BIOINFORMATIC ANALYSIS IDENTIFIES POTENTIALLY KEY DIFFERENTIALLY EXPRESSED GENES AND PATHWAYS IN ORBITAL ADIPOSE TISSUES OF PATIENTS WITH THYROID EYE DISEASE

F.F. Zhu¹, L.Z. Yang^{1,*}

¹Shanghai Ninth People's Hospital, affiliated Shanghai Jiaotong University School of Medicine - Division of Endocrinology and Metabolism, Shanghai, China

Abstract

Context. Thyroid eye disease (TED), an orbital inflammatory status, generally occurred in Graves' disease.

Objective. This study aimed to acquire further insight into molecular mechanisms of TED, especially several key involved genes and pathways.

Design. The microarray dataset GSE58331 including expression data for orbital adipose tissue samples, isolated from TED patients and normal controls, was downloaded from a publicly accessible Gene Expression Omnibus database. Differentially expressed genes (DEGs) were identified from 23 adipose tissues of TED patients *versus* 20 samples from normal controls.

Subjects and Methods. A protein-protein interaction network of DEGs was constructed by using Search Tool for the Retrieval of Interacting Genes and Cytoscape 3.6.0. Several hub genes/proteins were extracted from the protein-protein interaction network based on connectivity degree. Furthermore, we used the iRegulon plugin of Cytoscape3.6.0 to predict the transcription factors (TFs).

Results. A total of 678 DEGs (538 up- and 140 down-regulated genes) were identified in TED patients. Proopiomelanocortin (POMC), interleukin 2 (IL-2), G protein subunit gamma 3 (GNG3), CXC motif chemokine receptor 4 (CXCR4), toll like receptor 4 (TLR4), colony stimulating factor 1 receptor (CSF1R), lysophosphatidic acid receptor 3 (LPAR3), CXC motif chemokine ligand-8 (CXCL8), etc., were considered as the hub genes among the DEGs. There were 6 TFs predicted to be differentially expressed in regulating the DEGs related to TED. A total of 71 DEGs had been reported to be associated with TED in the Comparative Toxicogenomics Database.

Conclusions. Through this analysis, we have identified plenty of potential biomarkers and pathways which may have an important role in the pathogenesis of TED. However, these findings require verification by more detailed future experimental studies.

Key words: Thyroid eye disease, Differentially expressed gene, Protein-Protein Interaction, Orbital adipose tissue, Transcription factors Pathway.

INTRODUCTION

Thyroid eye disease (TED), an orbital inflammatory status, generally occurred in Graves' disease (1-3). It is mainly identified by a series of clinical disorders including redness and swelling of eyelid and conjunctiva, excessive tearing, retrobulbar discomfort, upper eyelid retraction, and even sight loss (4, 5). TED is a rare disease, and its estimated prevalence is approximately 0.25% (6, 7). Sex difference in its incidence has been noted with female having higher risk than male (6-9). Clinically, the severity of Graves' disease, genders, radiotherapy and clinical course of thyroid disorder do not influence the consequence of TED (10, 11). Due to the severe adverse effects and complication of existing therapies including orbital radiation and surgery as well as immunosuppression, the satisfying treatments for TED remain challenging (12-14).

Pathogenesis of TED is becoming increasingly clear, however, details still remains to be clarified. Hypertrophic orbital extraocular tissues and muscles of TED patients are consequences of the accumulation of hyaluronan (HA) and glycosaminoglycan (15, 16). Additionally, orbital fibroblast can be transformed into adipose tissue inducing orbital hyperplasia and hypertrophy (17, 18). Many genetic studies have demonstrated the links between genetic variants and TED. Park HJ *et al.* reported that ST8SIA1 variant was associated with the severity of TED (19). Currently, researchers have found many differentially expressed genes (DEGs) related to TED (15, 20-23). Moreover, advances in high technology have brought some new ways to explore the multifaceted molecular basis of TED. However, little attention has been paid to explore the links among the DEGs and the possible underlying molecular mechanisms.

*Correspondence to: Li-Zhen Yang MD, Shanghai Jiao Tong University School of Medicine, Shanghai Ninth People's Hospital, Division of Endocrinology and Metabolism, 639 Zhizaoju Road, Shanghai, China, 200011, China, E-mail: dryanginsh@yahoo.com

Bioinformatic analysis is a powerful tool to explore the characteristics of biology at a system level and the relationship of different functional elements, such as protein-protein interaction (PPI) network. The GSE58331 dataset was produced from Rosenbaum JT and his colleagues. The pathway enrichment and Gene Ontology (GO) analyses regarding the DEGs can be employed to recognize DEGs in TED patients *versus* normal individuals. Some genes related to TED have been annotated by literature mining. Our study tries to explore the underlying molecular mechanisms of TED.

MATERIALS AND METHODS

Source of database

In our study, a GSE58331 data set, which contained 175 samples (lacrimal gland and anterior orbital adipose tissues) from 83 individuals (25 cases with TED, 7 cases with sarcoidosis, 6 cases with Wegener granulomatosis, 25 cases with nonspecific orbital inflammation, and 20 cases with unknown orbital disorder), was obtained from the database of the Gene Expression Omnibus (GEO). In some cases, more than one biopsy samples were obtained from the same subject, and in some cases the samples were analyzed twice. Of note, orbital adipose tissues from 23 TED patients and 20 normal controls were included into the present study for subsequent analysis. Moreover, the annotation files for the platform of this microarray data, GPL570 Affymetrix Human Genome, were also obtained from GEO.

Data pre-processing

The genetic expression normalization and the related background corrections for the pre-processing procedure of raw CEL files were conducted via the affy package of Bioconductor in R3.2.2. Meanwhile, multiple probes corresponding to one gene symbol were simplified as a single one through using the averaging value. There were 54,000 probes in the microarray and a total of 9,660 genes symbol expression had no duplicate after processing.

Extraction of DEGs

The DEGs among TED and control samples were analyzed with a limma package in Bioconductor. The Student's t-test was performed to analyze the gene expression, especially P value as well as adjusted P value of gene expression data in the DEGs analysis. The genes which satisfied the corresponding conditions

were considered to be DEGs: adjusted P value < 0.05 and $|\log_2(\text{FC})| > 0.58$ equated with 1.5 FC. A heatmap of DEGs created by the gplots package in R visualized the expression values.

Gene ontology and enrichment analyses

After recognizing DEGs, the pathway enrichment and GO analyses were conducted. For the functional GO analysis, the datasets of Annotation, Visualization and Integrated Discovery and Kyoto Encyclopedia of Genes and Genomes (KEGG) were employed. The GO enriched analysis covers 3 contents as follows: biological processes, cellular components and molecular function. A P value < 0.05 was considered statistically significant.

Construct the PPI network

For exploring the underlying pathomechanisms of TED, DEG PPI network was constructed through a STRING dataset, which provided further messages by analyzing multi-source genomic data. The list of DEGs was mapped into PPIs. The combined score of the interaction between selected genes more than 0.4 was applied to establish PPI network. The genes were ranked by the connectivity degrees, and the top 15 were defined as key genes of PPI network. To visualize the PPI network, we employed a software of Cytoscape (version 3.6).

Transcription factors associated with TED

The comparative toxicogenomics dataset (CTD), a public accessible resource, can offer some useful messages regarding the relationships between disease and gene/protein across different species. The present study used the Graves Ophthalmopathy rather than TED as a keyword inputted into CTD to assess if there were unknown DEGs associated with TED/GO. Then the Cytoscape with iRegulon was used to evaluate the transcription related to genes. The parameters of the targets module and predicted regulators included minimal consistency between homologous genes (0.05) and maximal error detection rate of motif similarity (0.001). Finally, TFs and desired genes paired Normalized Enrichment Score > 3.0 would be selected.

RESULTS

DEGs in TED versus normal orbital adipose tissues

The present study identified 678 DEGs (538 up-regulated and 140 down-regulated) in TED samples

Figure 1. Heat map of DEGs. The gene expression data are presented in matrix format. Rows represent individual genes and columns represent each sample. Green color represents a lower expression and red color represents a higher expression. Black color indicates no differential expression.

Figure 2. Protein-protein interaction network of the DEGs. The network contains 606 nodes and 839 edges. The white nodes represent up-regulated genes and the gray nodes indicate down-regulated genes.

versus control samples according to the Student's t-test analysis. There were obvious differences of these genes expression between the two groups (Fig. 1).

GO and KEGG

The present study investigates if cellular functions were affected by these DEGs, hence KEGG pathway and GO were analyzed. The up-regulated DEGs enriched in the GO terms with positive regulation of cellular proliferation, positive regulation of T cell proliferation and calcium channel activity. Several enriched pathways of up-regulated DEGs included Rap1 and AKT/ phosphoinositide 3 kinase (PI3K-Akt) signaling pathways and cytokine-cytokine receptor interaction etc. (Table 1).

The DEGs down-regulation was related to the negative regulation by osteoclast differentiation, peptidyl-tyrosine phosphorylation, cell proliferation, metalloproteinase activity and Ras signaling pathway (Table 2).

PPI network

Based on the STRING database and > 0.4 than the scoring standard, we found the PPI network with 839 edges and 606 nodes. These edges and nodes represented the mutual effects between the up-regulated genes and down - regulated genes respectively (Fig. 2). Some hub genes extracted from the PPI network were as follows: CXC motif chemokine ligand-8 (CXCL8),

Figure 3. Transcription regulatory network of DEGs. The white nodes represent up-regulated genes and the gray nodes indicate down-regulated genes. Triangular nodes indicate transcription factors (TFs) and round nodes denote target genes

Table 1. Top 5 GO terms and pathways enriched by up-regulated DEGs (P <0.05)

Category	Term	Count	P-Value
BP	GO:0008284~positive regulation of cell proliferation	24	1.19E-03
	GO:0048286~lung alveolus development	6	1.33E-03
	GO:0001836~release of cytochrome c from mitochondria	5	2.17E-03
	GO:0042104~positive regulation of activated T cell proliferation	5	3.97E-03
	GO:0097070~ductus arteriosus closure	3	5.69E-03
CC	GO:0005576~extracellular region	67	1.39E-05
	GO:0005887~integral component of plasma membrane	58	9.24E-05
	GO:0005886~plasma membrane	130	5.63E-04
	GO:0030424~axon	13	7.87E-03
	GO:0031528~microvillus membrane	4	1.02E-02
MF	GO:0005262~calcium channel activity	8	1.61E-03
	GO:0005198~structural molecule activity	15	3.22E-03
	GO:0051400~BH domain binding	3	5.71E-03
	GO:0001664~G-protein coupled receptor binding	6	1.10E-02
	GO:0003779~actin binding	14	2.01E-02
PATHWAY	hsa04060: Cytokine-cytokine receptor interaction	14	3.12E-03
	hsa04151: PI3K-Akt signaling pathway	18	3.24E-03
	hsa04978: Mineral absorption	6	4.43E-03
	hsa04015: Rap1 signaling pathway	11	2.74E-02
	hsa04360: Axon guidance	8	3.10E-02

BP, biological process; CC, cellular component; MF, molecular function.

Table 2. GO terms enriched by down-regulated DEGs (P <0.05)

Category	Term	Count	P-Value
BP	GO:0045671~negative regulation of osteoclast differentiation	3	1.07E-02
	GO:0018108~peptidyl-tyrosine phosphorylation	5	1.82E-02
	GO:0044691~tooth eruption	2	1.95E-02
	GO:0009887~organ morphogenesis	4	2.25E-02
	GO:0008285~negative regulation of cell proliferation	7	4.59E-02
CC	GO:0016020~membrane	26	5.95E-03
	GO:0005794~Golgi apparatus	12	3.17E-02
	GO:0005576~extracellular region	18	4.27E-02
MF	GO:0008237~metallopeptidase activity	5	2.00E-03
	GO:0000978~RNA polymerase II core promoter proximal region sequence-specific DNA binding	8	9.01E-03
	GO:0005178~integrin binding	4	3.20E-02
	GO:0001875~lipopolysaccharide receptor activity	2	3.25E-02
	GO:0008131~primary amine oxidase activity	2	3.88E-02
PATHWAY	hsa04014: Ras signaling pathway	5	4.73E-02

BP, biological process; CC, cellular component; MF, molecular function

proopiomelano-cortin (POMC), interleukin 2 (IL-2), G protein subunit gamma 3 (GNG3), toll like receptor 4 (TLR4), colony stimulating factor-1 receptor (CSF1R), lysophosphatidic acid receptor 3 (LPAR3), CXC motif chemokine receptor 4 (CXCR4), vascular cell adhesion molecule 1 (VCAM1), relaxin 3 (RLN3), somatostatin (SST), phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3), cholecystokinin (CCK), somatostatin

receptor 2 (SSTR2) and integrin subunit beta 3 (ITGB3) (Table 3).

Transcription factors and genes

Currently, the CTD dataset stores 1733 marker/mechanism or therapeutic genes related to GO/TED (download time: 2018.03.17). Among them, the two marker genes associated with GO/TED were

stearoyl-CoA desaturase (SCD) and prostaglandin-endoperoxide synthase 2 (PTGS2).

Thyroperoxidase activity is associated with thyroid hormone level(24), and the supplement of thyroxine significantly increases peroxidation (25). In addition, peroxisome proliferator activated receptor γ may also play a role in TED(26). However, both PTGS2 and SCD were not differently expressed in this analyzed microarray dataset. Furthermore, based on CTD database, this study inferred that a total of 71 genes out of 678 DEGs were related to the development of TED (Table 4).

A transcription regulatory network of DEGs was re-engineered by the Cytoscape 3.6.0. with iRegulon. There were 6 important TFs obtained from the above network: forkhead box N4 (FOXN4), hepatocyte nuclear factor 4 alpha, myocyte enhancer factor 2A (MEF2A), MAF b ZIP transcription factor (MAF), serum response factor (SRF) and nescient helix-loop-helix 1(NHLH1) (NSE>3.0).

Table 3. The hub genes and corresponding degrees

Expression changes	Gene symbols	Degree
Down	TLR4	25
	CSF1R	21
	VCAM1	19
Up	IL8	45
	POMC	30
	IL2	30
	GNG3	26
	CXCR4	25
	LPAR3	20
	RLN3	19
	SST	17
	PIK3R3	17
	CCK	17
	SSTR2	17
	ITGB3	16

Table 4. DEGs associated with GO in CTD database

Expression changes	Genes
Down	ABCB11, HSDL2, AADAC, DCLK1, ADM, FIBIN, TLR4, PGRMC2, PLIN1, VCAM1, CEBPA, FABP4, MAOA, LEPR, ADAM9
	SLC17A8, MRPL12, EPN3, CHRNG, TRPM6, CD300LB, GCH1, IL23A, KCNMA1, GADD45G, MAP2K2, BAK1, UCP3, HAL, IGKC, PSAT1, TUBB1, MRPL9, DMP1, ITGB3, PRSS8, SLPI, SLC24A2, CYP2B6, PNLIP, FOSL1, XDH, SLC30A2, CXCR4, SLC02A1, ETFB, IL1R2, ABCC3, POMC, DAB1, BGN, TRPM8, CYR61, SLC12A8, CRP, NOCT, VEGFBTGF3, UCP1, ATF3, B3GNT4, FLNA, RAB1BL, BCL2A1, DGAT1, FGFR3, BDH1, PODX, CXCL8, STAR, AGER
Up	

DISCUSSION

Up to date, TED is still a severe disease, and some patients even lost their sight. The main reason is that we do not know enough about the deep pathological mechanisms of TED, including its genopathology. In this research, we employed the combined bioinformatic methods to explore gene expression profiles in orbital adipose tissues within dataset GSE58331. The results showed that 678 DEGs (140 down-regulated and 538 up-regulated) exist in TED orbital tissues compared to normal controls. The up-regulated DEGs are obviously related to calcium channel activity and regulation of cell proliferation. Furthermore, the down-regulated DEGs inhibit osteoclast differentiation, peptidyl-tyrosine phosphorylation, negative regulation of cell proliferation, membrane, metalloproteinase activity. The enriched pathways of up-regulated DEGs include Rap1 and PI3K-Akt signaling pathways, cytokine-cytokine receptor interaction, mineral absorption and axon guidance, while only Ras signaling pathway is enriched for down-regulated DEGs. Obviously, the up- and down-regulations of DEGs are involved in different pathways as well as GO terms.

TED is an inflammatory process within the orbital tissues (3,27-29). The highly expressed insulin-like growth factor-1 and thyrotropin receptors on extra-cellular region of orbital cells play important roles in onset of TED (30,31). The cumulative evidence demonstrates that the orbital fibroblasts proliferation and adipogenesis are activated by the binding of autoantibodies to these receptors, which then resulted in the remodeling characteristics of TED (17, 32). Furthermore, in orbital adipose tissue of TED individuals, various overexpressed cytokines and their receptors are indispensable for the links among orbital fibroblasts and immune cells (33, 34). In addition, PI3K-Akt signaling pathway is involved in up-regulation of hyaluronan production and adipogenesis within orbital

of TED patients (35, 36). Hence, the results of this research are similar to those of the previous studies.

The results of the PPI network analysis suggest that IL-8, POMC, IL2, GNG3, CXCR4, TLR4, CSF1R, LPAR3 and seven other genes may be the hub genes through the PPI network analysis. IL-8, an up-regulated DEG, is a pro-inflammatory cytokine secreted by leukocytes (37). Considerable studies have shown that IL-8 mRNA and protein elevated in serum or orbital tissue of TED. In addition, IL-8 gene polymorphism is strongly related to TED (38); IL-8 is both a chemo-attractant for leukocytes and a stimulant of fibroblast differentiation and promoted angiogenesis (15). Nowak *et al.* measured the serum samples of 39 TED patients before and after treatment with methylprednisolone and speculated that improvement of active TED was related to the decrease of IL-8 serum concentration (39). IL-2, a protein-encoding gene, also plays a role in the proliferation of leukocytes. Serum level of IL-2 may be implicated in the pathogenesis of TED (40).

POMC, an up-regulated DEG in the dataset, is a preproprotein undergoing a tissue-specific post-translational changes. In the corticotroph cells of the anterior pituitary, the major end products of POMC are adreno-corticotrophin and lipotropin beta. In other tissues, POMC generates some peptides playing roles in immune modulation and energy homeostasis. Such as, the POMC peptides in immunocytes are important for the fundamental immune functions like cytotoxicity, phagocytosis and chemotaxis (41).

GNG3 is a member of signal-transducing molecules acting as molecular switches inside cells. Besides, the GNG3 always determines the specificity of which signaling pathways are affected by this particular complex. Hynes TR *et al.* have found that inhibition of G-protein $\beta\gamma$ signaling produces the changes of the cytokine mRNA levels, which autoimmune diseases may benefit from (42). Many studies have strengthened that autoimmunity is one of the important features of pathogenesis of TED. These results obviously implicate GNG3 in the pathogenesis of TED through an autoimmune process.

CXCR4, one of up-regulated DEGs in the present study, produces chemokine receptor. Fernando R *et al.* have suggested that the emergence of CXCR4 cross talk is relevant to TED (43). TLR4 encodes toll like receptor 4, plays a role in stimulating immune reactions. Furthermore, TLR-9 rather than TLR-4 gene polymorphisms are related to increased susceptibility of TED (44), and may contribute to the pathogenesis of TED in children (45). The results of our study indicated

that TLR4, a hub gene, is down-regulated in TED orbital adipose tissues.

Moreover, the KEGG pathway analysis for up-regulated DEGs screened out five pathways as follows: PI3K-Akt, mineral absorption, Rap1 and axon guidance, and cytokine-cytokine receptor interaction. The down-regulated DEGs are mainly associated with Ras signaling pathway. Considerable studies showed the over-expressed cytokines (IL-1 β , 6, 10, 16, IFN- γ and TNF- α .) in orbital tissues in TED regulate the CD 40 expression and recruit T and B cells. Study of pathogenesis of TED revealed that PI3K-Akt signalling pathway is the most important intracellular process, which simultaneously regulates the hyaluronan synthesis and adipogenesis in orbit (15, 36). Ras signaling pathway is associated with cell differentiation and proliferation. It is commonly known that the enhanced differentiation and proliferation of adipocytes and fibroblasts are the most important features of pathogenesis of TED (15). Hence, it can be speculated that Ras signaling pathway is involved in TED.

Subsequently, the DEG transcription regulatory network indicates that 6 transcription factors (HNF4A, FOXN4, MEF2A, MAF, SRF and NHLH1) may be involved in regulating this network. For deep analysis of TED, we could examine the expression level of the 6 TFs in TED orbital adipose in the future experimental studies.

Overall, we have identified 678 DEGs through bioinformatic analysis on GSE58331. Among them, IL-8, POMC, IL2, GNG3, CXCR4, TLR4, etc. are the hub genes which are mostly relevant to pathogenesis of TED. Functional enrichment revealed that cytokine-cytokine receptor interaction, Ras and PI3K-Akt signaling pathways have significant roles in the onset and development of TED. Our findings were valuable for a deeper understanding about the pathogenetic mechanism of TED. Of course, further experiments will be needed to confirm the findings of the present study and to determine the potential functions of these genes relevant to TED.

Conflict of interest

The authors declare that they have no conflict of interest.

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