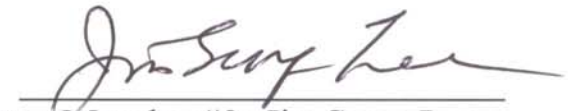

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ABSTRACT

Development of biomarkers for invasiveness of GH secreting pituitary adenoma

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MicroRNAs (miRNAs) have been implicated in the pathogenesis of many human tumors. miRNAs act not only as a tumor suppressor but also as an oncogene in some malignancies, including thyroid cancer, prostate cancer, and pancreatic cancer. However, few studies have been reported about the role of miRNAs on pituitary adenoma. It was hypothesized that miRNAs might be involved in tumor size and invasiveness of growth hormone (GH)

secreting pituitary adenoma. miRNA for microarray was extracted and purified from 6 GH secreting pituitary adenoma patients. Three of them had the pituitary adenoma of Hardy classification I (non-invasive) and the others had the one of Hardy classification IV (invasive). The significance of data from microarray was re-evaluated with in-vitro condition using rat GH-producing cell (GH3) line before and after estradiol (E2) treatment. There was significant difference in expression profile of miRNA according to the invasiveness of pituitary adenoma. miRNA-338 and miRNA-652 was significantly up-regulated but miRNA-432 was significantly down-regulated in invasive pituitary adenoma. Several candidate transcripts from microarray were further explored with GH3 cell line. The most strongly differentially expressed miRNA was miRNA-338. In E2 treated GH3 cell, the expression of miRNA-338 increased as rising concentration of E2 treatment. p27 expression in GH3 was down-regulated after miRNA-338 inhibitor treatment. These data suggest that specific miRNA, especially miRNA-338 might influence on the progression of GH secreting pituitary adenoma.

Key words : Pituitary adenoma, Acromegaly, micro RNA, Growth hormone, Biomarker, Invasion

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I. INTRODUCTION

Pituitary adenoma is one of the most common benign intracranial tumors. They usually grow noninvasively and rarely innervate adjacent structures[1,2]. Growth Hormone (GH) secreting pituitary adenomas comprise about 20% of all pituitary adenomas. About half of GH-secreting pituitary adenomas are histologically heterogeneous, expressing other hormones such as prolactin. Due to the importance and complex anatomy of the surrounding

tissue, determining the invasiveness of the tumor is essential in devising the treatment strategy preoperatively.

MicroRNAs (miRNA) are relatively small nucleic acids composed of 20-30 nucleotides. These noncoding RNAs are present throughout the genomes of animal, plants, and protozoa[3] and are classified as intergenic (between genes) or intragenic (within a gene).[4] Mature miRNAs are incorporated into the miRNA-induced silencing complex, where they associate with target messenger RNAs (mRNAs)[5]. They then regulate post-transcriptional expression of target genes predominantly by degrading the target mRNA or by inhibiting or enhancing protein translation[3]. Recent studies have shown the abnormal expression of certain miRNAs in various human cancers, such as prostate cancer and pancreatic cancer, indicating that some miRNAs may function as oncogenes or tumor suppressor genes[6-9]. More recently, abnormal miRNA expression has also been reported in pituitary tumors, suggesting that miRNAs might contribute to the development of pituitary adenoma[2,10]. However, few studies have been reported on potential roles for miRNAs on pituitary adenoma invasion and growth. In this study, it was investigated that miRNAs might regulate tumor size and invasiveness in pituitary adenoma

II. MATERIALS AND METHODS

Pituitary adenoma tissue collection

The use of patients tissue was approved by the Institutional Review Board for Human Research at Severance Hospital Yonsei University Health System. Human pituitary tumors were obtained from the patients as paraffin-embedded tissue samples from Severance Hospital, Seoul, Korea. Tumor size and aggressiveness were classified by preoperative magnetic resonance imaging finding, operation records, and clinical data after the operation. The clinical data was obtained from the pituitary tumor database of a single senior neurosurgeon (S.H, Kim). Three patients per group (invasive vs. non-invasive, Figure 1) were choosed and it was hypothesized that differential miRNA expression might influence tumor size and invasiveness in patients with GH-secreting pituitary adenoma. The patient clinical data is summarized in Table 1.

Figure 1. Preoperative sellar MRI of both groups; non-invasive (A) and invasive (B) GH-secreting pituitary adenoma.

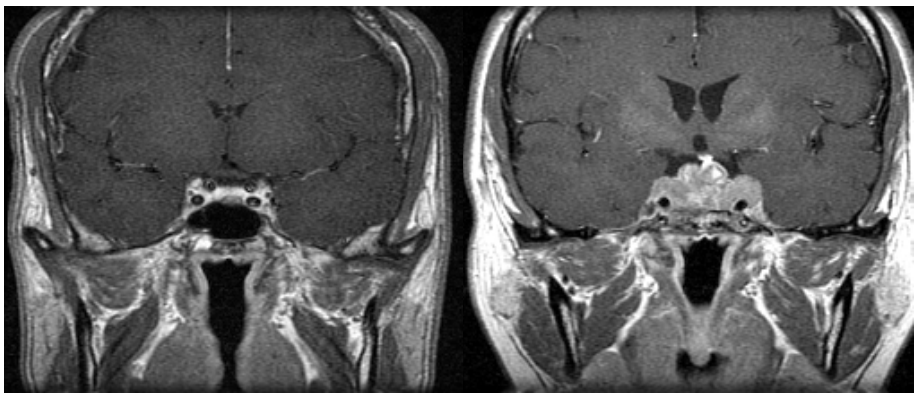

Table 1. Clinical summary of patients.

	Age/Sex	Hardy	Removal	OGTT preoperation	OGTT postoperation
1	31/F	IV	STR	24.71	3.3
2	48/F	IV	STR	9.71	4.06
3	31F	IV	STR	47.35	30.32
4	53/M	I	GTR	11.60	0.33
5	45/M	I	GTR	18.52	0.01
6	42/M	I	GTR	24.13	0.26

GTR, Gross Total Resection; OGTT, Oral Glucose Tolerance Test; STR, Subtotal Resection.

miRNA microarray chip processing and analysis of miRNA expression data

The miRNA Microarray System with miRNA Complete Labeling and hybridization Kit (Agilent Technologies) was used according to the manufacturer′s recommended protocol. The Agilent microRNA Spike-In Kit was used as an in-process control to measure labeling and hybridization efficiency. Briefly, 100 ng total RNA was dephosphorylated at 37°C for 30 min with calf intestinal phosphatase and denatured in 100% DMSO at 100°C

for 7 min. Samples were labeled with pCp-Cy3 by incubation T4 ligase at 16 °C for 2 h. The labeled RNA samples were dried in a Vacuum Concentrator for 1 h. Once samples were completely dried, they were prepared for hybridization by adding nuclease-free water, Hyb Spike-In solution, 10 X GE Blocking Agent and 2X Hi-RPM Hybridization Buffer. Arrays were hybridized in 45 μl of the mixture, and then rotated at 20 rpm for 20 h at 55 °C. Agilent Gene Expression Wash Buffers 1 (RT) and 2 (37°C) were used after hybridization as recommended by Agilent miRNA Microarray System protocol and were scanned on a Agilent Technologies G4900DA SureScan scanner at 3 μm resolution. Data were acquired using Agilent Feature Extraction software version 11.0.1.1. Microarray analysis using SurePrint G3 Human miRNA Microarray, Release 18.0, 8 × 60 K (Agilent, Inc., Santa Clara, CA) was carried out by Macrogen Co. (Seoul, Korea) according to the manufacturer's protocol.

GH3 cell culture

GH3 cells were purchased from American Type Culture Collection. GH3 cells were grown in DMEM/High glucose supplemented with 10% fetal bovine serum (FBS), 4 mM L-Glutamine, and Penicillin-Streptomycin Solution (HyClone). The medium was replaced 24 hours later with phenol red-free DMEM (Gibco) supplemented with 10% charcoal-stripped FBS and an antibiotic-antimycotic mixture for 24 h before treatment. Cells were then treated with 17β-estradiol (E2).

Validation of the miRNA by quantitative RT-PCR (qRT-PCR)

Cells were washed with cold PBS (pH 7.4), and small RNA was extracted using the mirVana miRNA Isolation Kit (Applied Biosystems®). The RNA Concentration was measured with a NanoDrop 200 spectrophotometer (Thermo Scientific), and 200 ng of the small RNA was used for the RT reaction with a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems®). Quantitative PCR analyses were performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems®) according to the manufacturer's protocols.

The Taqman RT primer sets used for experiments were rno-miRNA-338-3p (ID:000548), rno-miRNA-652-3p (ID:002352), and U6 snRNA (ID:001973). Primers used as negative controls were as follows:

Gapdh, sense 5′-GGATGGAATTGTGAGGGAGA-3′

antisense 5′-GAGGACCAGGTTGTCTCCTG-3′

Prl, sense 5′-CATCAATGACTGCCCCACTTC-3′

antisense 5′-CCAAACTGAGGATCAGGTTCAAA-3′

Transient transfection

GH3 cells were transfected with mirVana miRNA-338 mimic or inhibitor (Ambion®) using Lipofectamin200 (Invitrogen™). Transfection was performed according to the manufacturer's instructions.

Cell proliferation assay

GH3 cells were seeded at 4000 cells/well in 96-well plates for 24 h

after cultured in DMEM/High-Glucose media containing 10% FBS. Cells were treated as indicated for 4 days. Cell growth was determined using an MTS-based colorimetric assay. After adding 100 μl of MTS solution (final concentration, 0.5 mg/ml) to each well, the plates were incubated 4 h at 37 ℃. The absorbance was measured at 570 nm on an ELISA plate reader.

Western blot analysis

After transient transfection, cells were washed with cold PBS, and lysates were prepared using cell lysis buffer supplemented with complete protease inhibitor cocktail. Samples were centrifuged for 5 minutes at 12,000 rpm (clarifying spin), and supernatants were stored at 80°C for further processing. Protein levels for each sample were measured using a Bradford assay kit (Bio-rad Laboratories), and the samples were diluted to obtain identical protein concentrations. Proteins were separated by electrophoresis on a 12% SDS-PAGE gel and transferred to nitrocellulose membranes by application of a 500 mA current for 90 minutes. Membranes were incubated

overnight in 5% blotting-grade blocker nonfat dry milk (Bio-Rad Laboratories) in Tris-buffered saline plus 0.1% Tween 20 solution and incubated with specific antibodies. The primary antibodies used were anti-p27 (SC-528; rabbit, 1:1000, Santa Cruz biotechnology) and anti-β−actin (sc-47778; 1:10000, Santa Cruz biotechnology). Goat anti-rabbit IgG, (H+L)-peroxidase conjugated (Thermo scientific) was used as a secondary antibody for blotting.

Statistical analysis

Values shown represent the mean ± SD. Statistical significance was determined by Student's t-test and a p value < 0.05 was considered significant.

III. RESULTS

Differential miRNA expression between invasive and non-invasive pituitary adenoma

To identify the miRNA expression in pituitary adenoma, pair-wise comparisons between 2 groups were performed: invasive pituitary tumor (Hardy class IV, subtotal resection group) and noninvasive pituitary tumor (Hardy class I, gross total resection group) (Figure 1). Upon comparisons of the two groups, It was observed that significant changes in the expression profile of miRNAs correlating to the invasiveness of pituitary adenoma. Among them, 3 miRNA (hsa-miRNA-338, hsa-miRNA-432 and hsa-miRNA-652) were significantly different between invasive and noninvasive pituitary adenoma. (p value = 0.036, 0.025, and 0.033 respectively) (Figure 2). Two miRNAs (miRNA-338 and mi-RNA-652) were upregulated in invasive pituitary adenoma and miRNA-432 was downregulated in invasive pituitary adenoma. The most strongly differentially expressed miRNA was miRNA-338. Unfortunately, probes for miRNA-432 quantification were not availabe, we therefore decided to validate the microarray results by qRT-PCR. These experiments confirmed the expression of miRNA-338 in a GH3 cell line after E2 treatment; however, miRNA-652 did not show clear expression (Figure 3).

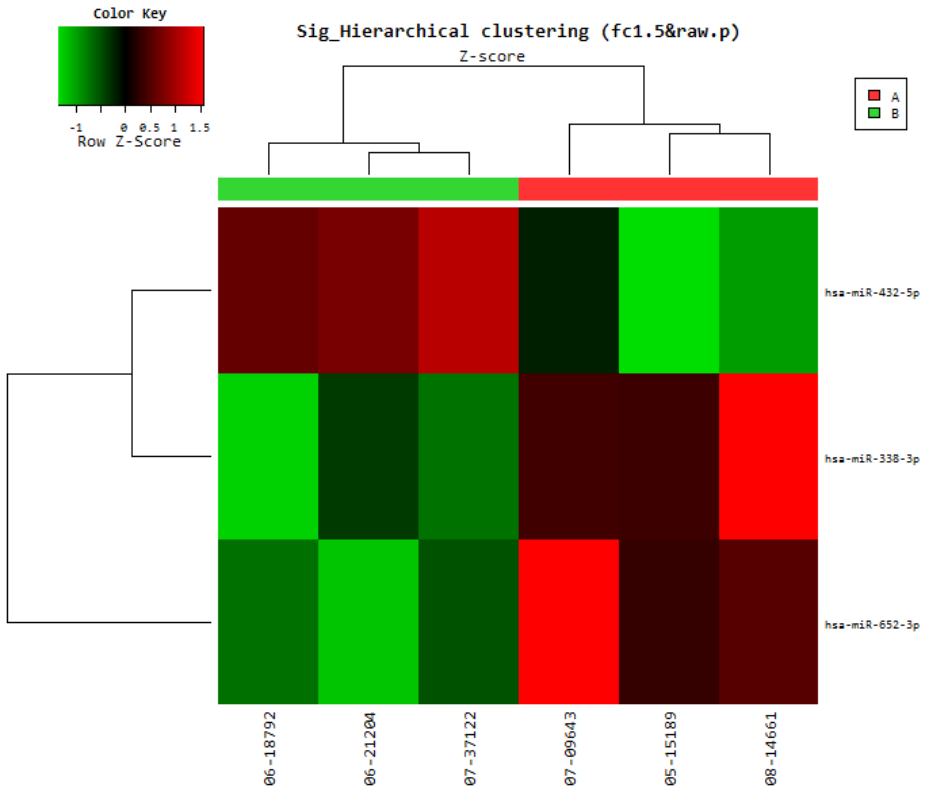

Figure 2. Heat map and unsupervised hierarchical clustering of the differentially expressed miRNAs in pituitary adenomas. Differentially expressed miRNAs between invasive pituitary adenomas versus noninvasive pituitary adenomas. A: invasive (Hardy IV); B: non-invasive (Hardy I).

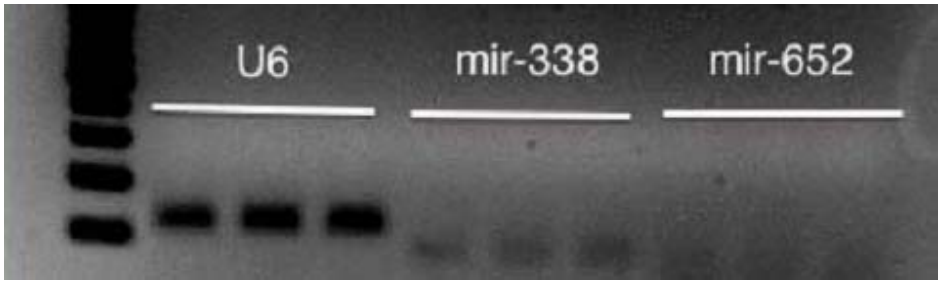

Figure 3. After the estradiol treatment and qRT-PCR, the cells were lysed and total protein extracts were resolved by SDS-PAGE. The results demonstrate the expression of miRNA-338, but do not clearly demonstrate miRNA-652 expression.

Increased miRNA-338 expression in proliferating GH3 cells

We next sought to resolve the effect of increasing concentrations of E2 treatment on mir-338 and mir-652 expression, as well as the time course of mir-338 and mir-652 upregulation after treatment We performed qRT-PCR analysis and the relative fold differences in mRNA levels in E2 and vehicle-treated control cells were calculated. The results from the time course

experiments showed that 10 nM E2 significantly increased miRNA-338 levels after 24 hours of incubation; however, miRNA-652 did not show any difference after E2 treatment (Figure 4). As a positive control we verified that we could observe an effect of E2 treatment in Gh3 cells effect on the expression of prolactin, a well-established E2 target (Figure 5). Because we did not observe a significant change in miRNA-652 levels after E2 treatment in GH3 cell lines, we decided to focus on miRNA-338 expression in these cells.

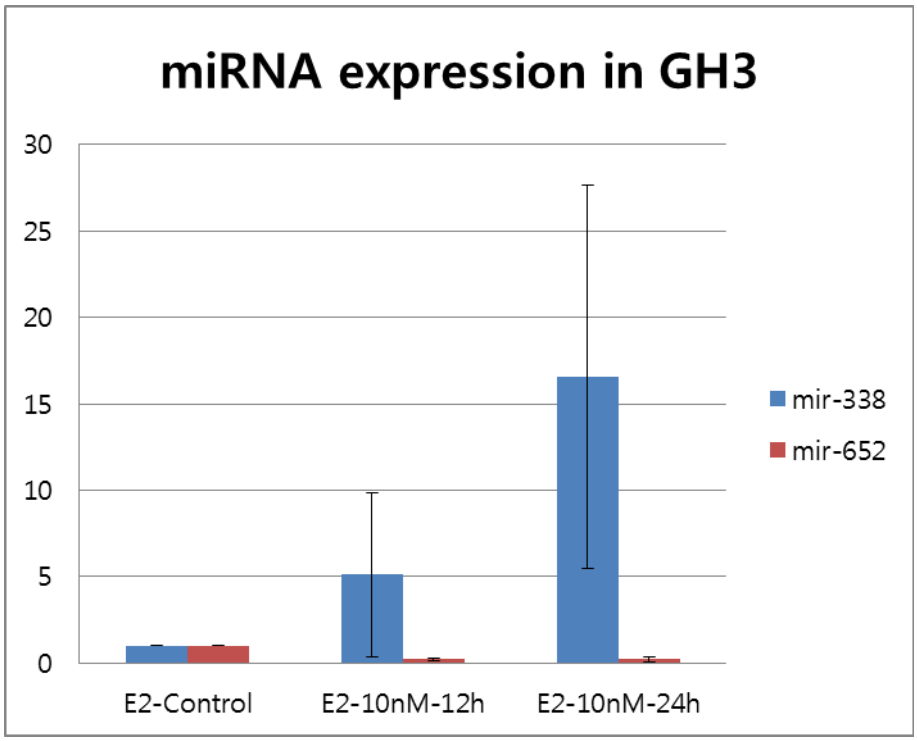

Figure 4. miRNA expression after estradiol treatment in the GH3 cell line.

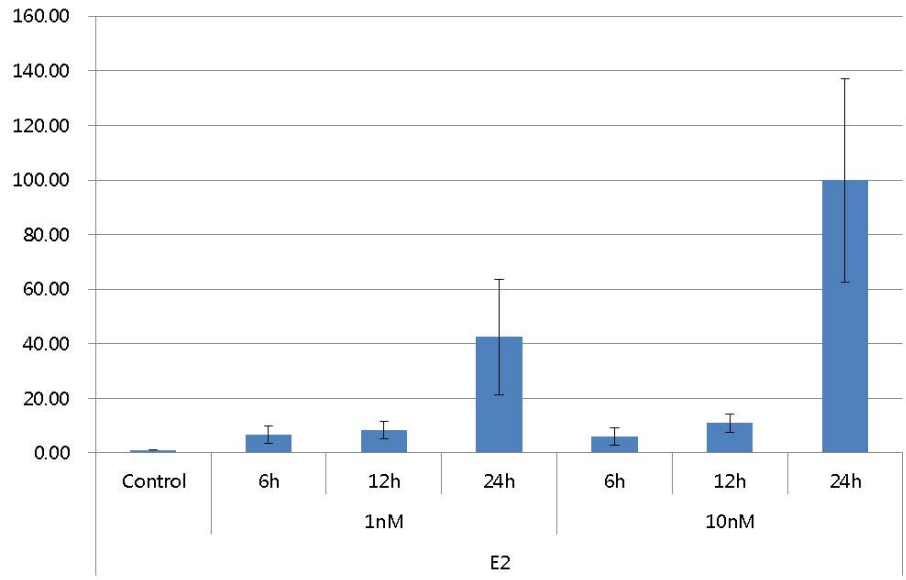

Figure 5. Prolactin expression after estradiol treatment in the GH3 cell line

p27 expression in GH3 cell line after miRNA-338 mimic and inhibitor

As shown in Figure 6, to confirm the results of the array experiments we determined that treatment of the GH3 cells with microRNA inhibitors or mimetics resulted in upregulation or downregulation of miRNA-338 expression, respectively. We seeded 6×10^5 cells on 6-cm plates. After culture for 24 h after cultured in DMEM/High Glucose containing 10% FBS, cells were treated with 30 nM mimetic or 100 nM inhibitor for 48 h. After mimetic treatment, miRNA-338 expression level was highly increased, whereas treatment with an inhibitor led to decreased miRNA-338 expression

levels. To confirm the tumorigenic effect of miRNA-338, we treated GH3 cell lines with miRNA-338 mimetics or inhibitors. Although the effects of the mimetic were not consistent, the inhibitor caused marked down-regulation of p27 (Fig. 6). These results suggest that miRNA-338 might be involved in pathways modulating the invasiveness of the GH secreting pituitary adenomas.

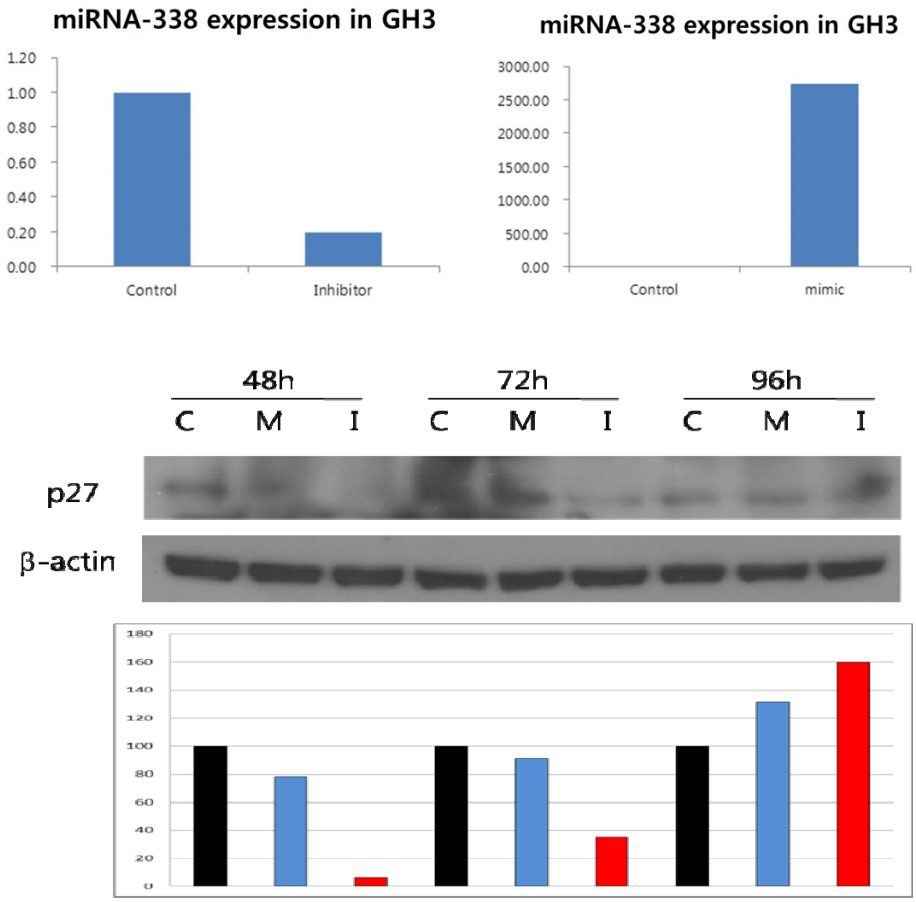

Figure 6. p27 expression in GH3 after with an miRNA-338 mimetic or inhibitor (C: control; M: mimetic; I: inhibitor)

IV. DISCUSSION

Pituitary adenoma is the one of the most common intracranial neoplasm[11]. Typically, pituitary adenoma consists of one of six adenohyopophysial cell types: lactotropes secreting prolactin (PRL), somatotropes secreting GH, corticotropes secreting adrenocorticotropic hormone (ACTH), mammosomatotrophs, gonadotropes secreting luteinizing hormone (LH) and follicle stimulating hormone (FSH), and thyrotropes secreting thyroid stimulating hormone (TSH). The molecular pathways involved in the neoplastic transformation or invasive of pituitary cells remain unknown. Most pituitary tumors are benign, but mass and hormonal effects can have severe consequences, including death[12].

Although GH-secreting pituitary adenomas are never malignant, they may cause significant morbidity, notably acromegaly, and increased mortality due increase levels of GH. Among many clinical indications, surgery is the preferred option for treating most patients with GH secreting pituitary

adenomas. Serum GH levels are controlled within an hour of the complete resection of GH secreting adenomas. Mass excision through the transsphenoidal approach is used most commonly, and, in the hands of experienced neurosurgeons, cures the majority of patients who harbor a well-circumscribed microadenoma with serum GH levels less than 40 μg/L. In general, IGF-1 levels normalize in 80% of patients who have microadenoma and approximately 50% of those who have a macroadenoma after transsphenoidal adenomectomy[13]. Outcome after surgery is closely related to the invasiveness of the pituitary adenoma. In the case of invasive pituitary adenoma, complete surgical excision is sometimes impossible, because adjacent structure invasion can cause surgical morbidity and sometimes mortality. Therefore, controlling the invasiveness of pituitary adenoma is an important determinant of remission after surgery. Nonsurgical options for adenoma remain quite limited. Despite the significant efforts made over the last decade, little is known about the genetic causes of invasive pituitary

adenoma and even less of this knowledge has been applied therapeutically.

Significant research suggests that miRNAs, which are small nucleotide, single stranded non-coding RNA molecules, are important for cancer pathobiology. They serve as post-transcriptional regulators of gene expression by base paring to target messenger RNAs[14]. Although there are several reports on the role of pituitary adenoma pathogenesis, the exact role of miRNA in pituitary adenoma pathogenesis or posttranscriptional mechanisms in pituitary adenoma are still poorly understood[1,10,15]. Recent research revealed that specific miRNAs are dysregulated in pituitary adenoma, and also regulate tumor size, growth, and invasion [10]. The limited expression of miRNA-140 is related to decreased cell growth suggesting that it may promote cell proliferation and tumorigenesis[16,17]. A recent study analyzed 9 ACTH secreting pituitary adenomas and reported that low miRNA-141 expression was associated with a higher likelihood of remission after surgery and suggests that miRNA-141 regulates pituitary adenoma genes involved in

tumor invasion[18]. In another study, researchers analyzed 98 pituitary adenomas and reported that let-7 expression was significantly lower in adenomas of Hardy grade III and IV than in hardy grade I and II, suggesting that let-7 might regulate the invasiveness of pituitary adenoma[19].

In this study, we investigated the role of miRNA in invasion of GH secreting pituitary adenoma. After a retrospective review of the Yonsei pituitary tumor database (single senior neurosurgeon series, Prof S.H, Kim), we selected tissue samples from 6 patients with invasive or non-invasive GH secreting pituitary adenoma. We performed microarray analysis of these samples and found there were several miRNAs (miRNA-432, miRNA-652, miRNA-338) which were correlated with the invasiveness of GH secreting pituitary adenoma. Among the implicated miRNAs, we focused on miRNA-338, as there were too little information regarding miRNA-432 and miRNA-652 to warrant further study. To confirm the expression of miRNA-338, we performed an *in vitro* study using the GH3 cell line (rat GH secreting pituitary

adenoma cell line). After confirmation of the expression of miRNA-338 in the GH3 cell line, we confirmed that miRNA-338 expression is directly proportional to the degree of GH3 proliferation. We then verified the role of miRNA-338 by treating the GH3 cell line with a mimetic or inhibitor of miRNA-338. Finally, the p27 expression after treatment with a mimetic or inhibitor of miRNA-338 were analized to evaluate the biological role of miRNA-338.

In previous work, miRNA-338 has been shown to be associated with human cancer[20-22], suggesting that it may play a role in tumor suppression or oncogenesis of pituitary tumors. However, its role in pituitary adenoma was previously unclear. In this study, several experiments were conducted to evaluate the expression and role of miRNA-338 in pituitary adenoma. Although these findings only demonstrate a correlation between miRNA338 and GH secreting pituitary adenoma, this is to the best of our knowledge the first report showing a potential role of miRNA-338 in pituitary adenoma. It

was found that miRNA-338 is expressed in a GH3 cell line using qRT-PCR and human GH secreting pituitary adenoma tissue. Most notably, it is significantly overexpressed in invasive GH secreting pituitary adenomas.

Our western blot analysis for p27, a cell cycle regulator, after treatment of GH3 cells with a mimetic or inhibitor of miRNA-338 imply that miRNA-338 could be involved in oncogenesis or invasive changes in pituitary adenoma. It is well known that. p27 is the gene encoding Cyclin-dependent kinase inhibitor 1B[23], which controls cycle progression in G1 by binding to proteins in the cyclin E-CDK2 or cyclin D-CDK4 families of cyclin dependent kinase inhibitors. It was shown that p27 expression was markedly decreased after miRNA-338 inhibitor treatment, thus implying that miRNA-338 regulates p27 expression by inhibiting tumor suppressor function of p27. This would subsequently cause adenoma proliferation and invasion. However, understanding the precise mechanism of inhibition of p27 remains a difficult challenge.

V. CONCLUSION

In conclusion, miRNA-338 influences the progression of GH secreting pituitary adenoma and may also regulate invasion and aggressiveness of GH secreting pituitary adenomas, acting as an oncogene. We have identified and experimentally validated p27 as a miRNA-338 target gene and suggest that both p27 and miRNA-338 interact and may play roles in GH secreting pituitary adenoma invasion.

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ABSTRACT (IN KOREAN)

성장호르몬 분비 뇌하수체선종의 침윤과 연관된 생체표지자의 개발에 관한 연구

지도교수 김 선 호

연세대학교 대학원 의학과

조 진 모

Micro RNA (miRNA)는 갑상선암, 전립선암, 췌장암등 많은 종류의 종양의 발생에 관련이 있는 것으로 생각되고 있으며, 이들 종양을 유발하거나 억제하는 과정에 관여한는 것으로

알려져 있다. 하지만 뇌하수체 종양에서 miRNA의 역할에 대해서는 일부 보고된 바가 있으나 아직 명확히 밝혀진 바가 없다. 저자는 특정 miRNA가 성장호르몬 분비 뇌하수체 선종에서 종양의 크기와 침습도와 연관이 있다는 가정을 세우고, 침습형성장호르몬 분비 뇌하수체 선종과 비침습형성장호르몬 분비 선종의 환자 조직 각 3명씩 총 6명의 조직을 이용해 두 군의 miRNA 의 발현에 차이가 있는지를 microarray 를 통해 확인하였다. 두군간 유의하게 발현의 차이를 보인 miRNA는 miRNA-338, miRNA-652, miRNA-432였으며, 이들 miRNA의 존재를 in vitro 상에서 확인하기 위해 쥐의 성장호르몬 분비 뇌하수체 선종 세포주인 GH3 세포주를 이용하여 실험하였다. GH3 세포주에 estradiol을 처리하며 GH3 세포를 증식시킨 후 miRNA의 발현을 관찰하였고, 침습형 성장호르몬 분비 뇌하수체 선종에서 의미있게 발현이 증가하였던 miRNA-338과 miRNA-652의

발현이 증가함을 확인하였다. 이 중 기술적으로 실험이 가능하고 차이가 확연한 miRNA-338를 대상으로 miRNA-338에 대한 mimic 과 inhibitor를 처리한 뒤 세포주기의 조절자로 알려진 p27에 대한 발현여부를 관찰한 결과, inhibitor를 처리후 p27의 발현이 현저히 감소하는 것을 확인 하였다. 결론적으로 이러한 결과들은 miRNA-338이 성장분비 뇌하수체 선종의 침습도에 영향을 줄 수 있는 것을 암시하는 것으로 향후 이에 대한 추가적 연구의 진행이 필요하다 하겠다.

핵심되는 말: 뇌하수체 선종, 말단비대증, microRNA, 성장호르몬, 생체표지자