The Involvement of the Fcεri Signaling Pathway in the Pathogenesis of Lacrimal Gland Benign Lymphoepithelial Lesions

Jing Li
Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University
Rui Liu
Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University
Mei Sun
Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University
Jinjin Wang
Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University
Nan Wang
Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University
Xuan Zhang
Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University
Xin Ge
Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University
Jianmin Ma (jmma@sina.com)
Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University

Research Article

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The involvement of the FcεRI signaling pathway in the pathogenesis of lacrimal gland benign lymphoepithelial lesions

Jing Li[1a], Rui Liu[1a], Mei Sun[1], Jinjin Wang[1], Nan Wang[1], Xuan Zhang[1], Xin Ge[1], Jianmin Ma[1*]

1Beijing Institute of Ophthalmology, Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University; Beijing Ophthalmology & Visual Sciences Key Laboratory, Beijing 100730, China

[a] **Co-first author:** Jing Li and Rui Liu

***Corresponding author:**

Jianmin Ma, Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University; Beijing Ophthalmology & Visual Sciences Key Laboratory, Beijing, China. 100730. E-mail: jmma@sina.com.

[Abstract]

Objective: Benign lymphoepithelial lesion of lacrimal gland (LGBLEL) is a common orbital inflammatory disease with unknown pathogenesis. This paper analyzed the role of the FcepsilonRI (FcεRI) signaling pathway in the pathogenesis of LGBLEL.

Methods: Transcriptome sequencing and proteome sequencing were performed on LGBLEL and orbital CH diagnosed by histopathology in Beijing Tongren Hospital, Capital Medical University, between July 2010 and October 2013. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were used to jointly analyze the differentially expressed genes and proteins related to FcεRI signaling pathway. Four LGBLEL and three orbital CH diagnosed by histopathology in Beijing Tongren Hospital, Capital Medical University, between October 2018 and August 2019 were randomly selected as the experimental group and the control group, respectively. RT-PCR, immunohistochemical staining, and western blotting were used to verify the genes and proteins related to the FcεRI signaling pathway.

Results: Combined transcriptome and proteome analysis showed that the FcεRI signaling pathway was up-regulated in LGBLEL (P=0.0040), and that the important proteins such as SYK, p38, JNK, PI3K, and ERK were highly expressed in LGBLEL tissues. RT-PCR results showed that the mRNA expression levels of SYK, p38, JNK, PI3K, and ERK were significantly increased in the LGBLEL group (P=0.0066, P=0.0002, P=0.0003, P<0.0001, P<0.0001, respectively). Immunohistochemical staining results showed that the protein expression levels of SYK, p38, JNK, PI3K, and ERK in LGBLEL tissues were significantly higher than in orbital CH. Western Blotting showed that the protein contents of p-SYK, p-p38, p-JNK, p-PI3K, and p-ERK were significantly higher than in orbital CH (P=0.0169, P=0.0074, P=0.0046, P=0.0157, P=0.0156, respectively).

Conclusion: The genes and proteins related to the FcεRI signaling pathway are up-regulated in LGBLEL, indicating that the FcεRI signaling pathway participates in

the pathogenesis of LGBLEL.

Keywords: Lacrimal gland; Benign lymphoepithelial lesions; Pathogenesis; FcεRI signaling pathway

Lacrimal gland benign lymphoepithelial lesion (LGBLEL) is an inflammatory disease that is common in middle-aged women. Symptoms of LGBLEL include swelling of the eyelids and diffuse enlargement of the lacrimal gland. The typical pathological manifestations are diffuse infiltration of lymphocytes and plasma cells in lacrimal gland tissue, atrophy and disappearance of glands, and hyperplasia of fibrous tissue [1]. At present, the pathogenesis of and mechanisms involved in LGBLEL are not clear, though several different hypotheses suggest that LGBLEL may be related to sex hormones, basal cell infiltration, autoimmune disease, and/or correlated with IgG4 levels [1–3]. Some cases of LGBLEL have been found that the expression of IgG4 in serum and tissue is increased. Therefore, LGBLEL with positive expression of IgG4 is considered to be IgG4-related ocular disease (IgG4-ROD) [4]. IgG4-ROD is an immune-associated inflammatory disease, whose etiology may be related to allergic diseases such as Sjogren's syndrome, allergic rhinitis, dry eye, and asthma [5,6].

Studies have found that complement mediated signaling pathways, T cell signaling pathways, and B cell signaling pathways are all involved in the pathogenesis of LGBLEL [1,7,8]. FcεRI is a high-affinity IgE receptor, which has been shown to be involved in type I hypersensitivities, such as allergic asthma [9]. Activation of the classical IgE-mediated FcεRI signaling pathway can directly lead to increased secretion of cytokines, such as interleukin (IL)-3, IL-4, IL-5, IL-13, and tumor necrosis factor α, leading to activation of helper T2 cells (Th2) and eosinophils, as well as aggravation of the inflammatory response, which is manifested by increased concentrations of serum IgG4 and IgE. IgE has a positive feedback effect on the FcεRI signaling pathway. Non-IgE-mediated allergic reactions can be caused by activation of IgG immune-related complexes and complement systems [10–12]. Activation of the FcεRI signaling pathway can lead to the release of inflammatory mediators and promote a chronic inflammatory response. If the inflammation continues, it can lead to long-term lacrimal tissue injury, fibrosis, remodeling, and even malignant transformation. Therefore, we hypothesize that IgE-mediated FcεRI signaling pathway may be involved in the pathogenesis of LGBLEL.

In this study, transcriptomics and proteomics were combined to analyze the role of the FcεRI signaling pathway in the pathogenesis of LGBLEL. RT-PCR, immunohistochemical staining, and Western Blotting were used to verify the expression of genes and proteins related to the FcεRI signaling pathway in LGBLEL tissues and to study the mechanism of action of the FcεRI signaling pathway.

Subjects and Methods

Subjects

In this study, nine LGBLEL and nine CH patients, diagnosed via histopathological examination by two experienced pathologists in Beijing Tongren Hospital, Capital Medical University, between August 2010 and March 2013 were

randomly selected for transcriptome sequencing. The LGBLEL group had a male-to-female ratio of 1:3.5 and ranged in age from 36 to 70 years, with a median age of 46 years. The male-to-female ratio of the CH group was 1:2, and ranged in age from 31 to 59 years with a median age of 52 years.

Six cases of LGBLEL and six cases of orbital CH, diagnosed via histopathological examination by two experienced pathologists in Beijing Tongren Hospital, Capital Medical University, between July 2010 and October 2013, were randomly selected for proteome sequencing. The LGBLEL group had a male-to-female ratio of 1:5, and ranged in age from 28 to 64 years, with a median age of 38.5 years. In the CH group, the male-to-female ratio was 1:2, and ranged in age from 31 to 55 years, with a median age of 51.5 years.

Four cases of LGBLEL and three cases of CH, diagnosed via pathologic examination by two experienced pathologists in Beijing Tongren Hospital, Capital Medical University, between October 2018 and August 2019, were randomly selected as the experimental group and control group, respectively, for a verification experiment. The LGBLEL group had a male-to-female ratio of 1:3 and ranged in age from 45 to 50 years, with a median age of 46 years. The control group had a male-to-female ratio of 2:1 and ranged in age from 45 to 53 years, with a median age of 48 years.

For experiments involving human participants (including the use of tissue samples), informed consent had been obtained. This study was done in accordance with the principles of the Helsinki Declaration and was approved by the Ethics Committee of Beijing Tongren Hospital, Capital Medical University.

Tissue and blood specimen collection

The pathological tissue samples of LGBLEL and orbital CH were collected by clinicians during surgery and transferred to the standardized laboratory for study. Portions of the pathological tissue specimens were cryopreserved for later use, while the other parts were soaked in 10% formalin for paraffin embedding and sectioning.

Joint analysis of transcriptome and proteome

The frozen tissue was ground into powder in liquid nitrogen, and 1 mL of lytic solution was added to every 50–100 mg of tissue for homogenization. The supernatant was centrifuged at 12,000 rpm at 4°C for 10 min and transferred to a new tube. The solution was mixed with an equal volume of 70% ethanol and put into an adsorption column. The solution was centrifuged at 4°C for 1 min. After the waste liquid was discarded, the adsorption column was put into a collection tube. The adsorption column was washed with 700 mL RW1 and 500 mL RPE and centrifuged. The waste liquid was discarded and transferred to a new collection tube and centrifuged at 4°C for 2 min. The adsorption column was removed and placed into a new tube, and 30 ml of RNase-free water was added. The RNA was centrifuged at 4°C for 1 min and stored for later use. An mRNA library was constructed and purified, and cDNA was synthesized. After PCR amplification, a library quality inspection was conducted. After the quality inspection, computer sequencing was performed to obtain the

original transcriptome data.

The protein was extracted via the RIPA method, and the concentration, total amount, and homogeneity of the protein were determined. After qualified quality inspection, a Q ExActive mass spectrometer from Thermo Scientific was used for secondary mass spectrometry identification and protein quantification. Samples were processed and labeled according to the Pierce TMT ® Mass Tagging Kits and Reagents kit and the AB Sciex iTRAQ™ Reagent kit instructions. Mixed labeled samples were used for C18 column grading. After the samples were fully dissolved, they were loaded into the liquid-mass spectrometry (LCMS) and sequenced by secondary mass spectrometry. The original proteome data were obtained, and the GO and KEGG combined analysis was performed.

Reverse transcription polymerase chain reaction (RT-PCR)

The pathological tissue was cut, lysate added, and RNA extracted. PCR primers were designed, and the cDNA of total RNA was synthesized via the reverse transcription method according to the RT kit instructions, and was used as a template for RT-PCR. The PCR reaction conditions were 95°C/3 min, 95°C/30 s, 55°C/20 s,72°C/20 s, 40 cycles, with GAPDH used as the internal reference. Primer sequence information is shown in Table 1.

Table 1. Sequences of real-time PCR primers

Gene	Primer Sequence 5’ -3’
GAPDH	F: GCCTTCCGTGTCCCCACTGC
	R: GGCTGGTGGTCCAGGGGTCT
Lyn	F: GCCTTGTACCCCTATGATGG
	R: CTATTTCCTGGTGCCAAAAG
SYK	F: TGAAGCAGACATGGAACCTG
	R: CAATTTGCTCAGATTCTTCCC
P38	F: GGTTACGTGTGGCAGTGAAG
	R: CAATGTTGTTCAGATCTGCCC
ERK	F: TGTTGCAGATCCAGACCATG
	R: AGGTCTTCTTGTGATGGGGA
JNK	F: TCAGAATCAGACTCATGCCA
	R: CATCTGAATCACTTGGCAAAG
PI3K	F: CAACAACTGCATCTTCATCG
	R: TCTCTTCTCCGTTCTTGAGG

Immunohistochemical staining

The diseased tissue sections were dewaxed, incubated at room temperature for 5–10 min, washed with distilled water, and soaked in PBS for 5 min. Drops of primary antibody were added, and the tissue sections incubated overnight at 4°C. The tissue was then washed three times with PBS, biotin-labeled secondary antibody was added after 5 min, and the tissue sections incubated at 37°C for 30 min. The tissue was washed again three times with PBS, DAB stained, rinsed with water, hematoxylin stained, mounted, and imaged under a microscope (Olympus CX41, Japan). The

primary antibodies used for the immunohistochemical staining of proteins related to FcεRI signaling pathway were Lyn, Syk, p38, JNK, PI3K, and ERK.

Western blotting

The diseased tissue was cut into pieces, lysate was added, the tissue was centrifuged, and the protein concentration was determined. An electrophoretic gel was prepared, and the protein samples were mixed with 5× Loading Buffer and treated at 100°C for 5 min. One hundred micrograms of total cell protein were collected from each well, and electrophoresis was performed with a constant current power source of 15 mA per gel. The transfer tank was filled with electro-transfer solution to start the film transfer, and the ECL system was used for western blotting.

Data processing and statistical analysis

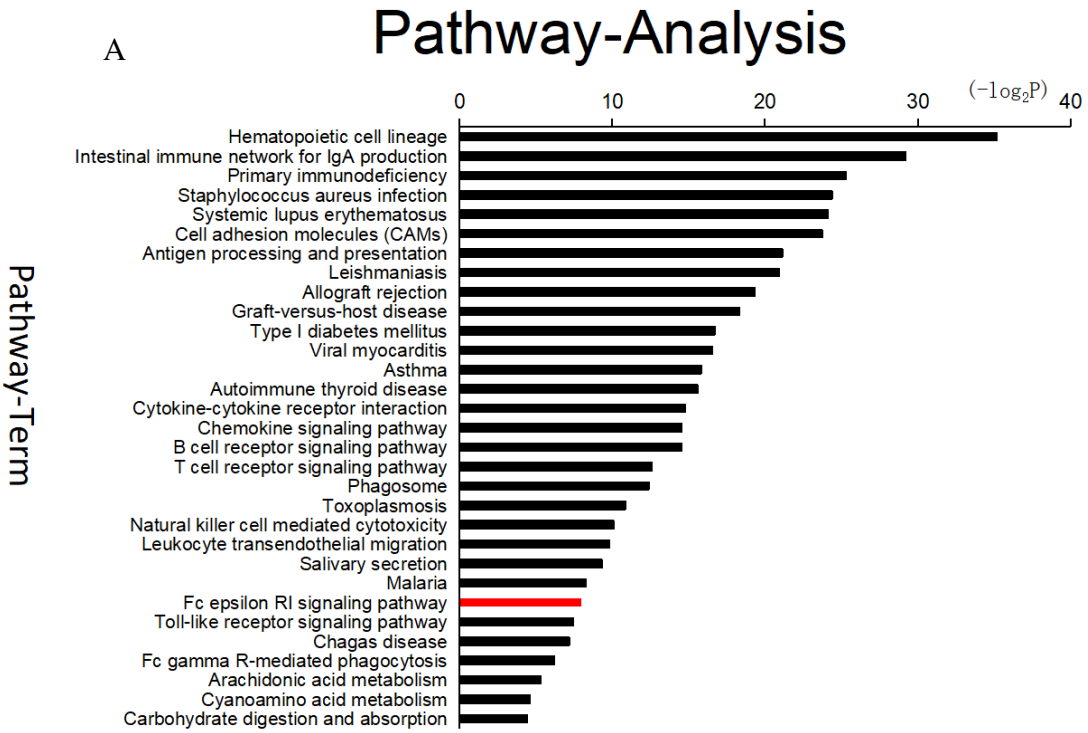
Statistical analysis was performed using SPSS Version 18.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). An unpaired T test was used to analyze the differences in RNA content and protein concentration between the LGBLEL and CH groups. $P<0.05$ was considered statistically significant.

Results

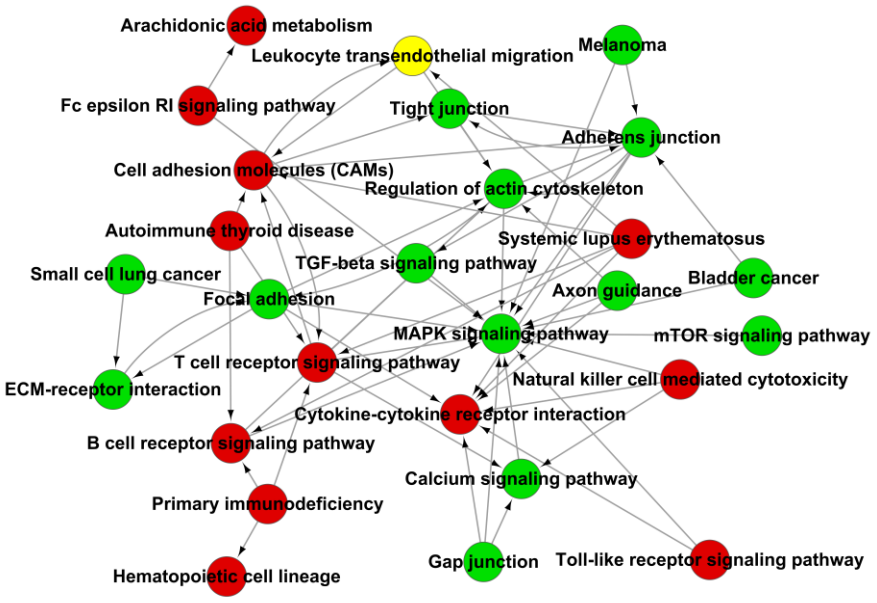
Results of combined transcriptome and proteome analysis

Transcriptome analysis showed that, compared with the orbital CH group, 31 signaling pathways were up-regulated and 25 signaling pathways were down-regulated in the LGBLEL group (Figure 1A and B). The FcεRI signaling pathway was up-regulated in the LGBLEL group ($P=0.0040$) (Figure 1C). The transcriptome combined with the proteome analysis indicated that important proteins related to the FcεRI signaling pathway were highly expressed in LGBLEL tissues, including SYK, p38, JNK, PI3K, and ERK (Figure 1D).

A



B



C	Path ID	Path Term	DifGene	P-Value	FDR	-log2P
	hsa04664	Fc epsilon RI signaling pathway	17	0.0040	0.0299	7.9569

D

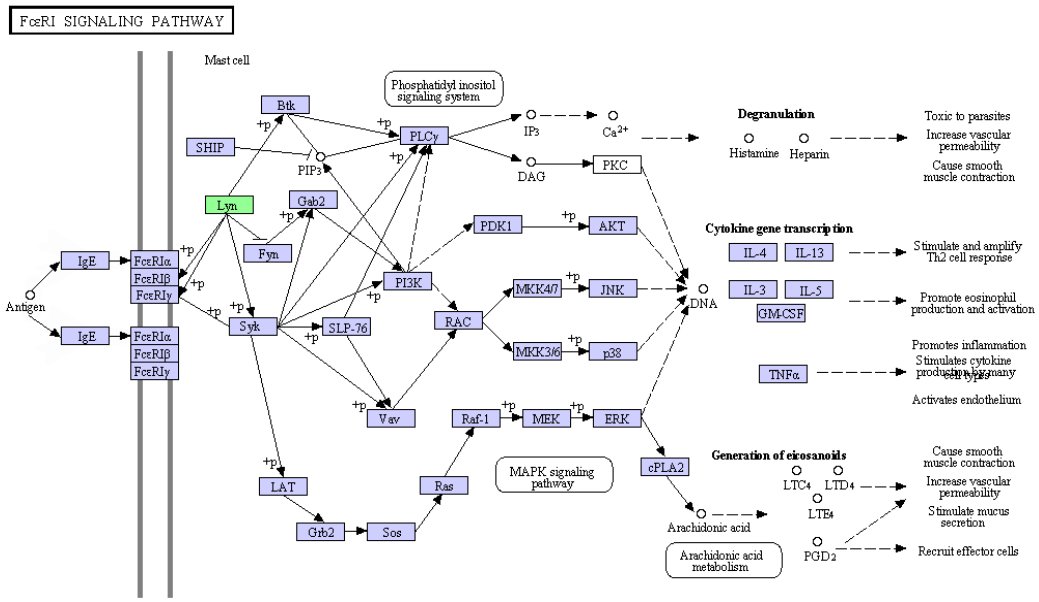


Figure 1. The results of transcriptome combined with proteomics analysis. A: Transcriptome analysis showed that 31 LGBLEL signaling pathways were up-regulated. B: The interconnection diagram of important signaling pathways in LGBLEL. C: Transcriptomics analysis showed that the FcεRI signaling pathway was up-regulated in LGBLEL compared with CH (P=0.0040). D: The expression of protein related to the FcεRI signaling pathway. Green represents up-regulation, purple represents down-regulation.

The mRNA expression levels of SYK, p38, JNK, PI3K, and ERK related to the FcεRI signaling pathway were increased in LGBLEL

The mRNA expression levels of SYK, P38, JNK, PI3K, and ERK related to the FcεRI signaling pathway were significantly increased in the LGBLEL group compared with the orbital CH group ($P=0.0066$; $P = 0.0002$; $P = 0.0003$; $P < 0.0001$; $P<0.0001$, respectively) (Figure 2).

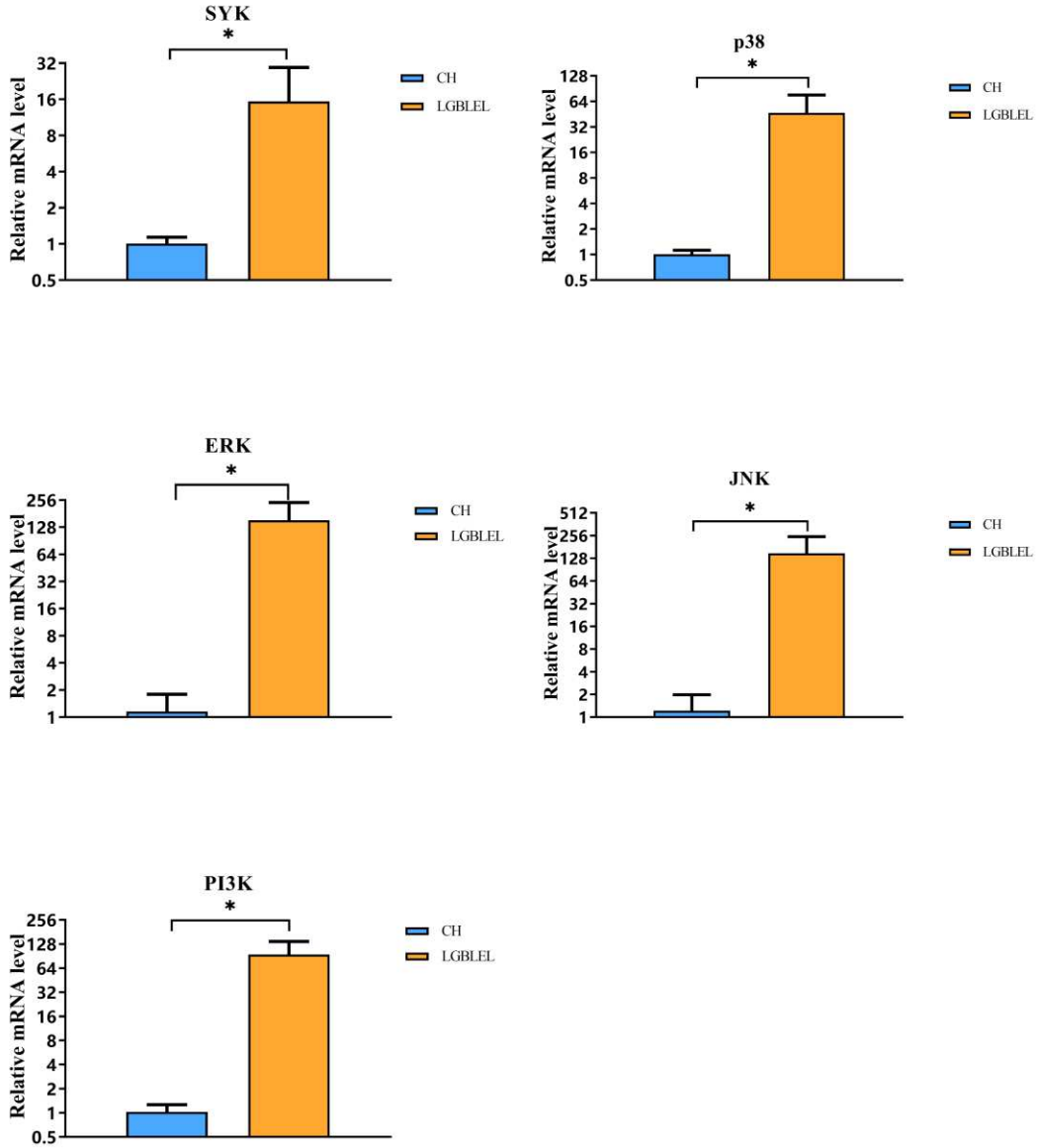
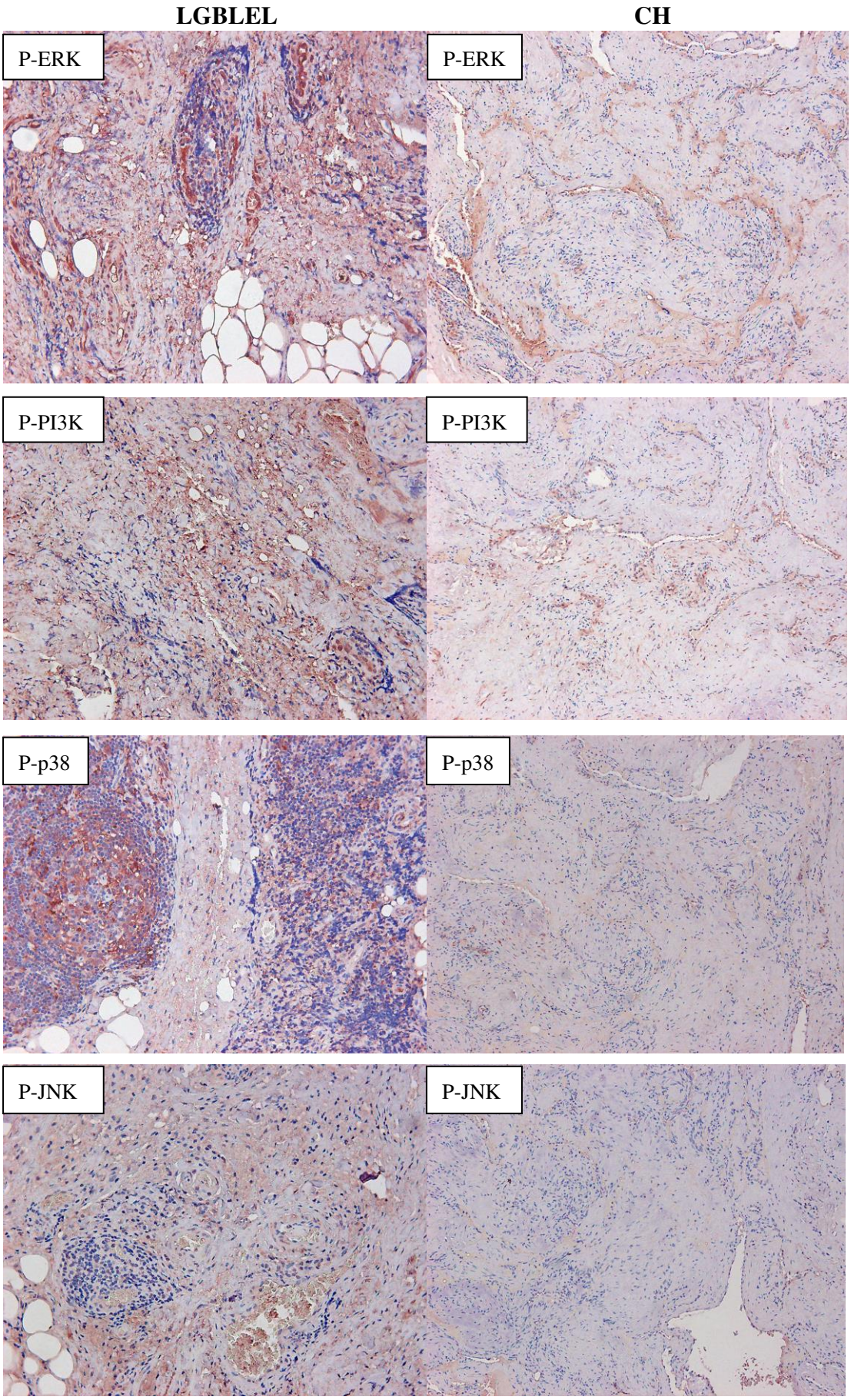


Figure 2. The mRNA expression levels of important genes in the FcεRI signaling pathway. Compared with CH, the mRNA expression levels of SYK, P38, JNK, PI3K and ERK in LGBLEL tissues were significantly increased (P=0.0066, P=0.0002, P=0.0003, P<0.0001, P<0.0001). "*" means statistically significant.

Immunohistochemical staining showed higher protein expression levels of SYK, p38, JNK, PI3K, and ERK in LGBLEL tissues than in orbital CH tissues

The results of immunohistochemical staining showed that SYK, p38, JNK, PI3K, and ERK proteins were positively expressed and the expression levels were significantly higher in LGBLEL tissues than in orbital CH. The positive protein was stained brown and yellow (Figure 3).


LGBLEL
CH
P-ERK
P-ERK
P-PI3K
P-PI3K
P-p38
P-p38
P-JNK
P-JNK

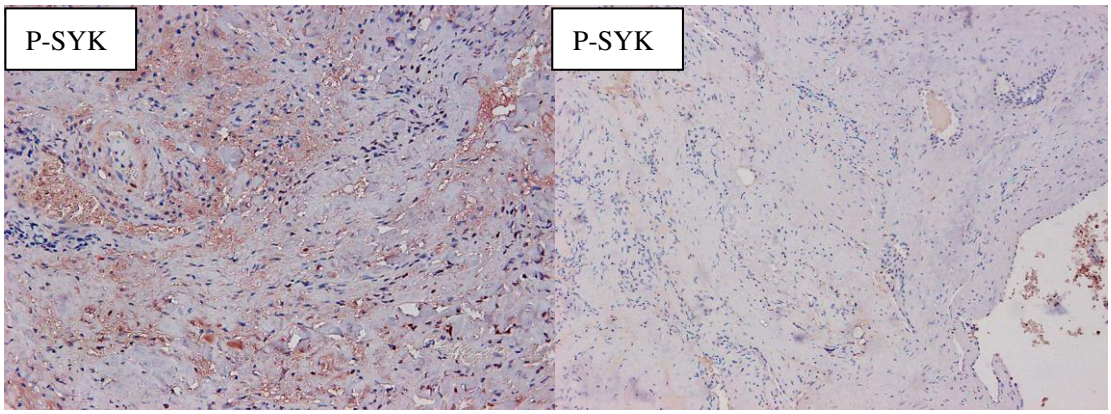
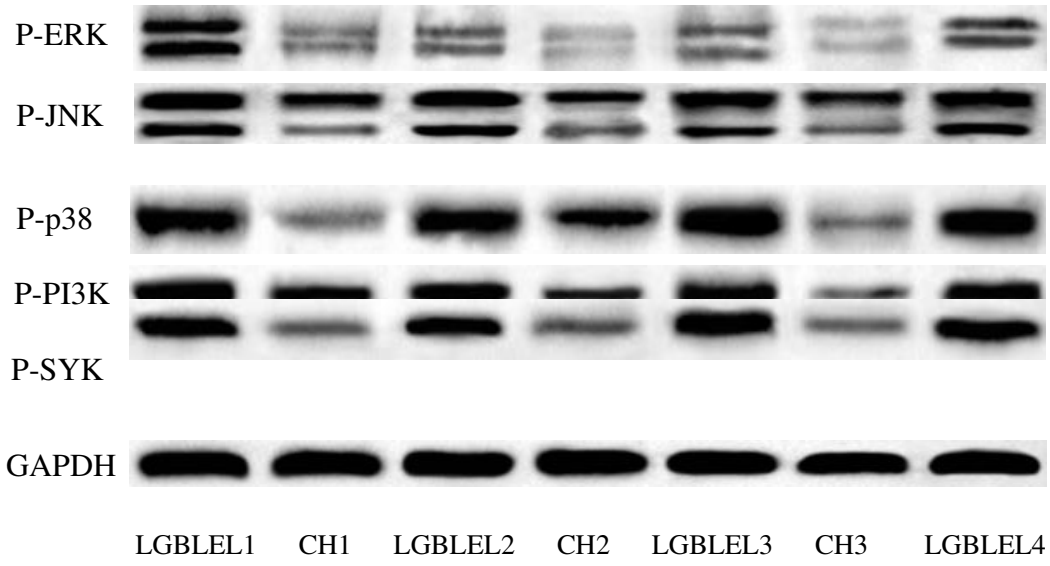


Figure 3. Immunohistochemical staining of important proteins related to FcεRI signaling pathway in LGBLEL and CH. Syk, p38, JNK, PI3K and ERK proteins were brownish yellow in LGBLEL tissues (SP method, magnification X200), and its expression levels were higher than those in orbital CH (SP method, magnification X100).

Western blotting showed higher p-SYK, p-JNK, p-p38, p-PI3K, and p-ERK protein expression in LGBLEL tissues

According to the results of western blotting and protein content detection, p-SYK, p-p38, p-JNK, p-PI3K, and p-ERK proteins were highly expressed in LGBLEL tissues and were significantly higher than in the CH group (Figure 4A). The p-SYK, p-p38, p-JNK, p-PI3K, and p-ERK protein contents in LGBLEL tissues were significantly higher than in CH (P=0.0169; P=0.0074; P=0.0046; P=0.0157; P=0.0156, respectively) (Figure 4B).

A



B

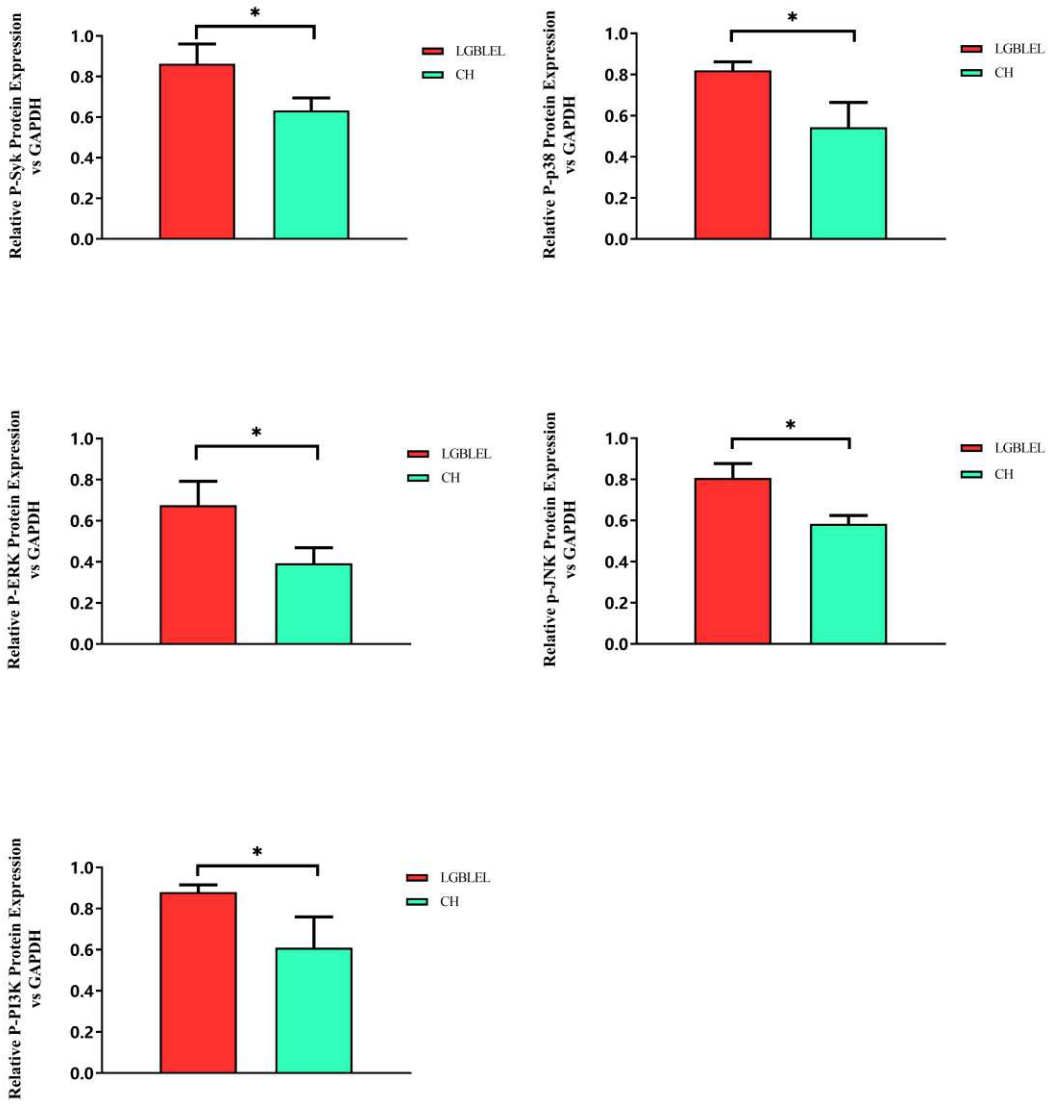


Figure 4. Comparison of protein immunoblotting and protein content between LGBLEL and orbital CH. A: Western blotting showed higher expression of proteins related to FcεRI signaling pathway in LGBLEL than in orbital CH. B: The protein contents of p-SYK, p-p38, p-JNK, p-PI3K and p-ERK were significantly higher than those of orbital CH (P=0.0169; P = 0.0074; P = 0.0046; P = 0.0157; P = 0.0156).

Discussion

The FcεRI signaling pathway has been proven to be associated with allergic responses, inflammatory responses, and autoimmune diseases, such as rheumatoid arthritis and osteoarthritis [13–15]. Allergic diseases such as allergic rhinitis, chronic sinusitis, and asthma are common causes of chronic inflammatory lesions and associated with the activation of mast cells and basophil granulocytes. LGBLEL is a chronic inflammatory disease, which clinical studies have indicated may be related to sinusitis, allergic rhinitis, and other diseases. Adzavon et al.[16] found that the FcεRI signaling pathway may be involved in the mechanism leading to malignant development in LGBLEL. The occurrence of malignant transformation was one of the

pathological processes of LGBLEL; therefore, the FcεRI signaling pathway was examined in this study to further verify its role in the LGBLEL pathogenesis.

The combined transcriptome and proteomics analysis showed that the FcεRI signaling pathway was involved in the pathogenesis of LGBLEL, and RT-PCR was used to quantitatively analyze the important genes related to the FcεRI signaling pathway. The mRNA expression levels of SYK, p38, JNK, PI3K, and ERK were found to be increased in LGBLEL tissues. Western blotting and immunohistochemical staining confirmed that SYK, p38, JNK, PI3K, and ERK protein expressions were significantly higher in LGBLEL tissues than in orbital CH. This study confirmed the involvement of the FcεRI signaling pathway in the pathogenesis of LGBLEL.

Syk is a cytoplasmic tyrosine kinase that is involved in many cellular signaling processes and drives immune inflammation [17]. Studies have shown that SYK, JNK, and ERK play important regulatory roles in B cell receptor signaling pathways [18]. Syk, PI3K, JNK, and p38 can jointly affect T cell activation and participate in the T cell receptor signaling pathway [19–21]. Syk and JNK can participate in complement system-mediated phagocytosis, T cell activation, and inflammatory responses [22,23]. In previous studies, it has been confirmed that the B cell receptor signaling pathway, the T cell receptor signaling pathway, and the complement signaling pathway are involved in the LGBLEL pathogenesis [1,7,8]. The B cell receptor, T cell receptor, and FC receptor are classical immune-related receptors [17]. Therefore, we hypothesized that these signaling pathways may interact with FcεRI-mediated signaling pathways to promote inflammatory infiltration of lacrimal tissue, leading to lacrimal gland enlargement and fibrosis.

IgG4-RD is considered to be a Th2 cell-dominated disease, and cytokines, such as IL-4 and IL-13, can promote IgG4 conversion [24]. Studies of IgG4-RD have shown infiltration of a large number of CD4+ and CD25+ Treg cells in the affected tissues and an increase in the number of CD4+ and CD25+ Treg cells in the blood, suggesting activation of Treg cells in IgG4-RD [25]. Treg cells and Th2 cells can produce inflammatory mediators, such as IL-10, IL-4, IL-5, IL-13, and transforming growth factor β, leading to chronic inflammation. IgE-mediated activation of the FcεRI signaling pathway can directly lead to increased secretion of cytokines, such as IL-3, IL-4, IL-5, IL-13, and tumor necrosis factor-α, leading to Th2 cell activation, eosinophilic activation, and aggravation of the inflammatory response [10,11]. The release of inflammatory mediators can activate the B cell receptor pathway, T cell receptor pathway, and complement signaling pathway, leading to lacrimal gland injury, tumor-like hyperplasia, and dysfunction.

In this study, we demonstrated the role of IgE-mediated FcεRI signaling in the pathogenesis of LGBLEL. Studies have shown that in the IgG-mediated replacement pathway, IgG acted on a variety of immune cells by cross-linking with FcγR [12]. Beutier et al. showed that FcγRIII receptors were the main receptors for IgG-dependent passive systemic allergic reactions induced by IgG1, IgG2a, and IgG2B antibodies, and that macrophages, mast cells, basophils, and neutrophils participated in the IgG-mediated pathway through the recruitment of FcγR [26]. IgG immune complex can also cause the release of complement C3A, C5a, and C5b-9,

which then activates mast cells and basophils, causing cell degranulation and release of soluble mediators [27]. Due to the elevated expression of IgG and its subtypes in serum of some LGBLEL patients, the IgG-mediated replacement pathway and complement system may also play important roles in the pathogenesis of LGBLEL.

Due to the difficulty in obtaining normal lacrimal gland tissue, this study used orbital cavernous hemangioma as the control group, which is consistent with previous studies [7,8]. The role of the IgG-mediated substitution pathway and complement system in the pathogenesis of LGBLEL and the relationship between the FcεRI signaling pathway and other signaling pathways need to be further studied and verified. In addition, the expression level of inflammatory mediators still needs to be determined in serum from LGBLEL patients. The pathogenesis of LGBLEL is complex and involves multiple signaling pathways. Based on previous studies, the results of this study indicate that the FcεRI signaling pathway is involved in LGBLEL pathogenesis and provides new evidence for understanding the pathogenesis of LGBLEL.

Abbreviation

LGBLEL: lacrimal gland benign lymphoepithelial lesions
FcεRI: FcepsilonRI
CH: cavernous hemangioma
GO: gene ontology
KEGG: kyoto encyclopedia of genes and genomes
IgG4-ROD: IgG4-related ocular disease
RT-PCR: reverse transcription polymerase chain reaction

Ethics approval and consent to participate

This article does not include the patients' name, portrait and other private information. Informed consent was obtained from the patient for publication of this article and any accompanying images.

Consent for publication

All authors read and approved the final manuscript to public.

Availability of data and materials

Not applicable

Conflict of Interest Statement

Jing Li, None; Rui Liu, None; Mei Sun, None; Jinjin Wang, None; Nan Wang, None; Xuan Zhang, None; Xin Ge, None; Jianmin Ma, None.

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Authors' contributions

Jing Li and Rui Liu analyzed and wrote the manuscript; Mei Sun, Nan Wang, Jinjin Wang and Xuan Zhang helped collect and analyze data; Jianmin Ma and Xin Ge read and criticized the manuscript. All authors critically read and edited the manuscript. All authors read and approved the final manuscript.

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Not applicable

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Ethics certificate

首都医科大学附属北京同仁医院科研项目临床研究伦理审批件

编号：TRECKY2019-093

项目名称	Fc ε RI 信号通路在泪腺良性淋巴上皮病变发病中的作用机制研究 Research on FcεRI signaling pathway in the pathogenesis of benign lymphoepithelial lesions of the lacrimal gland		
项目来源	国家自然科学基金		
科　室	眼科研究所	研究负责人	李静
会议审查材料	1. 项目简介 2. 研究方案　（版本号：V1.0，版本日期：2019 年 5 月 6 日） 3. 知情同意书（版本号：V1.0，版本日期：2019 年 5 月 6 日）		
会议时间	2019 年 8 月 1 日 13：00-16：00		
会议地点	北京同仁医院 行政楼 702 会议室		
参会人数	出席：8 人；　投票：8 人；　回避：0 人；		
投票结果	☑ 同意：1 票　☑ 作必要的修正后同意：7 票 ☐ 作必要的修正后再审：____票　☐ 不同意：____票		
审查结果	批准☐；　作必要的修正后批准☑；　作必要的修正后再审☐；　不批准☐		

审查意见：

经伦理委员会审查，建议受试者排除儿童，并完善《知情同意书》告知内容和文字表述后，批准按照已审查的《研究方案》开展项目。

（副）主任委员（签章）：

首都医科大学附属北京同仁医院 伦理委员会

2019 年 8 月 1 日

备注：1）如发生严重不良事件，请在获知后 24 小时内报告至伦理委员会；2）如修改研究方案及相关研究材料，请及时提交伦理委员会审查；3）如为跨年度研究，请及时提交年度报告至伦理委员会；4）研究结束后，请及时以书面形式通知伦理委员会。5）本审批件有效期自签发后 1 年。

声明：本伦理委员会组成及操作遵守中国药物临床试验质量管理规范(GCP)、ICH GCP 及中国相关法律法规。

Figures

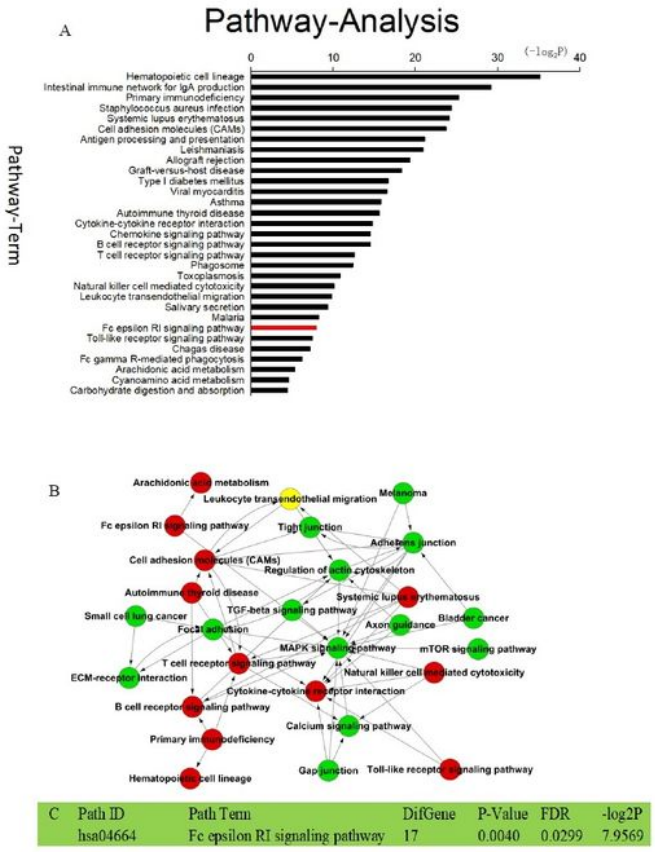

Figure 1

The results of transcriptome c ombined with proteomics analysis. A: Transcriptome an alysis showed that 31 LGBLEL signaling pathways were up regulated. B: The interconnection diagram of important signaling pathways in LGBLEL. C: Transcriptomics analysis showed that the Fc ε RI signaling pathway

was up regulated in LGBLEL compared with CH (0.0040). D: The expression of protein related to the Fc ε RI signaling pathway G reen represents up regulation, purple represents down regulation.

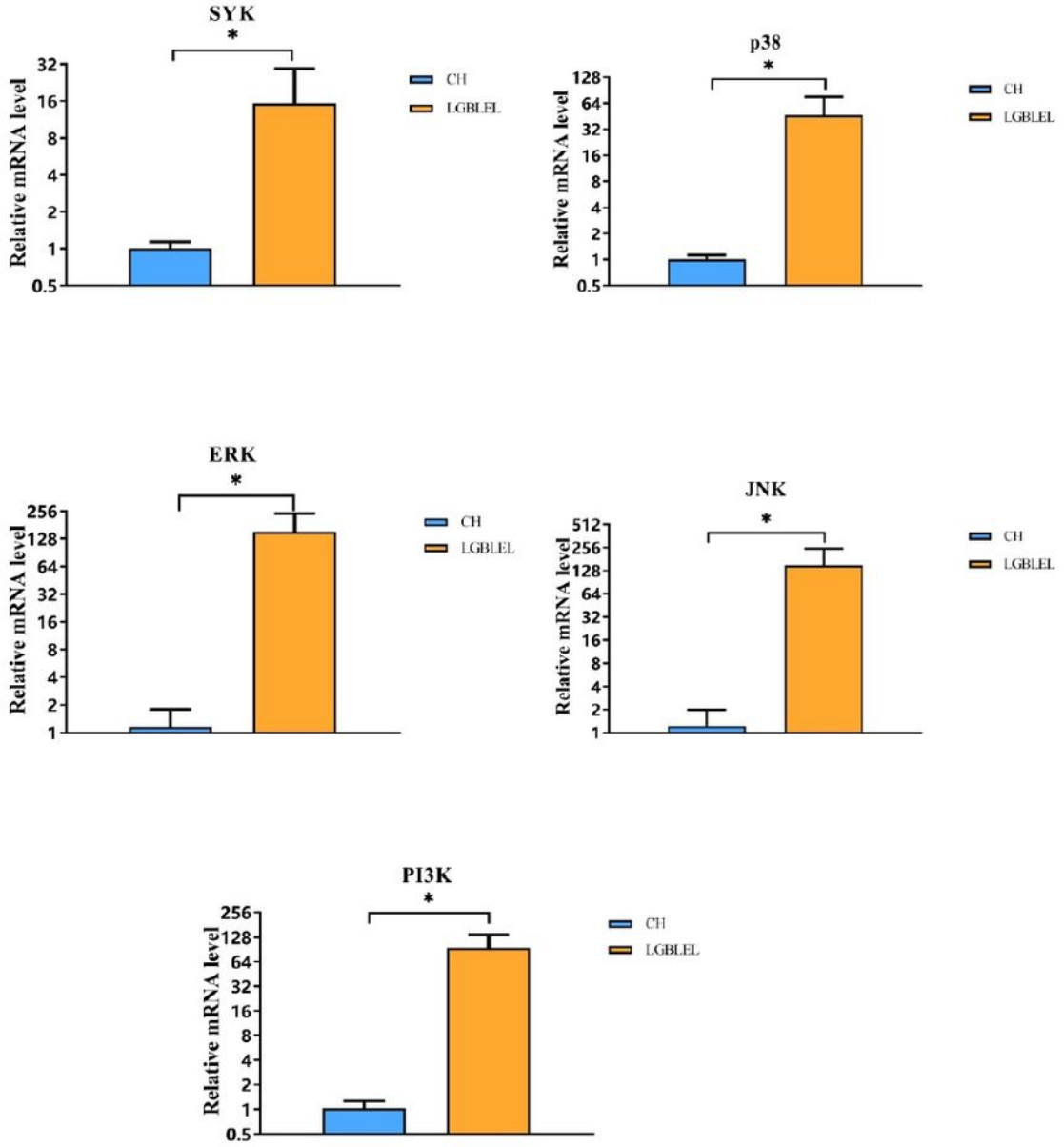

Figure 2

The m RNA expression levels of important genes in the Fc ε RI signaling pathway. Compared with CH , the mRNA expression levels of SYK, P38, JNK, PI3K and ERK in LGBLEL tissues were significantly increased (P=0.0066, P=0.0002 , P=0.0003, P<0.0001, P<0.0001). "*" means statistically significant.

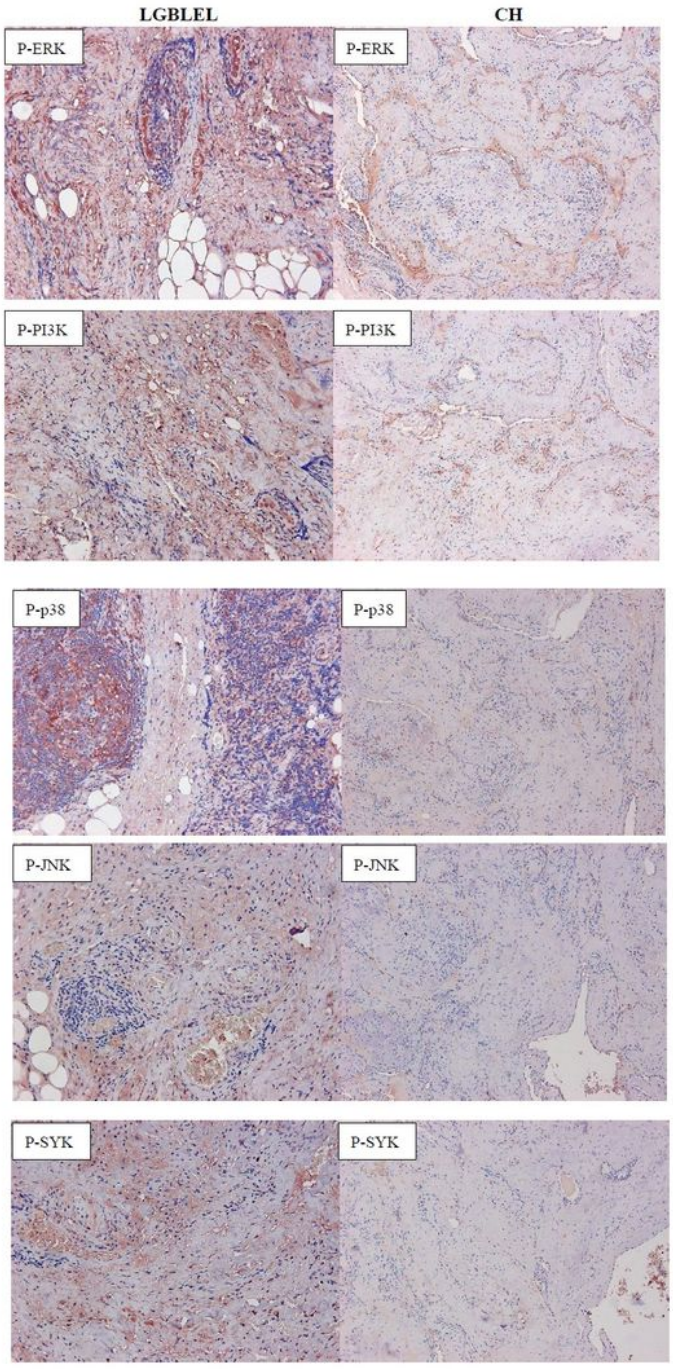

Figure 3

Immunohistochemical staining of important proteins related to Fc ε RI signaling pathway in LGBLEL and CH . Syk, p38, JNK, PI3K and ERK proteins were brownish yellow in LGBLEL tissues (SP method, magnificatio n X200), and its expression levels were higher than those in orbital CH (SP method, magnification

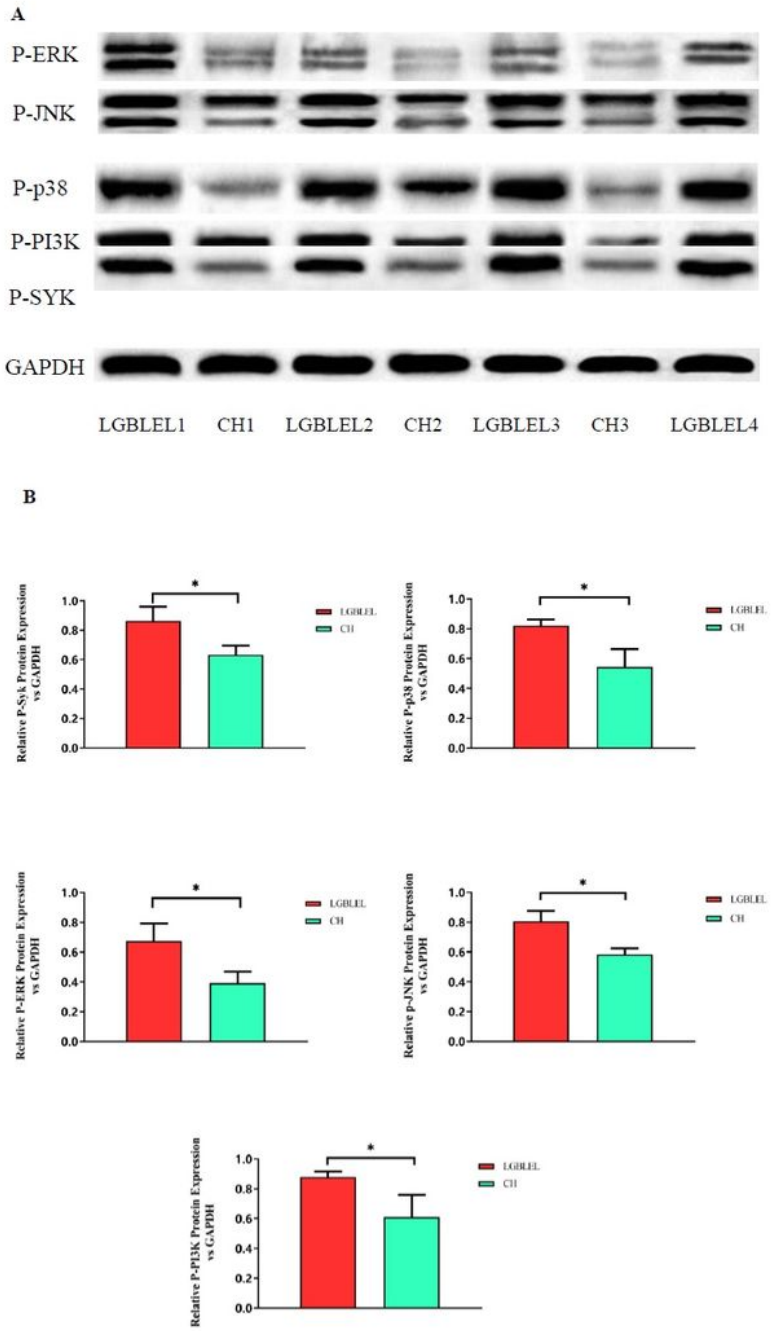

Figure 4

Comparison of protein immunoblotting and protein content between LGBLEL and orbital CH . A : Western blotting showed higher expression of proteins related to Fc ε R I signaling pathway in LGBLEL than in orbital CH . B: The protein contents of p SYK, p p38, p JNK, p PI3K and p ERK were significantly higher than those of orbital CH (P=0.0169; P = 0.00 74; P = 0.0046; P = 0.0157; P = 0.0156).