Target Identification for Thyroid-Associated Orbitopathy through High Throughput RNA sequencing

Wonjin Kim

Department of Medicine

The Graduate School, Yonsei University

Target Identification for Thyroid-Associated Orbitopathy through High Throughput RNA sequencing

Directed by Professor Eun Jig Lee

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Wonjin Kim

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This certifies that the Doctoral Dissertation of
Wonjin Kim is approved.

Thesis Supervisor: Eun Jig Lee

Thesis Committee Member#1: Young Suk Jo

Thesis Committee Member#2: Yong-Wook Cho

Thesis Committee Member#3: Jin Sook Yoon

Thesis Committee Member#4: Sangwoo Kim

The Graduate School
Yonsei University

December 2022

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<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Target Identification for Thyroid-Associated Orbitopathy through High Throughput RNA sequencing

Name

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Eun Jig Lee)

Background: Thyroid-Associated Orbitopathy (TAO) is an autoimmune disorder which is a potentially sight-threatening ocular disease. Because of complexity and autoimmune features of TAO, its pathogenesis remains to be elucidated. The objective of this study was to explore potential biomarkers through high throughput RNA sequencing method.

Method: We applied RNA-sequencing (RNA-seq) to orbital tissue from five TAO subjects who performed orbital decompression surgery for the treatment of TAO and five non-TAO orbital tissues obtained from subjects having other orbital surgery. RT-PCR was performed to validate the gene expression level, we used the orbital tissues obtained from five other TAO patients and four non-TAO controls.

Results: We identified 184 consistently differentially expressed genes comprising 120 up-regulated and 64 down-regulated genes. From those genes, top 10 up-regulated genes and 14 up-regulated genes which were enriched in three pathways, TGF-beta, TNF, and WNT pathways, found by KEGG analysis, were selected. Finally, 24 up-regulated hub genes were used for further validation. Among them, SOCS3 and NR4A1 were revealed as higher expression genes in TAO orbital tissue compared to non-TAO orbital tissue. SOCS3 gene showed greater expression levels in orbital tissues from TAO patients than non-TAO subjects.

Conclusions: In this study, we revealed genes associated with TAO pathophysiology using

high-throughput RNA-seq technology that most of up-regulated genes showed their roles in the regulation of cell transcription and immune processes. Furthermore, genes which found altered levels of expression in TAO subjects might have important role in the disease phenotype and might be the therapeutic target.

Key words: thyroid-associated orbitopathy; Graves' disease; RNA sequencing.

Target Identification for Thyroid-Associated Orbitopathy through High Throughput RNA sequencing

Wonjin Kim

Department of Medicine
The Graduate School, Yonsei University

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I. INTRODUCTION

Graves' disease (GD) is one of autoimmune thyroid disease which thyroid autoantibodies bind to the thyrotropin receptor on thyroid follicular cells, therefore they activate thyroid gland function and induce the production of excess thyroid hormone [1,2]. Up to 60% of GD patients develop a manifestation localized to the eye, known as thyroid-associated orbitopathy (TAO) [1,3,4]. These ocular symptoms and thyroid dysfunctions develop simultaneously or within 18 months of each other in 85% of cases [5,6]. Clinical features of TAO include upper eyelid retraction and edema, proptosis, and erythema of periorbital tissues. As a result of those morphological changes, TAO patients go through intense pain and inflammation, diplopia, eye retraction and sight-threatening compressive optic neuropathy [7].

Because of its complexity of disease pathogenesis, a choice of the best treatment is difficult. Glucocorticoids have been used as the first-line treatment of TAO for several decades [8], because of their anti-inflammatory and immunosuppressive actions, either alone or with orbital radiotherapy. However, proptosis and extraocular muscle involvement with fibrotic changes are poorly responsive [9,10]. Another flaw of glucocorticoid therapy is the long-term side effects, including liver to

xicity and cardiovascular events [11-15].

Orbital radiotherapy is another choice of treatment which showed improvements in diplopia in the combined treatment with glucocorticoids [16]. However, radiotherapy is contraindicated in subjects who have diabetes mellitus or hypertension, which can be at a risk of development of retinopathy [17]. After orbital radiotherapy, there might be worsening of soft tissue inflammation which usually relieved with combined glucocorticoids treatment.

To overcome those side effects of conventional treatment, several research are released and ongoing in order to identify novel therapeutic targets. Rituximab, a chimeric human and mouse anti-CD20 monoclonal antibody, has been tested in TAO and showed promising results [18-20]. Immunosupressants like cyclosporine, azathioprine, intravenous immunoglobulin, and ciamexone have been proven its effectiveness in TAO patients. However, drug side effects and high cost are big burdens to complementing corticosteroids [21].

In severe cases of TAO accompanied with optic neuropathy or irresponsive to medical treatment, decompression surgery of adipose and/or other tissues around the orbit is indicated [22], but the surgical outcome might be unsatisfactory.

The pathogenesis of TAO remains elusive, but it is currently believed that it is related to the autoimmune response in which autoantibodies against thyroid-stimulating hormone receptor and insulin-like growth factor 1 (IGF-1) receptor cross-react with orbital fibroblasts [23]. Several chemokines, such as IL-6, IL-4, IL-16, IL-1B, and monocyte chemotactic factor-1, are released from the activated orbital fibroblasts which further recruit T lymphocytes to the orbit [24], and finally results the perpetuation of orbital inflammation [25].

Previous transcriptomics studies relied on hybridization-based microarray technologies and offered a limited capacity to fully catalogue and quantify the diverse RNA molecules that are expressed from wide ranges of genomes [26]. The introduction of high-throughput next-generation sequencing technologies revolutionized transcriptomics by allowing RNA

analysis through cDNA sequencing at massive scale [27]. RNA-sequencing (RNA-seq) has proven to be useful in the quantitative measurements of gene expression from very small amounts of cellular materials [26]. Few studies about gene expression in TAO subjects were reported using RNA-seq technology [28-30]. Hao *et al*.[28] used extraocular muscle from TAO mouse model and performed RNA-seq and found out SRC was the most significant differentially expressed gene between TAO and controls. Tao *et al*.[29] performed RNA-seq using the orbital adipose-derived stem cells from human active TAO patients who underwent surgery for compressive optic neuropathy or orbital rehabilitation. In another study [30], they used orbital adipose tissue from Caucasian TAO patients who had active phase of disease status and revealed several associated pathways and markers of TAO. Most previous studies were performed in active status of TAO and non-Asian subjects, thus we selected majority of patients with stable in thyroid function and eye status, also with the same ethnicity, Korean.

In the present study, we used RNA-seq to identify the differential gene expression patterns between TAO patients and non-TAO orbital tissues and to find out the potential diagnostic and therapeutic targets for TAO.

II. MATERIALS AND METHODS

Patients

Orbital adipose/connective tissue explants were obtained from severe TAO patients (n = 5; three men and two women, aged 28-61 years) undergoing surgical decompression for severe proptosis associated with increased orbital fat volume, and tissues from five control individuals (three men and two women, aged 30-73 years with no history of TAO or other autoimmune diseases) were obtained in the course of aging lower eyelid blepharoplasty or excision of orbital mass (**Table 1**). Clinical information including age, sex, duration and treatment of GD, duration of TAO, treatment for TAO, and thyroid functions were collected

(**Table 1**). The clinical activity score[31] was determined by a board-certified ophthalmologist based on history and physical examination findings. They were all less than 4 (i.e., all the TAO patients were not in an active inflammatory disease status). Orbital decompression surgery is usually not performed in the active stage, because surgery itself can aggravate proptosis and inflammation. Therefore, all patients were euthyroid status at the time of surgery and had not been treated with radiation or corticosteroids for at least 3 months. The protocol for obtaining orbital adipose/connective tissue was approved by the Institutional Review Board of Severance Hospital, and written informed consent was signed from all patients (IRB No. 4-2014-0292).

Table 1. Clinical characteristics of subjects in the study

	Case 1	Case 2	Case 3	Case 4	Case 5
Age (years)	28	33	61	29	30
Sex	Male	Male	Male	Female	Female
Smoking history	Yes	No	Yes	Yes	no
Duration of TAO prior to surgery (months)	12	156	58	9	8
Treatment for GD	Carbimazole 10mg	Methimazole 5mg	Methimazole 5mg	Methimazole 10mg	Methimazole 20mg
Treatment for TAO	IV steroid	None	PO steroid IV steroid Radiation therapy	Artificial eye drop	none
CAS score (0-10)	3	3	4	3	4

Thyroid function	Euthyroid	Subclinical hypothyroid	Subclinical hypothyroid	Euthyroid	Hyperthyroid
Thyroid stimulating antibody at the time of surgery	Positive	Positive	Positive	Positive	Positive

GD, Graves' disease; TAO, Thyroid-associated orbitopathy; IV, intravenous; PO, per oral; BMI, body mass index; CAS, clinical activity score.

Cell Culture and Differentiation Protocol

Tissue explants are minced and placed directly in plastic culture dishes in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), penicillin (100 U/mL), and gentamycin (20 ug/mL), allowing preadipocyte fibroblasts to proliferate. After fibroblasts have grown out from the explants, monolayers are passaged serially by gently treating with trypsin/EDTA, and cultures are maintained in 80-mm flasks containing DMEM with 10% FBS and antibiotics. Cell cultures are grown in a humidified 5% CO2 incubator at 37℃. The strains are stored in liquid N2 until needed, and they are used between the third and seventh passage.

RNA preparation and sequencing

Total RNA was isolated from orbital tissues using Isol-RNA Lysis Reagent (5 PRIME, Gaithersburg, MD, USA) according to the manufacturer's instructions. First-strand cDNA synthesis from 1 μg total RNA was performed using ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan).

Analysis of RNA-seq data

For RNA-seq data, the distribution of expression values was plotted to identify the peak in the distribution, which can help to estimate the noise in the system. The values were regularized by adding the noise to each gene's expression level before the ratios were calculated. This ensures that genes with low expression do not contribute to the list of genes with large fold changes so that the signature genes can be chosen from the significantly higher and differentially expressed genes ($p<0.05$). The Panther analysis program (http://www.pantherdb.org) was performed to illustrate the top 184 gene expression profiles in TAO and non-TAO orbital tissues.

For the selection of hub genes, we used Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to determine pathways involved in TAO. KEGG pathway analysis revealed that the DEGs were enriched in three pathways, including pathways involved in TGF-beta pathway, WNT pathway, and TNF pathway. From those three pathways, we selected up-regulated genes from each pathway: eight for TGF beta pathway, two for WNT pathway and five for TNF pathway. With those 15 genes, top 10 up-regulated genes from TAO patients were added. Finally, 24 genes except one repeated gene were selected for further analysis. The GeneMANIA online website tool was used to analyze the interconnection of gene clusters.

qRT-PCR

To validate differential gene expression results, qRT-PCR assays for 24 hub genes were performed using a SYBR Green Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols, using the primers described in **Table 2**. We used the orbital tissues obtained from five other TAO patients who performed orbital decompression surgery and four non-TAO subjects that underwent other orbital surgery in order to find out the target genes from selected hub genes. To validate the expression levels of target gene, additional orbital tissues from 18 TAO patients and 16 non-TAO subjects were used.

Table 2. Primer Sequence for RT-PCR

Gene	Sequence (5'→ 3')	Product length	Primer No
FOS		82	1
Forward primer	CAGACTACGAGGCGTCATCC		
Reverse primer	TCTGCGGGTGAGTGGTAGTA		
FOSB		255	1
Forward primer	TGCGCCGGGAACGAAATAA		
Reverse primer	CTGAGCCCGGCAAATCTCTC		
PTGS1		146	1
Forward primer	TCTTGCTGTTCCTGCTCCTG		
Reverse primer	GTCACACTGGTAGCGGTCAA		
JUN		125	5
Forward primer	CCAACTCATGCTAACGCAGC		
Reverse primer	CTCTCCGTCGCAACTTGTCA		
JUNB		260	1
Forward primer	ACTTTTCTGGTCAGGGCTCG		
Reverse primer	GGGTGTCACGTGGTTCATCT		
ID1		255	1
Forward primer	AAACGTGCTGCTCTACGACA		
Reverse primer	GGAACGCATGCCGCCT		
ID2		271	1
Forward primer	TGAAAGCCTTCAGTCCCGTG		
Reverse primer	TGGTGATGCAGGCTGACAAT		
ID3		117	1
Forward primer	AGCGCGTCATCGACTACATT		
Reverse primer	TGACAAGTTCCGGAGTGAGC		
ID4		78	9

Forward primer	TGTGCCTGCAGTGCGATATG		
Reverse primer	CTTTCTTGTTGGGCGGGATG		
SGK1		222	1
Forward primer	TTACTCCAGGATGAGGGGCA		
Reverse primer	GGGCCAAGGTTGATTTGCTG		
IGLL5		290	1
Forward primer	AAGTGGGTTGTGAGACCCCT		
Reverse primer	GGTCCCAGTTCCGAAGACAT		
SIK1		176	1
Forward primer	CTGGCTCGCCAGGTGTG		
Reverse primer	TGGTGCTGTAACTGGAGCAG		
RGS1		136	2
Forward primer	GAGTTCTGGCTGGCTTGTGA		
Reverse primer	ATTCTCGAGTGCGGAAGTCA		
BMP5		134	1
Forward primer	AGCACCAGAAGGATACGCTG		
Reverse primer	GCTTTGGTACGTGGTCAGGA		
BMPR1B		239	1
Forward primer	AGCAAGCCTGCCATAAGTGA		
Reverse primer	CACAGGCAACCCAGAGTCAT		
MYC		96	1
Forward primer	CCCTCCACTCGGAAGGACTA		
Reverse primer	GCTGGTGCATTTTCGGTTGT		
ATF3		147	2
Forward primer	TGATGCTTCAACACCCAGGC		
Reverse primer	GGATGGCAAACCTCAGCTCT		
CXCL2		298	4
Forward primer	TAAAAGGGGTTCGCCGTTCTC		

Reverse primer	GGGGACTTCACCTTCACACTTT		
GDF6		76	1
Forward primer	ACTTGCCCGCCATGGATAC		
Reverse primer	CCGGGCAAATCCCACAGAAA		

III. RESULTS

Profiles of Gene expression in TAO

Total 184 genes annotated underwent appropriate filtering and the differentially expressed genes (DEG) have been identified. Among them, 120 up-regulated and 64 down-regulated genes were identified ($P < 0.05$ and fold change > |1|, Table 3). Biologic process enrichment analysis showed a large prominence of transcription, metabolic process and cell death (Table 4).

We used those genes for further analysis, and their gene expression is illustrated in Figure 1. The circular diagram reveals the percent of genes involved in different biological processes. Most genes were involved on cellular processes (24.2 %), biologic regulations (18.0 %) followed by metabolic processes (14.9 %) in TAO patients. About 6.7 % of genes are involved in immune system process.

Table 3. Screening of 184 commonly differentially expressed genes, including 120 up-regulated and 64 down-regulated genes in orbital tissues of TAO.

DEGs	Genes
Up-regulated	FOSB, NR4A1, FOS, RGS1, IL6, IGLL5, SIK1, NR4A3, ATF3, NR4A2, EGR1, EGR3, CSRNP1, APOLD1, PTGS2, MS4A1, SOCS3, SNAI1, ITLN1, ELK2AP, JUNB, VWA8, EGR2, DUSP1, CYR61, DUSP5, CD79A, F2RL3, CD177, HBEGF, CTH, SOX17, RGS2, ADAMTS1, IER2, C8orf4,

	ADAMTS4, SGK1, JUN, C11orf96, CD69, CXCL2, BTG2, FUT1, ZFP36, ID1, ZNF331, ID3, SLC25A25, MAFF, RGS16, LOC284454/MIR24-2, SLC2A3, WISP1, KLF2, CH25H, USP32P1, TREM1, KLF4, RNF122, GRASP, HLA-DRB5, CDKN1A, NFKBIZ,NXPE3, SLC19A2, DLL1, BMPR1B, PDE4B, ADRB1, GADD45B, DUSP4, GDF6, SELE, CADPS, RSPO2, CTGF, HES1, MYC, RASD1, NFIL3, CXCR4, TIPARP, CHODL, ANKRD36BP2, THBD, NRARP, CEBPD, FOSL2, VAT1L, MCL1, PTGFR, RHOB, HYAL2, TSC22D1, ADRB2, CCRL2, IRF4, SERTAD1, SLC16A9, BMP5, IGJ, CCDC144A, LRRN1, PPP1R15A, SLPI, PIM2, BHLHE40, PIGA, CLDN5, HBA2, PHLDA1, ID2, IER3, CREM, ADAM28, CA3, ZFAND5, ARRDC3, ID4, CNR1
Down-regulated	ADRBK2, C2, MMP2, KCNB1, TF, GAS7, UCHL1, PLBD2, TNXB, WIF1, CLCA2, PRUNE2, AHNAK2, SIGLEC1, CNTN1, IL2RA, KCNJ5, DKK2, THBS2, MRO, MAL2, PTPRQ, TOP2A, SPOCK1, MASP1, LINC01252, BMP7, FAM118A, DHRS9, LBP, ELOVL6, SHOX2, CD209, ZNF385B, APOC1, LINC01239, COL1A1, ANO3, CFP, PKD1L2, MARCO, ECM1, SCN2A, UBD, PAPPA2, COBL, UNC13C, EGFL6, MOXD1, FCER2, CPXM1, CCL13, SSC5D, SPP1, FRZB, FASN, GALNT5, SCD, ABCB5, CETP, COL6A6, CCL18, KRT4

Table 4. Significantly enriched biologic processes among the differential expression genes.

Pathway ID	Number of gene	Name of pathways	P-value
GO:0045449	62	Regulation of transcription	1.09E-07
GO:0051252	48	Regulation of RNA metabolic process	3.47E-07
GO:0006350	48	Transcription	2.15E-05

GO:0006355	46	Regulation of transcription, DNA-dependent	1.23E-06
GO:0007166	35	Cell surface receptor linked signal transduction	0.010935955
GO:0010033	32	Response to organic substance	9.01E-10
GO:0006357	30	Regulation of transcription from RNA polymerase II promoter	1.83E-08
GO:0010605	25	Negative regulation of macromolecule metabolic process	1.15E-05
GO:0042981	25	Regulation of apoptosis	5.05E-05
GO:0043067	25	Regulation of programmed cell death	5.90E-05
GO:0010941	25	Regulation of cell death	6.25E-05

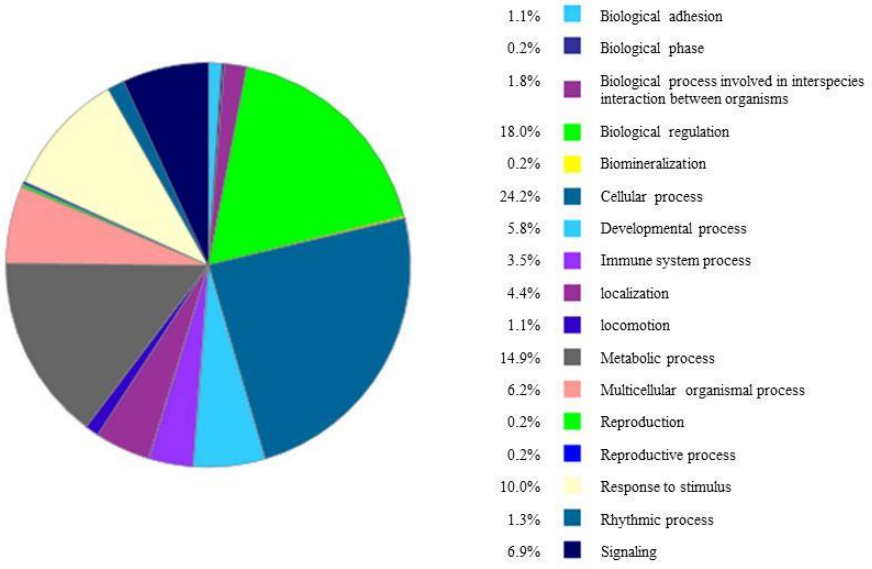

Figure 1. Genes expressed in orbital tissues from TAO patients.

KEGG pathway analysis

KEGG is a collection of databases encompassing data of genomes, pathways, diseases, and chemical substances. KEGG pathway analysis revealed that the DEGs were associated several pathways. Among them, we selected TGF-beta pathway which was one of top 10 ranked pathways. Then, WNT and TNF pathways which had close crosstalk with TGF-beta signaling pathway were included. Finally, three pathways, TGF-beta, WNT, and TNF pathways were chosen for further analysis. In the TGF- beta pathway, ID1, ID2, ID3, ID4, GDF6, BMPR1B, MYC, and BMP5 found to be up regulated. In the WNT signaling pathway, SOCS3 and SGK1 were up-regulated and DKK2 and PPARGC1B showed down-regulation. In the TNF pathway, five genes, PTGS2, SOCS3, CXCL2, JUN, and JUNB represented up-regulation.

Highly expressed genes in TAO

Then, we selected the top 10 up-regulated genes from TAO subjects (Table 5). The fold changes of top 10 up-regulated genes were between 3.60 – 5.05. FOSB showed 5.05-fold which showed the highest gene expression. NR4A1 and FOS were followed by 4.42 and 4.36 folds, respectively. The expression levels of each gene are described in the Table 4.

Table 5. Differentially expressed genes from orbital tissues of TAO.

Function	Gene	Fold Change	P Value
Regulation of transcription	FOSB	5.05	<0.05
	NR4A1	4.42	<0.05
	FOS	4.36	<0.05
Immune response	RGS1	4.13	<0.05
	IL6	4.11	<0.05
	IGLL5	4.07	<0.05
Regulation of cell differentiation	SIK1	3.86	<0.05
Regulation of transcription	NR4A3	3.82	<0.05

	NR4A2	3.60	<0.05
	ATF3	3.60	<0.05

Network analysis

Using the GeneMANIA online tool, we found complex interactions between 24 hub genes. Seventy percent of genes showed co-expression, and 17.64% found physical interactions.

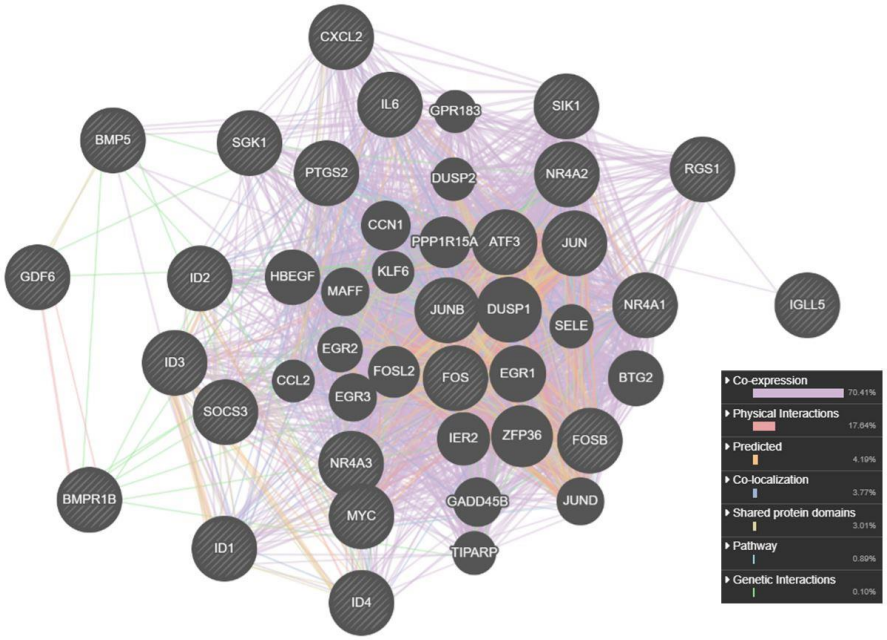

Figure 2. The network of 24 hub genes constructed by GeneMANIA.

Validation: RT-PCR

We selected 24 hub genes from top 10 DEGs and KEGG pathway analysis. SOCS3, NR4A1, FOS, FOSB, RGS1, ATF3, and CXCL2 showed significant expression in TAO subjects compared to non-TAO orbital tissue. Among them, NR4A1 and SOCS3 expression levels were remarkably upregulated in TAO samples compared to non-TAO samples (Figure 3).

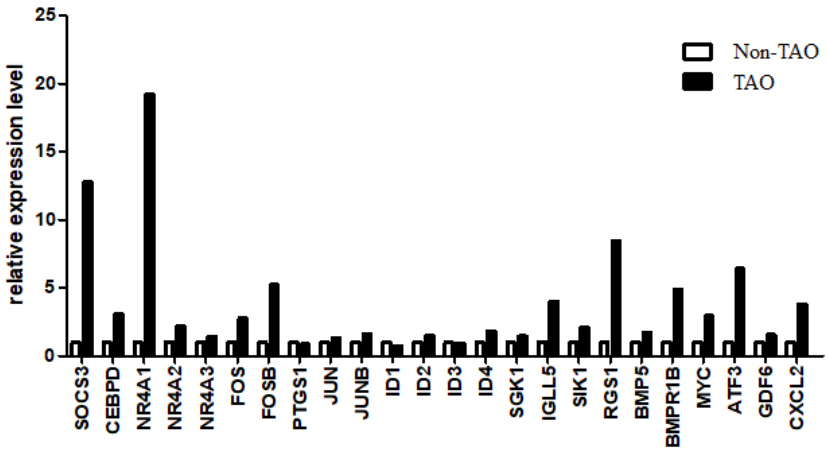

Figure 3. Expression levels from 24 hub genes in the orbital tissues from TAO patients compared with non-TAO orbital tissue.

Increased Gene Expression of SOCS3 in TAO Tissues

To investigate the potential role of those genes, we measured the expression level of SOCS3 in orbital tissues obtained from TAO and non-TAO subjects. The RT-PCR results showed that gene expression levels of SOCS3 were higher in TAO tissues (n=17) compared with non-TAO (n=15) subjects (Figure 4). Also, relative gene expression levels were greater in TAO patients (Figure 5).

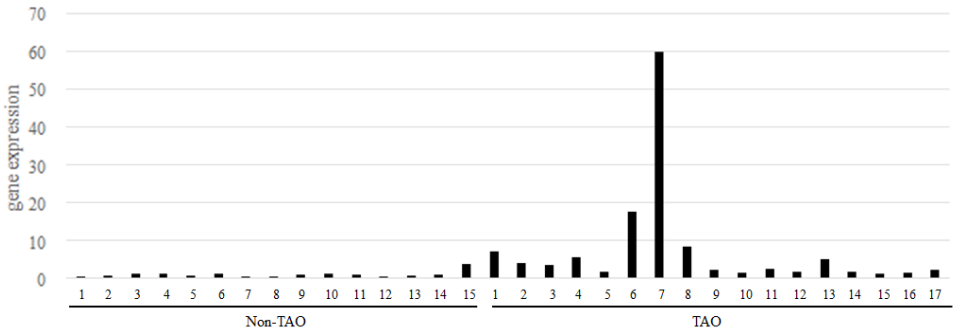

Figure 4. Gene expression level of SOCS3 in TAO and non-TAO tissues.

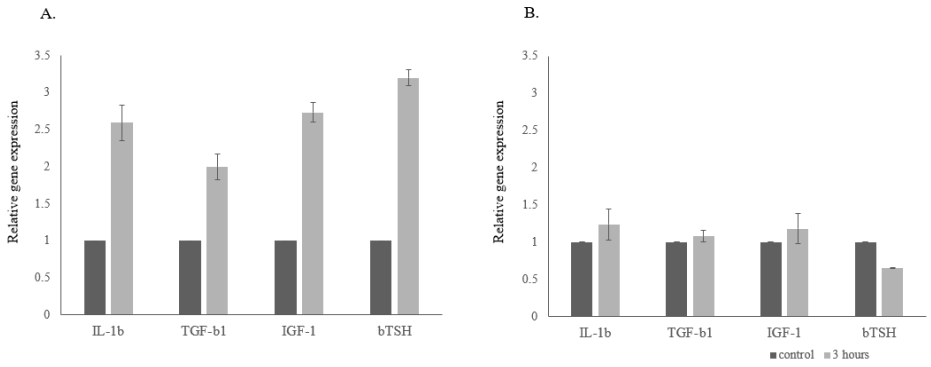

Figure 5. Relative gene expression of SOCS3 in (A) TAO patients and (B) non-TAO subjects.

IV. DISCUSSION

In this study, we revealed genes associated with TAO using high throughput RNA sequencing technology. Most of up-regulated genes were involved in the regulation of transcription, and metabolic processes. Among them, top 10 DEGs showed they are involved in regulation of transcription and/or immune responses. As we used those 10 DEGs with selected 14 DEGs which were enriched in three pathways, TGF beta pathway,

WNT pathway, and TNF pathway, for further analysis, NR4A1 and SOCS3 were highly expressed in the orbital tissues from TAO compared to non-TAO, control. In further RT-PCR results, SOCS3 showed greater gene expression in orbital tissued of TAO patients.

TAO is a complex disease resulted from autoimmune process which results from the production of autoantibodies to the thyrotropin receptor. It has many susceptible genes [32-34] and associated with T cells [35,36] and B cells [37]. Because of its nature of the autoimmune disorders, it is well-known that inflammatory and immunologic pathways were activated. Yin *et al*.[38] reported overexpression of the antigen presentation pathway and active innate and adaptive immune signaling networks using mRNA-sequencing method in the thyroid tissues from GD patients. They emphasized the influences of autoimmune responses to the innate and adaptive immune systems in GD.

Previous studies reported several transcriptomic analyses in TAO, mostly using microarray. Microarray is a useful technique for gene expression profiling. In a single gene expression profiling method, the expression levels of thousands of genes can be simultaneously determined compared to real-time PCR. Various studies about associated genes in periorbital tissues or adipose tissues of TAO were reported using microarray. Han *et al*.[39] demonstrated an interaction between both Th1 and Th2 cytokines and IL-1ß-driven PGHS-2 and HAS gene expression in orbital fibroblasts of TAO which showed the cytokine milieu existing in TAO. Adipocyte-related immediate early genes were overexpressed in intraorbital adipose tissue of active TAO patients, such as CYR61, BTG2, ZFP36, EGR1 and DUSP1[40], which were also up-regulated DEGs in our study. They assumed that CYR61 may have a role in orbital adipogenesis, so it can serve as a marker of disease activity. BTG2 and DUSP1 might have a role in antiproliferative effects which were found in other cell systems [40]. Other study identified the potential role of CASQ2 which triggers autoimmunity events [41]. These studies have provided various associated genes of TAO.

In this study, two genes, NR4A1 and SOCS3, among 24 hub genes, showed higher expression levels in the periorbital adipose tissues of TAO compared to non-TAO orbital

tissues. NR4A1, nuclear receptor subfamily 4 groups A member 1 which is also known as Nurr77, TR3, and NGFI-B, is a transcription factor which is stably highly expressed in tolerant T cells. It plays a key role in mediating inflammatory responses. Overexpression of NR4A1 is known to inhibit effector T cell differentiation, so that deletion of NR4A1 overcomes T cell tolerance, exaggerates effector function, so it enhances immunity against tumor or chronic virus [42]. In other study [43], temporary upregulation of TGF-ß induces NR4A1 expression in normal wound healing process, which terminates TGF-ß signaling to prevent prolonged and uncontrolled activation of fibroblast. Several studies also reported NR4A1 is associated with inflammation and fibrosis of various organs [44-46].

For moderate-to-severe and active TAO, the combination of cyclosporine and oral glucocorticoids is a valid second-line treatment [8]. Cyclosporine is a calcineurine inhibitor (CNI) immunosuppressant which is known as a regulator of regulatory T cell differentiation, expansion and function [47,48]. Sekiya *et al*. [49] reported that NR4A1 plays a pivotal role in regulatory T cell differentiation in the presence of CNI, cyclosporine. NR4A1 was not suppressed by treatment of CNI, so it can mediate regulatory T cell differentiation in the presence of CNI. So, upregulated NR4A1 might be one of the targets for the treatment of TAO.

SOCS3, suppressor of cytokine signaling 3, is known as an important regulator of inflammatory disease [50]. It is a negative modulator of the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway [51]. STAT3 is recognized as a master transcription factor which controls the lineage commitment to Th17. Activated STAT3 induces the expression of transcription factors in Th17 differentiation, and the ablation of STAT3 in T cells impairs Th17 cell differentiation, skewing them toward anti-inflammatory regulatory T cells [52]. SOCS3-medicated control of signaling from cytokine receptors can have profound effects on the regulation of immunity and inflammation by affecting the development, activation and homeostatic functions [53]. SOCS proteins, especially SOCS1 and SOCS3, are reported that they are associated with several autoimmune diseases, including rheumatoid arthritis, type 1 diabetes, systemic lupus

erythematosus, psoriasis and multiple sclerosis [54]. It regulates cytokine or hormone signaling usually preventing diseases, but in some cases it aggravates diseases [55].

In the thyroid cancer cells, SOCS3 expression was significantly associated with reduction in tumor growth *in vivo* [56]. Ezra *et al*.[57] reported that IGF-1 signaling genes were dominantly expressed in the orbital fat tissues of TAO subjects using microarray. Those genes were IGF-1, SOCS3 and IRS2 which are IGF-1 receptor binding/signaling genes. This study also revealed that SOCS3 was one of the up-regulated genes in subjects of TAO compared to non-TAO tissues which might be function as a cytokine-induced negative regulator of cytokine signaling.

Although microarray is a good method to measure the expression levels of large numbers of genes simultaneously, this method has limitations, such as reliance upon existing knowledge about previous genome sequence, high background levels owing to cross-hybridization [58,59], and difficulty to reproduce expression levels across different experiments. Taking all these disadvantages into account, RNA-seq compensates those limitations which allows the entire transcriptome to be analyzed in a very high-throughput and quantitative way [60]. It investigates the entire transcript and gives total expression levels of each gene and ratio between different isoforms or splice variation [61]. As RNA-seq is considered a new technology overviewing various gene expressions, several studies with various diseases or organs were performed.

Tao *et al*.[29] used orbital adipose-derived stem cells from TAO patients and reported the upregulated genes in adipogenesis. They also found downregulation of early neural crest markers and ectopic expression of HOXB2 and HOXB3 which demonstrate dysregulation of pathways of development and tissue patterning. RNA-seq performed in thyroid tissues of GD patients showed overexpression of the antigen presentation pathway of HLA and related genes, and active innate and adaptive immune signaling pathways [38]. Orbital adipose tissue from three TAO patients showed 328 DEGs associated with active TAO many of which were responsible for mediating inflammation, adipogenesis, cytokine signaling, glycosaminoglycan binding and IGF-1 signaling [30]. As TAO has complicated

disease pathophysiology involving inflammation and/or adipogenesis, most studies performed in Graves' patients showed higher expression of genes associated with inflammation and/or adipogenesis or related signaling pathways.

The present study had several limitations. First, the sample size was too small. Despite this limitation in sample size, this study identified significant changes in expression of genes using high throughput RNA sequencing technology in the orbital tissues from TAO subjects. Second, orbital tissues obtained from the patients of TAO may not directly reflect *in vivo* status. There are several different inflammatory and/or oxidative mechanisms happens *in vivo* tissues [62,63], cultured orbital tissues might be incomplete to analyze whole disease entity. Third, patients were not within the same status of disease which might affect status of orbital tissues. Moreover, differences of age, sex and other factors would have influenced the results. We couldn't perform additional experiments with adjustment of those factors due to time limit, so further studies as designed with same clinical conditions of disease status might be needed.

V. CONCLUSION

In conclusion, we revealed genes associated with TAO using high-throughput RNA sequencing technology which was involved in the regulation of cell transcription and immune processes. Among twenty-four hub genes, SOCS3 revealed higher gene expression in TAO patients compared with non-TAO subjects. These data might suggest new directions for future studies and lead to potential diagnostic or therapeutic targets for TAO patients.

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APPENDICES

<Abbreviations: in alphabet order>

DEG: differentially expressed genes

FBS: fetal bovine serum

GD: Graves' disease

IGF-1: insulin-like growth factor

KEGG: Kyoto Encyclopedia of Genes and Genomes

RNA-seq: RNA-sequencing

TAO: Thyroid-associated orbitopathy

High Throughput RNA sequencing을 통한 갑상선 안구병증 관련 표적 물질 발굴

<지도교수 이 은 직>

연세대학교 대학원 의학과

김 원 진

배경: 갑상선 안구병증은 갑상선 기능항진증의 가장 많은 원인인 그레이브스병과 함께 발생할 수 있는 안구의 질환으로, 안구통증, 복시, 시야장애 등의 다양한 질환의 경과를 가질 수 있다. 갑상선 안구병증은 자가면역질환의 하나로, 아직까지 질환의 병태생리가 명확히 밝혀져 있지 않다. 따라서 본 연구에서는 갑상선 안구병증의 진단 또는 치료의 유전적인 바이오마커를 확인하고자 high throughput RNA 시퀀싱을 이용하여 분석 하였다.

방법: 갑상선 안구병증을 진단받고 안와 감압술(orbital decompression surgery)를 시행받은 환자 5명의 안구 조직과 다른 안과적 질환으로 안와 수술을 받는 대조군 5명의 안구 조직을 이용하였다. 확인된 유전자들의 유전자 발현의 정도를 검증하기 위해 RT-PCR 방법을 사용하였다.

결과: 총 184개의 유전자를 확인하였고, 그 중 120개의 상향 조절 유전자(up-regulated gene)와 64개의 하향 조절 유전자(down-regulated gene)을 확인하였다. 184개의 유전자 발현과 연관된 경로를 확인할 수 있는 KEGG 분석을 이용하여 TGF-베타 경로, TNF 경로, WNT 경로가 연관성 있는 경로였고, 이 경로와 연관된 14개의 상향 조절 유전자와 전체 184개 중 상위 10개 상향 조절 유전자를 선택하여 추가 분석을 시행하였다. 24개의 중추 유전자(hub gene)들을 RT-PCR로 검증하였을 때, SOCS3와 NR4A1이 대조군 대비하여 갑상선 안구병증 환자들의 안구 조직에서의 발현이 높았다. SOCS3의 경우 갑상선 안구병증에서의 유전자 발현이 상향되어 있음을 추가적으로 확인할 수 있었다.

결론: 갑상선 안구병증의 병태생리에 관여할 수 있는 여러 가지 유전자들의 발현을 RNA-시퀀싱 방법을 통해서 확인할 수 있었고, 대부분이 세포의

전사과정이나 면역반응에 관여하는 유전자임을 알 수 있었다. 추후 추가적인 실험과 분석을 통하여 갑상선 안구병증의 질환의 특성, 진단적 마커 또는 치료 타겟이 될 수 있는 마커 유전자를 확인할 수 있는 연구가 필요할 수 있겠다.

핵심 되는 말: 갑상선 안구병증, 그레이브스병, RNA 시퀀싱, 유전자

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