Lysosome-Related Genes Are Regulated in the Orbital Fat of Patients with Graves' Ophthalmopathy

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Purpose. The molecular mechanism involved in the hypertrophy of the orbital fat in patients with Graves' ophthalmopathy or thyroid eye disease (TED) remains unclear. Comparison of genome-wide expression profiles may help in the search for the gene sets involved in TED.

Methods. Twenty-five orbital adipose tissue specimens were obtained, from which the RNA was isolated. Four of the tissue specimens (from four individuals, two with TED and two control subjects) were subjected to cDNA microarray analysis. The data were analyzed by the gene set enrichment analysis (GSEA) to survey the biological pathways involved in the pathogenesis of TED. Messenger RNA levels of some top-ranked genes in GSEA-selected pathways are validated by quantitative PCR (QPCR).

Results. The expression of specific gene sets related to lytic vacuoles, lysosomes, and vacuoles were different between the specimens obtained from patients with TED and control subjects ($P < 0.001$). These three gene sets overlapped. For QPCR, four top-ranked genes were selected from these overlapping gene sets and another one that related to visual failure, using 21 independent samples of patients with TED ($n = 15$) and control subjects ($n = 6$). The results showed that ceroid-lipofuscinosis, neuronal 2, late infantile (*CLN2*; $P = 0.044$) and ceroid-lipofuscinosis, neuronal 3, juvenile (*CLN3*, which related to visual failure; $P = 0.012$) were significantly downregulated in the orbital fat of patients with TED. The expression of the β subunit of hexosaminidase A (*HEXB*) was reduced as well, but the change did not reach statistical significance ($P = 0.058$).

Conclusions. Lysosome-related genes, such as *CLN2*, *CLN3*, and *HEXB*, may be involved in the pathogenesis of adipose tissue hypertrophy in TED. (*Invest Ophthalmol Vis Sci.* 2008; 49:4760–4764) DOI:10.1167/iovs.08-2020

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The clinical manifestations of Graves' disease include thyrotoxicosis and several extrathyroidal signs, such as ophthalmopathy, dermopathy, and acropachy.[1] Between 25% and 50% of patients with Graves' disease may exhibit ocular complications, which are properly termed Graves' ophthalmopathy or thyroid eye disease (TED). Patients with TED have clinical manifestations such as lid retraction, proptosis, soft tissue swelling, strabismus, and compressive optic neuropathy.[2] The swollen soft tissues within the bony orbit displace the globe forward and impede venous outflow from the orbit. The swollen soft tissues are the results of adipogenesis and glycosaminoglycan accumulation, a result of the activation of local fibroblasts secondary to inflammation.[3] Although it is widely accepted that Graves' disease is an autoimmune process and that the primary antigen in the thyroid is the TSH receptor,[4,5] the pathogenesis of TED remains poorly understood. Most believe that it is also an autoimmune process and involves TSH and IGF-1 receptors and other as yet unidentified shared autoantigen(s) in the thyroid and orbital tissues.[3,6,7]

In TED, proptosis appears to be related to the orbital adipose tissue[8] and muscle volume.[9] The expansion of adipose tissue volume results from the accumulation of glycosaminoglycans and the emergence of newly differentiated fat cells within these tissues.[3,7,10] Studies for the mechanism of proptosis using the orbital fibroblast culture model help to reveal the mechanism of adipocyte differentiation in vitro. However, they fail to approach the pathophysiology of adipose tissue, which is composed of several cell types, in the orbits of patients with TED. Identification of the differentially expressed genes in the orbital fat from patients with TED has the potential to provide new insight into the molecular mechanisms involved in the pathologic process and to provide new therapeutic targets for treatment of this disease.

Kumar et al.[11] performed gene expression profiling of orbital adipose tissue and found that secreted frizzled-related protein-1 (sFRP-1) was upregulated in orbital fat from patients with TED, which enhanced adipogenesis in orbital preadipocyte cultures. However, this kind of single gene expression profiling in array analysis failed to show the pathophysiology of adipose tissue as a whole in TED. In this study, we applied a more powerful analytical method, referred to as gene set enrichment analysis (GSEA),[12] to search for gene sets that may be involved in the pathogenesis of TED. Before we applied GSEA, single gene expression profiling had been used to find possible target genes, but the top-ranked genes failed to show a significant difference when they were tested with quantitative real-time PCR (QPCR). In this study, we combined computational screening using GSEA with experimental follow-up to detect and validate the relevant genes.

Materials and Methods

Human Orbital Adipose Tissues

Orbital adipose tissues were obtained from 17 patients with severe TED who underwent orbital decompression surgery and 8 patients with no history of Graves' disease who underwent elective orbital

TABLE 1. Demographics and Clinical Characteristics of Patients with TED and Control Subjects

Characteristics	TED (n = 17)	Control (n = 8)
Age (y)	31.3 ± 6.2 (22–45)*	30.6 ± 10.4 (22–53)*
Sex		
Female	15 (88.2%)	7 (87.5%)
Male	2 (11.8%)	1 (12.5%)
BMI	22.0 ± 2.1 (18.6–26.4)*	22.0 ± 2.3 (19.5–24.5)*
Proptosis (mm)	23.4 ± 1.5 (21–26)*	NA
Clinical activity score (CAS)	3.4 ± 1.1 (2–6)*	NA
TBII (%)	24.3 ± 6.0 (17.4–41.9)*	NA
Duration of TED (mo)	58.9 ± 28.3 (30–20)*	NA
Drug history		
ATD	17 (100%)	NA
Steroid	8 (47.0%)	NA
Thyroxine	7 (41.2%)	NA
Radioactive iodine	3 (17.6%)	NA
Thyroidectomy	1 (5.9%)	NA
Smoking	0	NA

* Data are expressed as mean ± SD (range).

surgery. The demographic and clinical characteristics of patients are listed in Table 1. Patients enrolled in the study were those with moderate to severe proptosis measured by a Hertel exophthalmometer. Those with a high clinical activity score (CAS) may receive steroid treatment first and will not undergo surgical intervention until they have been observed by an ophthalmologist for at least 3 months. These patients did not receive other immunosuppressive agents or orbital radiotherapy. All patients were in euthyroid status at least for 3 months. TED orbital adipose tissues were obtained while patients with TED underwent orbital decompression surgery. The normal orbital adipose tissues were retrieved from the same anatomic site in patients with no history of Graves' disease who underwent elective orbital surgery. Seven of them had benign mixed tumor of the lacrimal gland, the other one had a benign cyst. One was a man (12.5%, and the percentage of men in the disease group was 11.8%). Their mean age was 30.6 ± 10.4 years (mean ± SD, range 22–53), which was not different from that of the patients with TED. The orbital tissues were soaked in liquid nitrogen for 3 seconds, then put in a sterile container and immediately put in liquid nitrogen for transfer to a −70°C freezer before RNA isolation. This study was approved by the Institutional Review Board of the National Taiwan University Hospital. Written informed consent was obtained from each patient, and the protocol adhered to the tenets of the Declaration of Helsinki.

RNA Isolation

Total RNA was isolated from the tissue samples (RNeasy Lipid Tissue Mini kit; Qiagen, Valencia, CA) according to the manufacturer's protocol. The optical density 260/280 ratios of all samples were >1.8.

Gene Array Sample Preparation, Hybridization, and Scanning

The tissue samples that were used for array analyses were taken from patients with severe proptosis with prominent fat volume expansion. Microscopically, the size of the adipocytes was enlarged and an edematous change was also found in the adjacent area. The quality of total RNA samples from orbital tissues were examined in a bioanalyzer and the 28s/18s ratios of the four samples were >1.5. We chose two TED and two control samples from different individuals by excluding those had been treated with steroid or other immunosuppressive agent. Two TED samples were taken from two patients who had severe proptosis with predominant extraocular fat volume expansion but less extraocular muscle volume expansion. These qualified total RNA samples were processed with one-cycle target labeling, and control reagents to fragmented cRNA were then hybridized with a microarray (Human Genome U 133A Array; Affymetrix, Santa Clara, CA), which contains 22,215 human gene probes. After washing and staining (Fluidics Station 400; Affymetrix), the arrays were scanned (GeneArray 2500 scanner).

Microarray Data Normalization and Analysis

A gene microarray (Microarray Suite, version 5.0; Affymetrix), was used as the first tool for data analysis. This software allowed for calculation of the signal and determination of whether each probe set was present. To test for sets of related genes that may have been systematically altered in the orbital fat of patients with TED, we used GSEA,[12] which combines information from the members of previously defined sets of genes (e.g., biological pathways) to increase the signal-to-noise ratio (SNR) and thus, to improve the statistical power.[12,13] First, all genes were ranked according to the difference in expression using the SNR between those in patients with and without TED. The null hypothesis of GSEA predicted that the rank ordering of the genes in a given comparison would be random with regard to the diagnostic categorization of the samples. The alternative hypothesis was that the rank ordering of the pathway members would be associated with the specific diagnostic criteria used to categorize the groups of affected individuals. Then, we measured the extent of association by a nonparametric, running-sum statistic termed the enrichment score (ES), and recorded the maximum ES (MES) over all gene sets in the actual data

TABLE 2. Primers for QPCR of Candidate Genes

Genes	Forward Primer	Reverse Primer
CLN2	GACCTACGGAAACCCA	AGTTGTATCGCTTACGGA
LAMP3	AGGTTCTAAACGGAAGC	AGACGGTCAAATAGGC
HEXB	TCTGCTCCTTGGTACT	CTGTCATAGGCGTCATC
MCOLN1	TGCGACAAGTTTCGAG	CGGACATACGCATACC
CLN3	CCACGACATCCTTAGC	ATCCCACTGACGAGAA
β-Actin	TTCTACAATGAGCTGCG	AGGTAGTCAGTCAGGT

TABLE 3. Summary Results of GSEA of the mRNA Expressions between Subjects with TED vs. Controls

GOID	No. of Gene	*P*	Name	Term Type
323	143	<0.001	Lytic vacuole	Component
5764	143	<0.001	Lysosome	Component
5773	146	<0.001	Vacuole	Component
8143	13	0.022	Poly(A) binding	Function
375	137	0.039	RNA splicing, via transesterification reactions	Process
377	137	0.039	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	Process
398	137	0.039	Nuclear mRNA splicing, via spliceosome	Process

set from the patients with TED. To assess the statistical significance of the MES, we used permutation testing of the GO (Gene Ontology; a collaborative database, available at www.geneontology.org) labels of the genes. We chose to permutate the gene labels instead of phenotypes because a small array number makes phenotype permutation impractical. Furthermore, gene label permutation is one of the standard options in the GESA software. Specifically, we compared the MES achieved in the actual data to that which occurred in each of 1000 permutations that shuffled the GO labels of the genes. The significance of the MES score was calculated as the fraction of the 1000 random permutations in which the top pathway gave a stronger result than that observed in the actual data.

Quantitative PCR

QPCR for assessment of selected genes was performed by using individual orbital adipose tissue samples from patients with TED ($n = 15$) and control subjects ($n = 6$). The samples used for QPCR were different from those used for microarray analysis. Total real-time PCR reactions were performed in a 10-μL total reaction mixture (1 μL of cDNA, 1 μL of LightCycler FateStart DNA MasterSYBR Green I, 1 μL of each of the 5-μM forward and reverse primers shown in Table 2, 5.8 μL of H_2O and 1.2 μL of 25 mM $MgCl_2$ stock solution) in a thermocycler (LightCycler; Roche, Penzberg, Germany). The PCR program was initiated with 10 minutes at 95°C before 40 thermal cycles. Calculation of the relative expression ratios of target genes in patients with TED versus control subjects in comparison to a reference gene (β-actin) was made by using $2^{-\Delta\Delta Ct}$.[14] The Student's *t*-test was used to assess statistically significant differences between the groups of tissue samples.

RESULTS

Gene Array Analysis

We used the GSEA procedure, which does not directly detect genes, but tests all categories in the gene ontology database to analyze the array result. First, we filtered out those genes with expression values <100 in all four arrays, leaving 17,123 genes. Then, we applied GSEA combined with the Gene Ontology (GO) database. We arbitrarily chose the term with several genes were between 10 and 200. There were 1439 terms remaining for further analysis. After 1000 permutations, we identified seven gene sets with significance (Table 3). The genes in Gene Ontology ID (GOID) 323 are overlapped with GOID 5764 and 5763, and so we grouped them into a set. The genes, in terms of components, were primarily related to lytic vacuoles, lysosomes, and vacuoles and were expressed significantly differently between orbital tissues from patients with TED and control subjects.

QPCR Analysis of Lysosome-Related Genes

Based on the GSEA results, we targeted the category of genes in GOID 323, 5764, and 5763. There were four genes in this category that were expressed highly differently between the patients with TED and control subjects—that is, ceroid-lipofuscinosis, neuronal 2, late infantile (*CLN2*; $P < 0.001$), lysosomal-associated membrane protein 3 (*LAMP3*; $P < 0.001$), mucolipin (*MCOLN1*; $P < 0.001$), and the β subunit of hexosaminidase A (*HEXB*; $P = 0.002$). In addition to the four highly differently expressed genes, we picked up ceroid-lipofuscinosis, neuronal 3, juvenile (*CLN3*; $P = 0.049$; Table 4) in the same category for QPCR analysis, because the first manifestation of its deficiency is visual failure.[15] QPCR analysis was performed using samples distinct from those used in the gene array studies. The levels of *CLN2* ($P = 0.044$) and *CLN3* ($P = 0.012$) mRNA, normalized to β-actin mRNA, were significantly lower in the orbital adipose tissue samples from patients with TED than from the control subjects. Although the level of *HEXB* expression was not significant ($P = 0.058$), there was a lower trend in the TED group (Fig. 1).

DISCUSSION

Expression profiling using DNA microarray has been performed extensively and has enabled researchers to generate hypotheses with respect to several disease mechanisms. However, the large number of genes tested, the high variability between individuals, and the limited samples sizes in human studies has made it difficult to distinguish true differences from noise. Thus, consideration of the variability in gene sets of biological pathways, rather than individual gene changes, is more sensitive and more useful for understanding biological changes. The standard GSEA procedure is one of the choices. By using the GSEA method, we determined that the expressions of specific gene sets related to lytic vacuoles, lysosomes, and vacuoles were substantially different ($P < 0.001$) between

TABLE 4. Details of the Selected Individual Genes in GOID 323, 5764, 5773

Gene Name	Gene Symbol	Location	*t*	*P*
Ceroid-lipofuscinosis, neuronal 2, late infantile (Jansky-Bielschowsky disease)	*CLN2*	11p15	−32.8707	0.000462
Lysosomal-associated membrane protein 3	*LAMP3*	3q26.3–q27	−31.734	0.000496
Mucolipin	*MCOLN1*	19p13.3–p13.2	−23.9679	0.000868
Hexosaminidase, beta polypeptide	*HEXB*	5q13	−15.9726	0.001948
Ceroid-lipofuscinosis, neuronal 3, juvenile (Batten, Spielmeyer-Vogt disease)	*CLN3*	16p12.1	−2.93752	0.04949

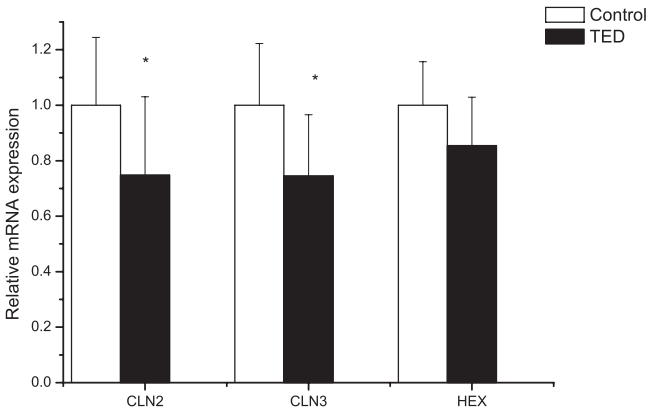

FIGURE 1. mRNA expression of lysosome-related genes. Relative expression of *CLN2*, *CLN3*, and *HEXB* in orbital adipose tissue samples from patients with TED ($n = 15$) and control subjects ($n = 6$). $P = 0.044$, 0.012, and 0.058, respectively, for TED compared with control groups.

orbital adipose tissues obtained from patients with and without TED. Furthermore, although the GOID of lytic vacuole-, lysosome- and vacuole-related gene sets were different, most of the genes listed within these three gene sets were overlapping. Ultrastructural studies using transmission electron microscopy have observed the formation of vacuoles in extraocular muscles and cultured orbital fibroblasts from patients with TED.[16,17] In the early stage of adipocyte differentiation, small vacuoles appeared before the accumulation of lipids (laboratory observation). We believe that the biologically meaningful and statistically significant categories are very unlikely to be false positives.

Many overlapping genes were found under the categories GOID 323, 5763, and 5764; approximately 140 genes were found. We were interested in specific lysosomal storage disease-related genes, because our colleagues found exophthalmos in a patient with lysosomal storage disease in whom the disease regressed with enzyme replacement therapy.[18] For selecting genes to be validated by QPCR, we went back to check the comparisons of individual genes within GOID 323, 5763, and 5764 between patients with TED and control subjects, to narrow down our targets. Among the approximately 140 genes, the top-ranked four genes related to lysosomal storage disease (*CLN2*, *LAMP3*, *MCOLN1*, and *HEXB*), along with *CLN3*, though not top ranked, were selected for QPCR. Deficiency of *CLN3* not only caused lipofuscinosis but also had visual failure as its first manifestation, and we were interested in the late-onset visual problem. *LAMP3* and *MCOLN1* expression did not show a difference, but the expression of *CLN2*, *CLN3*, and *HEXB* were downregulated in the orbital fat of patients with TED. *CLN2* and *CLN3* are involved in neuronal ceroid lipofuscinoses (NCLs), a group of neurodegenerative disorders characterized by the accumulation of autofluorescent inclusion bodies in many cell types that resemble ceroid and lipofuscin. Classically, the NCLs have been divided into three main childhood types (infantile, late infantile, and juvenile) and one adult type. Juvenile NCL, caused by mutation in the *CLN3* gene, typically manifests from 6 years of age with visual failure.[19,20] *CLN3* encodes a 43-kDa polypeptide localized to the lysosomal compartment, and its exact function is unknown.[21] *CLN2* encodes a lysosomal enzyme, tripeptidyl peptidase I (TPP1).[22,23] Mutation in the *CLN2* gene causes late infantile NCL, manifesting from 2 years of age with seizures. Gene therapy with the *CLN2* has resulted in safe long-term TPP1 expression in rats and nonhuman primates.[24] Our study showed downregulation of *CLN2* and *CLN3* in orbital fat of patients with TED. It would be interesting to determine whether the treatment for NCL could be applied to the treatment of TED. *HEXB*, another lysosome-related gene, encodes the β subunit of hexosaminidase A and is involved in the breakdown of gangliosides as well as glycosaminoglycans. Accumulation of glycosaminoglycans is seen in the tissues of mice with hexosaminidase deficiency.[25] In our study, the expression *HEXB* tended to be downregulated in the orbital fat of patients with TED. We thought that the hexosaminidase deficiency because of *HEXB* downregulation might cause the accumulation of glycosaminoglycans and result in orbital fat volume expansion in patients with TED. Although the degree of downregulation of these three genes, at around a 30% decrease, were not as high as a main factor in a disease could be, but we believe that subtle but broad changes of components of one functional pathway may be truly important in the pathogenesis of disease.

None of these lysosomal genes (*CLN2*, *CLN3*, and *HEXB*) have been reported to be associated with TED. Our study suggests that lysosome-related genes, such as *CLN2*, *CLN3*, and *HEXB*, may have contributed to orbital fat volume expansion by increasing adipocyte size or increasing edematous change by the accumulation of glycosaminoglycans in patients with TED.

There are more than 50 acid-dependent hydrolases (e.g., proteases, lipases, glycosidases) within in the limiting membrane named lysosomal-associated membrane proteins (*LAMP*s),[26] which exist in different types of cells. One of our studies revealed the expression of *CD68*, a *LAMP*, was increased in the orbital fat of patients with TED.[27] Adipose tissue is a heterogeneous tissue that contains not only adipocytes but also fibroblasts and inflammatory cells. The adipose tissue disease in TED may be associated with the regulation of lysosome-related genes in different types of cells. Our work shows that lysosome-related genes, such as *CLN2*, *CLN3*, and *HEXB*, may be involved in the pathogenesis of orbital adipose tissue hypertrophy in patients with moderate to severe TED.

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