

Association of TCR–signaling pathway with the development of lacrimal gland benign lymphoepithelial lesions

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Abstract

• **AIM:** To identify the association of the T cell receptor (TCR) signaling with the development of benign lymphoepithelial lesions (BLEL) of the lacrimal gland.

• **METHODS:** We collected affected lacrimal gland tissues from 9 patients who underwent dacryoadenectomy in the Capital Medical University Beijing Tongren Hospital Eye Center between August 2010 and March 2013 and were confirmed to have lacrimal gland BLEL by histopathological analysis. Tumor tissues from 9 patients with orbital cavernous hemangioma were also collected and used as control. Whole genome gene expression microarray was used to compare gene expression profiles of affected lacrimal gland tissues from patients with lacrimal gland BLEL to those from of orbital cavernous hemangiomas. Differential expression of TCR pathway genes between these tissues was confirmed by polymerase chain reaction (PCR) and immunohistochemistry.

• **RESULTS:** Microarray analysis showed that in lacrimal glands with BLEL, 32 signaling pathways were enriched in the upregulated genes, while 25 signaling pathways were enriched in the downregulated genes. In –depth analysis of the microarray data showed that the expression of 27 genes of the TCR signaling pathway increased significantly. To verify the differential expression of three of these genes, CD3, CD4, and interleukin (IL)–10, reverse transcription–PCR (RT–PCR) and immunohistochemistry assays were performed. RT–PCR analysis showed that CD3 and CD4 were expressed in the lacrimal glands with BLEL, but IL–10 was not expressed. Immunohistochemistry confirmed that CD3 and CD4 proteins were also present, but IL–10 protein

was not. CD3, CD4, or IL–10 expression was not found in the orbital cavernous hemangiomas with either RT–PCR or immunohistochemistry.

• **CONCLUSION:** TCR signaling pathway might be involved in the pathogenesis of lacrimal gland BLEL.

• **KEYWORDS:** lacrimal gland; benign lymphoepithelial lesion; whole genome gene expression microarray; T cell receptor–signaling pathway

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INTRODUCTION

Benign lymphoepithelial lesions (BLEL), also termed Mikulicz disease, are relatively rare, lacking specific symptoms. Thus, it is often overlooked or misdiagnosed by ophthalmologists^[1,2]. In 1888, a Polish physician, Mikulicz, first reported such a case, depicting a patient with bilateral swelling of the lacrimal glands, as well as salivary glands, and this disease was later named after him as the Mikulicz disease. In the 1950s to 1970s, histopathological analysis revealed that expansion of lymphocytes and replacement of glandular tissues by lymphoid tissues were the major histopathological features of this disease. In the 1990s, the World Health Organization (WHO) officially named this condition, a "BLEL"^[3,4].

Recently, we examined a group of patients with lacrimal glands confirmed to be affected by BLEL by histopathological analysis. We noted that only occasionally patients also presented with affected submandibular, sublingual, and parotid glands. Thus, we proposed a novel name for this condition, BLEL affecting only the lacrimal glands or "BLEL of the lacrimal gland." Lacrimal gland BLEL can affect people of all ages, races and genders, but it most frequently affects middle-aged women, having a protracted course with recurrent attacks and the potential to become malignant^[1,3,5-7]. Studies indicate that low estrogen and high IgG4 are associated with lacrimal gland BLEL, but the exact mechanisms of its pathogenesis are unclear^[8-13].

Table 1 Baseline clinical characteristics of patients in the experimental and control groups

| Groups | Mean age (a) | Gender | | Affected eyes | | | IgG4 level | |
|--------------------|--------------|--------|---|---------------|---|-----------|------------|--------|
| | | M | F | L | R | Bilateral | High | Normal |
| Experimental group | 50 | 2 | 7 | 2 | 0 | 7 | 9 | 0 |
| Control group | 46.4 | 3 | 6 | 5 | 4 | 0 | 0 | 9 |

T cell receptor (TCR) signaling pathway is an important component of the immune response [14,15]. TCR is a protein complex of integral membrane proteins involved in T cell activation during antigen presentation. Activation of T cells triggers a distinct immune response to eliminate the antigen source, thereby protecting the body [16,17]. Gene microarray (or gene chip) technology has assisted clinical studies of disease, offering a high throughput assay with high affinity and accuracy. An extensive review of literature (Medline Search) did not reveal any research on pathogenic mechanisms of BLEL [18]. Thus, our work represents for the first time, the gene expression profile of lacrimal gland tissues from BLEL patients with gene microarray, which was confirmed with microarray data to ascertain whether TCR signaling is involved in BLEL pathogenesis. This work offers a novel experimental basis for understanding the etiology and pathogenesis of lacrimal gland BLEL.

MATERIALS AND METHODS

Materials

Sources of specimens We collected affected lacrimal gland tissues from 9 patients who underwent dacryoadenectomy in the Capital Medical University Beijing Tongren Hospital Eye Center between August 2010 and March 2013 and were confirmed to have lacrimal gland BLEL by histopathological analysis. Tumor tissues from 9 patients with orbital cavernous hemangioma were also collected and used as control. This study was approved by the Ethics Committee of the Capital Medical University Beijing Tongren Hospital, and written consent was given by all participating patients.

Patients in the experimental group (lacrimal gland BLEL) were 36-70 years-of-age (mean 50y; 2 males). BLEL affected left eyes of 2 patients and 7 patients had both eyes afflicted. Circulating IgG4 were high in all 9 patients. Patients in the control group (aged 31-59 years-of-age; mean 46.4y; 3 males). Also, 5 patients had hemangioma of the left eye; 4 had right eyes afflicted. All control patients had normal circulating IgG4 (Table 1).

Major reagents The following reagents were used: polymerase chain reaction (PCR) mix kit (Sangon Biotech, Shanghai, China); RNA reverse transcription kit (New England Biolabs, Ipswich, MA, USA); rabbit anti-human CD3 and rabbit anti-human CD4 antibodies (ZSGB-Bio, Beijing, China); mouse anti-human IL-10 antibody (Abcam, Cambridge, MA, USA); universal two-step immunohistochemistry kit, horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG, and HRP-conjugated goat anti-rabbit IgG (ZSGB-Bio).

Methods

Whole genome gene expression microarray

Specimen preparation Surgically removed tissues were washed in phosphate buffered saline to remove blood and then each tissue was cut into two halves. One half was put into a cryopreservation vial and stored frozen in liquid nitrogen; the other half was fixed in 10% formalin, and then given to Sangon Biotech for paraffin embedding and section cutting.

RNA preparation Buffer RLT (600 μL) and 6 μL of β-mercaptoethanol were added into a microcentrifuge tube. Then, 40 mg of frozen tissue was ground into powder in liquid nitrogen and then the powder was transferred into the tube described above. Lysate was centrifuged at 4°C for 3min (12 000 rpm/min), and the supernatant was transferred to a new tube and mixed with an equal volume of 70% ethanol. The above solution was loaded into a column in two batches, centrifuged (10 000 rpm/min) at 4°C for 1min to remove the flow through. The column was washed sequentially with 700 μL of RW1, transferred to a new collection tube, and dried by centrifugation at 4°C for 2min. The column was transferred to a new tube and added 30 μL of RNase-free water. RNA was eluted by centrifugation (10 000 rpm/min) at 4°C for 1min and stored frozen in liquid nitrogen.

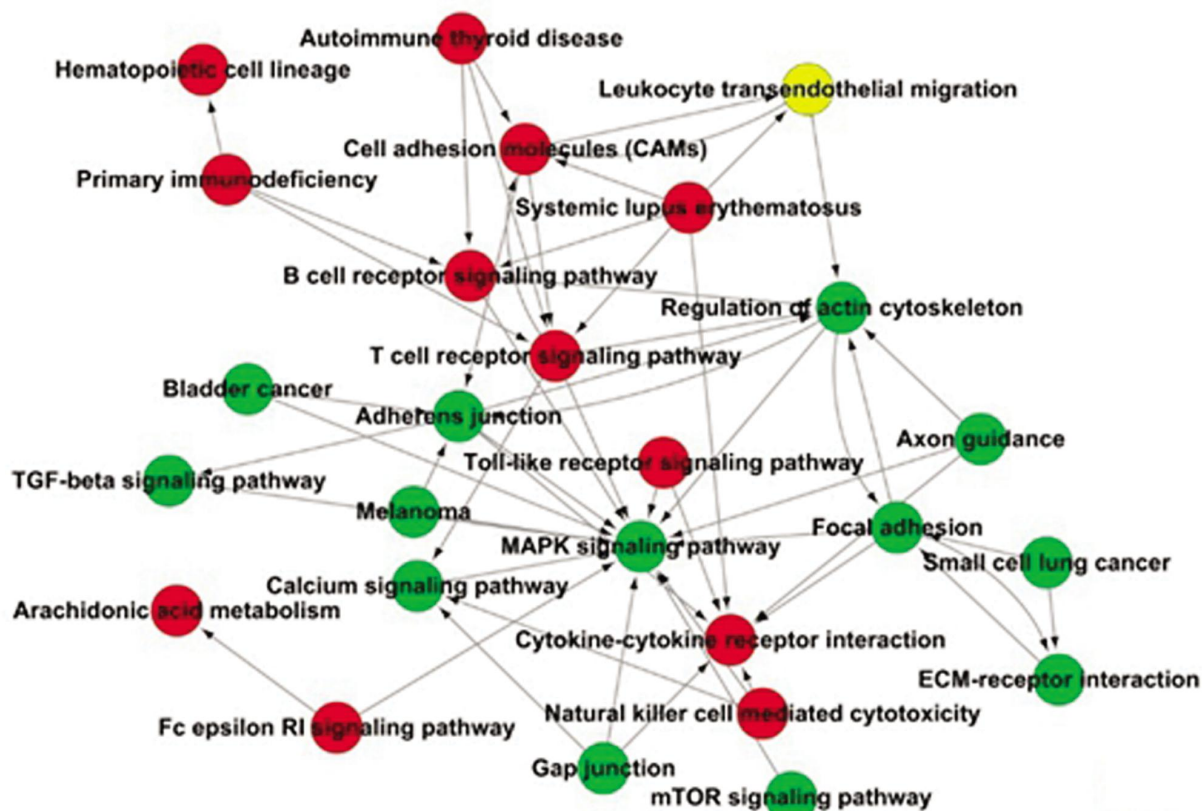
mRNA samples mRNA were given to Novel Bioinformatics Company (Shanghai, China) for whole-genome gene expression microarray analysis. Genes that were differentially expressed between the experimental and control group were annotated using the Kyoto encyclopedia of genes and genomes (KEGG) database. Significance of all pathways were calculated using a hypergeometric distribution based Fisher exact test. Multiple hypothesis testing results were calibrated to obtain the false discovery rate (FDR). Significantly overrepresented pathways among the differentially expressed genes were selected using $P < 0.05$ as the cut off for statistical significance.

Reverse transcription–polymerase chain reaction analysis

Microarray analysis showed that TCR pathway genes were significantly upregulated. Then, we selected genes CD3, CD4, and IL-10 to verify microarray results. RNA was prepared as described above. Primers were designed with the Dnaman software, and their sequences are shown in Table 2. RNA was reverse transcribed into cDNA following kit instructions and then used in PCR reactions. The PCR program included the following steps: 1) 95°C for 5min; 2) 25 cycles of 95°C 30s, 54°C 30s, and 72°C 30s; 3) 72°C 10min; 4) 4°C hold step. PCR products were separated *via* agarose gel electrophoresis, and then photographed under ultraviolet light.

Table 2 Primers used for RT-PCR

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') | GenBank accession number | Product size (bp) |
|-------|------------------------|------------------------|--------------------------|-------------------|
| CD3 | TCCCAACCCAGACTATGAGC | CAAGACTAGCCCAGGAAACAG | NM_000733 | 158 |
| CD4 | GGGCTTCCTGTCTCTCTTCATT | GCACCACTTTCTTCCCTGAGT | NM_000616 | 301 |
| IL-10 | ATGCCCAAGCTGAGAACCAA | TCTCAAGGGGCTGGGTCAGCTA | NM_000572 | 351 |



| | | | | | | | | | | |
|---------|---------|--------------------------------------|----|-----|-----|------|-----------|-----------|-----------|-----------|
| PAK3 | PAK3 | p21 protein (Cdc42/Rac)-activated | 27 | 554 | 107 | 5353 | 0.0001424 | 0.0015836 | 2.4381895 | 12.777318 |
| IL10 | IL10 | interleukin 10 | 27 | 554 | 107 | 5353 | 0.0001424 | 0.0015836 | 2.4381895 | 12.777318 |
| CD3D | CD3D | CD3d molecule, delta (CD3-TCR c | 27 | 554 | 107 | 5353 | 0.0001424 | 0.0015836 | 2.4381895 | 12.777318 |
| RASGRP1 | RASGRP1 | RAS guanyl releasing protein 1 (c | 27 | 554 | 107 | 5353 | 0.0001424 | 0.0015836 | 2.4381895 | 12.777318 |
| CTLA4 | CTLA4 | cytotoxic T-lymphocyte-associater | 27 | 554 | 107 | 5353 | 0.0001424 | 0.0015836 | 2.4381895 | 12.777318 |
| MAP3K14 | MAP3K14 | mitogen-activated protein kinase k | 27 | 554 | 107 | 5353 | 0.0001424 | 0.0015836 | 2.4381895 | 12.777318 |
| NFKBIE | NFKBIE | nuclear factor of kappa light polype | 27 | 554 | 107 | 5353 | 0.0001424 | 0.0015836 | 2.4381895 | 12.777318 |
| ITK | ITK | IL2-inducible T-cell kinase | 27 | 554 | 107 | 5353 | 0.0001424 | 0.0015836 | 2.4381895 | 12.777318 |
| PDCD1 | PDCD1 | programmed cell death 1 | 27 | 554 | 107 | 5353 | 0.0001424 | 0.0015836 | 2.4381895 | 12.777318 |
| TNF | TNF | tumor necrosis factor | 27 | 554 | 107 | 5353 | 0.0001424 | 0.0015836 | 2.4381895 | 12.777318 |
| LCP2 | LCP2 | lymphocyte cytosolic protein 2 (Sf | 27 | 554 | 107 | 5353 | 0.0001424 | 0.0015836 | 2.4381895 | 12.777318 |
| CD4 | CD4 | CD4 molecule | 27 | 554 | 107 | 5353 | 0.0001424 | 0.0015836 | 2.4381895 | 12.777318 |

Figure 1 The results of Microarray analysis was significant differences between experimental and control groups.

Immunohistochemistry Paraffin sections were heated to melt the paraffin, deparaffinized in xylenes, and then hydrated with an ethanol gradient. Sections were blocked in the blocking reagent for 30min, and then incubated with primary antibodies (rabbit anti-human CD3 1:50, rabbit anti-human CD4 1:50, or mouse anti-human IL-10 1:400) in a humidified chamber overnight at 4°C. Sections were then incubated with universal secondary antibodies, followed by DAB color developing solution, mounted with resin and coverslips. The stained slides were observed and photographed with an inverted microscope.

RESULTS

Whole-genome Gene Expression Analysis Microarray analysis indicated that, comparing the experimental and

control groups, 32 signaling pathways were overrepresented in genes that were upregulated, while 25 pathways were downregulated. In-depth analysis showed that the TCR pathway was significantly overrepresented, and 27 genes of this pathway were significantly upregulated. The most highly upregulated genes were CD3, CD4, and IL-10 (Figure 1).

Polymerase Chain Reaction Amplification Results

Reverse transcription-PCR analysis showed that in the experimental group the CD3 gene was expressed, as evidenced by the presence of the expected 158 bp PCR product (Figure 2A). RT-PCR for the CD4 gene also yielded the expected 301 bp product in the experimental group (Figure 2B). However, IL-10 was not expressed; the expected 351 bp PCR product was not seen (Figure 2C). None of these genes was expressed in the control group (data not shown).

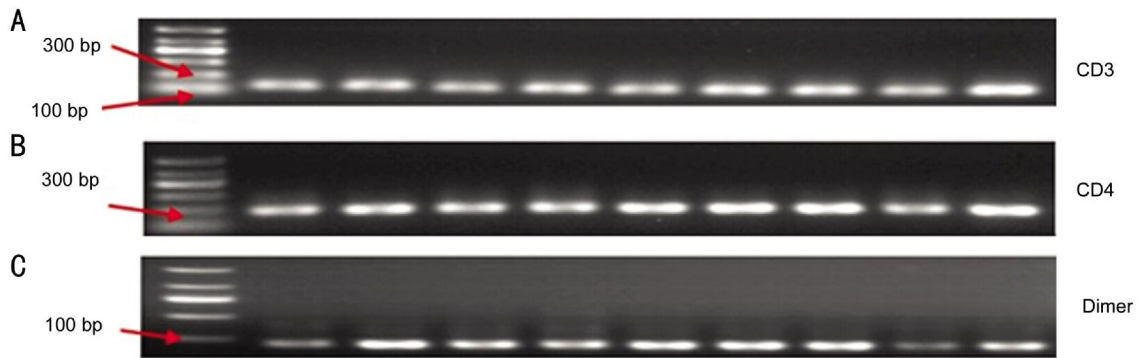


Figure 2 RT-PCR analysis showed that 2 RNAs were expressed.

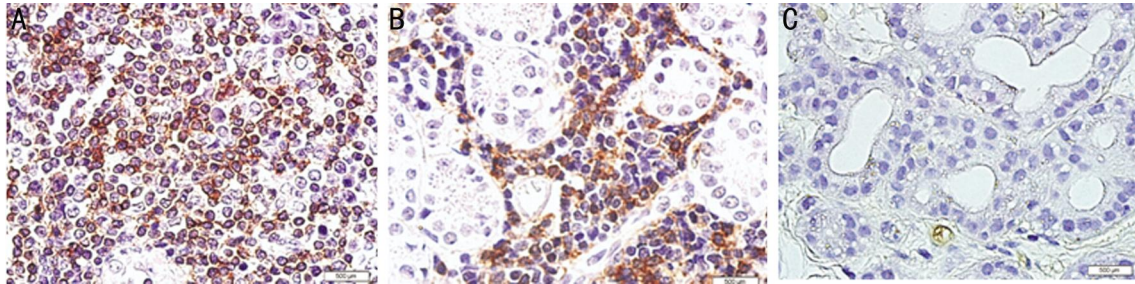


Figure 3 Immunohistochemistry analysis showed that only 2 proteins were positive and another one was negative.

Immunohistochemistry Immunohistochemistry analysis showed that CD3 and CD4 proteins were present in the experimental group (Figure 3A, 3B), but IL-10 protein was not detectable (Figure 3C). Negative staining was observed for all three proteins in the control group (data not shown).

DISCUSSION

Lacrimal gland BLEL causes suffering and contributes to health care costs. It usually first manifests as unilateral or bilateral painless swelling of the eyelids, where diffuse enlargement of the lacrimal gland in the affected eyelids can be detected by magnetic resonance imaging [1,2]. This disease is generally considered an auto-immune disease, but the exact mechanisms of its pathogenesis are still not fully understood[3,4].

In this study, we used tumor tissues from patients with orbital cavernous hemangioma as controls based on the following considerations: 1) orbital cavernous hemangioma is known to be a congenital blood vessel abnormality, and unlike BLEL, there is no inflammation or involvement of the immune system, thus using it as control would not interfere with our analysis of the experimental group; 2) orbital cavernous hemangioma is a more common disease, thus easier to obtain specimens; 3) normal orbital tissues are difficult to obtain.

TCR signaling initiates from TCRs on the T cell plasma membrane and it plays important roles in the immune response. The main function of TCR is to activate T cells after recognition of specific antigens. Activated T cells then mediate the cellular immune response to remove the antigens and protect the body. Lacrimal gland BLEL is thought to be caused by immune dysfunction, but it is not known whether TCR signaling is involved in its pathogenesis.

Gene microarray, or gene chip technology, involves placing a large quantity of gene fragments or oligonucleotides (known as probes) onto a solid phase, usually made of glass or silicon, at a high density. After hybridization with a sample containing nucleic acids, signal intensity for each probe can be recorded, thus the expression of many genes in the sample can be obtained. This technique provides a useful tool to systematically study all genes and pathways involved in the pathogenesis of lacrimal gland BLEL, facilitating our understanding of its pathogenic mechanisms. Here, we chose whole-genome gene expression microarray because it provides the most extensive coverage of genes to be tested[18]. We acquired gene expression profiles of lacrimal tissues from 9 patients with lacrimal gland BLEL, and compared them with those of tumor tissues from 9 patients with orbital cavernous hemangioma. We found that 32 signaling pathways were enriched in upregulated genes and 25 pathways were enriched in downregulated genes in the diseased lacrimal glands. In-depth analysis of the upregulated pathways showed that 27 genes of the TCR pathway were significantly upregulated ($P < 0.05$), suggesting that activation of this signaling pathway may be associated with the development of BLEL.

Because gene microarray analysis can yield false positive results, we confirmed microarray results by other measures. In this study, microarray analysis showed that CD3, CD4, and IL-10 were the most highly upregulated genes. In T cells, CD3 and CD4 are located on the plasma membrane, whereas IL-10 is on the nuclear membrane. Due to the difference in their location, their roles in the pathogenic process might also differ.

CD3 is an inflammatory factor, comprised of 6 peptide chains, found only on T cells. It binds to TCR through ionic bonds and transduces activation signals from antigen-bound TCR^[19]. CD4 is mainly present on T helper (Th) cells and is the co-receptor of TCR on Th cells, thus it is involved in antigen-binding and signal transduction^[20]. IL-10 is predominantly produced by Th2 cells and it both directly and indirectly inhibits cytokine secretion from Th1 cells, and facilitates the development of mature T cells, naive T cells, and thymocytes. As the main effector of Th2 cells, IL-10 is involved in multiple immune processes, including antigen presentation, thus it has unique roles in immunoregulation and autoantibody production^[21]. For these reasons, CD3, CD4, and IL10 were selected as representative factors of TCR pathway to confirm microarray results.

The etiology and pathogenesis of lacrimal gland BLEL is not yet clear. Most investigators consider it an inflammatory disorder caused by autoimmunity^[3,4]. To date, no reports have been made to explain the association of abnormal expression of TCR pathway genes with the development of BLEL. Here we used PCR and immunohistochemistry assays to measure CD3, CD4, and IL-10 expression in affected tissues of lacrimal gland BLEL. Data show that in affected tissues, CD3 and CD4 mRNA and protein are both higher than in control tissues, but neither IL-10 mRNA nor its protein was present in diseased tissues. This suggests that CD3 and CD4 may be involved in the pathogenesis of BLEL, but IL-10 is likely not involved. The study is a good attempt to investigate the role of TCR signaling pathway in BLEL, but it has some limitations. For example, the experiment only investigated three effector molecules. We will investigate on the effect of other effector molecules including tumor necrosis factor (TNF) in BLEL.

In summary, we used whole genome gene expression microarray to identify pathways overrepresented in differentially expressed genes in the lacrimal gland tissues affected by BLEL. Our data suggest that genes of the TCR signaling pathway, particularly CD3 and CD4, may be involved in the pathogenesis of lacrimal gland BLEL.

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