# The Role of the Complement System Classical and Alternative Pathways in the Pathogenesis of Lacrimal Gland Benign Lymphoepithelial Lesions

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**Research**

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# Abstract

**Objective:** The complement system plays an important role in chronic inflammation and autoimmune diseases. On the basis of previous studies, this paper further analyzed the role of the complement system classical pathway and alternative pathway in the pathogenesis of lacrimal gland benign lymphoepithelial lesions (LGBLEL).

**Methods:** Six cases of LGBLEL and six cases of orbital cavernous hemangioma (CH), diagnosed by histopathology in Beijing Tongren Hospital, Capital Medical University, between July 2010 and October 2013 were randomly selected for proteomic analysis. Gene ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were used to analyze the signaling pathways of differentially expressed genes and proteins. Four LGBLEL and three orbital CH cases, 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 complement system signaling pathway.

**Results:** The expression of complement system signaling pathway in LGBLEL tissue was significantly different ($P<0.0001$) compared with orbital CH, and C3, C5 and C9 were differentially expressed genes. RT-PCR results showed that mRNA expression levels of C1qA, C3, C5 and C9 related to the complement signaling pathway were higher in LGBLEL tissues than in orbital CH ($P<0.0001$). Immunohistochemical staining results showed that C1qA, C3, C5 and C9 protein expression was significantly higher in LGBLEL tissues than in orbital CH. Western blotting showed that the levels of C1qA, C3, C5 and C9 proteins were significantly higher in LGBLEL tissues than in orbital CH ($P=0.0008$; $P=0.0375$; $P=0.0306$; $P=0.0073$, respectively).

**Conclusion:** Both the classical and alternative pathways of complement system are involved in the pathogenesis of LGBLEL.


# Introduction

Lacrimal gland benign lymphoepithelial lesion (LGBLEL) is a chronic inflammatory lesion in which the main clinical manifestation is bilateral painless enlargement of the lacrimal glands accompanied by dysfunction [1,2]. With the classification and increased understanding of “IgG4-related disease” in recent years, some scholars have noted a correlation between the expression level of IgG4 and the pathogenesis of LGBLEL, as well as the similarities between the pathological manifestations of LGBLEL and IgG4-related ocular disease (IgG4-ROD). Therefore, LGBLEL with IgG4-positive expression is considered to be an IgG4-ROD [3, 4].

The complement system is an important component of the innate and adaptive immune system and plays an important role in a variety of disorders and diseases, such as inflammation, autoimmune diseases and immunodeficiency diseases, and is considered to be a key factor in aseptic chronic

inflammatory diseases [5, 6]. The activation pathways of the complement system can be divided into three types according to the different activators: the classical pathway, the mannosebinding lectin pathway and the bypass pathway [7]. The complement system has been found to be involved in the development of IgG4-related disease. IgG activates C3 via the classical pathway and initiates the amplification and activation of downstream complement C5-C9. IgG4 can directly activate C3 through the bypass pathway, and sequentially activate downstream C5-C9, which ultimately plays a role in immune regulation [8, 9]. Preliminary studies analyzed the possible involvement of the complement system in the pathogenesis of LGBLEL through transcriptome sequencing and revealed a relationship between the complement system and the pathogenesis of LGBLEL [10]. On this basis, the current study intends to further examine the role of the complement system in LGBLEL through proteomics.

# Subjects And Methods

### Subjects

In this study, six cases of LGBLEL and six cases of orbital CH, diagnosed by histopathological examination by two experienced pathologists in Beijing Tongren Hospital, Capital Medical University, between July 2010 and October 2013, were randomly selected for proteomic analysis. The LGBLEL group had a male-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. For the verification experiment, four cases of LGBLEL diagnosed by 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 three cases of orbital CH were selected as the control group. In the experimental group, the male to female ratio was 1:3 and ranged in age from 45 to 50 years, with a median age of 46 years. In the control group, the male to female ratio was 2:1 and ranged in age from 45 to 53 years, with a median age of 48 years. All patients were fully aware of the purpose of this study, and informed consent was received. This study was approved by the Ethics Committee of Beijing Tongren Hospital, Capital Medical University.

### Tissue and blood samples

Specimens of LGBLEL and orbital CH were collected intraoperatively by clinicians and quickly transferred to a standardized laboratory for processing and storage. 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.

### Proteomic analysis

Proteins were extracted from tissues and were identified and quantified by secondary mass spectrometry after quality inspection. Samples were processed and labeled according to the instructions in the Pierce TMT ® Mass Tagging and Reagents kit and the AB Sciex iTRAQ™ Reagents kit. Mixed labeled samples were used for C18 column grading. After the samples were fully dissolved, they were loaded into the

liquid mass spectrometry system for secondary mass spectrometry sequencing, and the original files were generated for GO and KEGG analysis.

### Reverse transcription polymerase chain reaction (RT-PCR)

The pathological tissues were cut, lysate was added and RNA was extracted. PCR primers were designed, and cDNA of total RNA was synthesized via reverse transcription, according to RT kit instructions, and the cDNA 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.

### Immunohistochemical staining

Diseased tissue was 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 incubated overnight at 4°C. The tissue was washed three times with PBS, biotin-labeled secondary antibody was added after 5 min, and the tissue was incubated at 37°C for 30 min. The tissue was again washed three times with PBS, 5 min each time, DAB stained, rinsed with water, hematoxylin stained for mounting, imaged under a microscope (Olympus CX41, Japan). The primary antibodies used for protein immunohistochemistry related to the signaling pathway of the complement system were C3, C5, C9 and C1qA.

### Western blotting

The diseased tissue was cut into pieces, and lysate was added. The tissue was then centrifuged, and the protein concentration was determined. An electrophoretic gel was prepared as follows: the protein samples were mixed with 5× loading buffer and treated at 100°C for 5 min. One-hundred micrograms of total cell protein was 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 electrotransfer 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 (Graph Pad Softarelnc., La Jolla, CA, USA). An unpaired t-test was used to analyze differences in RNA content and protein concentration between the LGBLEL group and control group. $P<0.05$ was considered statistically significant.

## Results

### Proteome analysis showed the differential expression of the complement system signaling pathway in LGBLEL

Proteomic analysis showed that the complement system signaling pathway in LGBLEL tissues was differentially expressed (P<0.0001) (Figure 1A), compared with orbital CH. The differentially expressed genes included C3, C5, C9 and C1q. The expressions of important proteins C3, C5 and C9 in the complement system signaling pathway were up-regulated, while the expression of C1q was down-regulated (Figure 1B).

### The mRNA expression levels of C1qA, C3, C5 and C9 related to complement system signaling pathway were increased in LGBLEL

The mRNA expression levels of C1qA, C3, C5 and C9 in the complement system signaling pathway were significantly increased in LGBLEL compared with orbital CH (both P<0.0001) (Figure 2).

### Immunohistochemical staining showed increased expression levels of C1qA, C3, C5 and C9 proteins in LGBLEL

Immunohistochemical staining results showed that the expression levels of C1qA, C3, C5 and C9 were significantly higher in LGBLEL (SP method, magnification X200) than in orbital CH (SP method, magnification X100) and are stained brownish yellow (Figure 3).

### Western blotting showed that C1qA, C3, C5 and C9 proteins were highly expressed in LGBLEL

According to the western blotting results, the protein expression levels of C1qA, C3, C5 and C9 were higher, and the protein content was significantly higher in LGBLEL compared with orbital CH (P=0.0008; P=0.0375; P= 0.0306; P=0.0073, respectively) (Figure 4A and B).

## Discussion

Evidence has shown that the activation of complement system is involved in the pathogenesis of IgG4-related disease and that IgG and IgG4 play important roles in the process of complement activation [11]. Sugimoto et al. found that IgG4 may be involved in complement activation in patients with IgG4-related hypotememia [12]. Muraki et al. reported that 36% of patients with IgG4-associated autoimmune pancreatitis had decreased serum complement C3 and C4, suggesting that the complement activation system is involved in the pathogenesis of IgG4-associated autoimmune pancreatitis [13]. Fukui et al. reported a significant increase of C5a in IgG4-related disease and suggested that C5a should be used as a therapeutic target, though this conclusion is controversial [14, 15]. Breville et al. reported a rare case of complement-mediated vascular microthrombosis secondary to IgG4-related disease, suggesting that IgG4 autoantibodies could induce complement-mediated vascular microthrombosis [16], and Montanez et al. showed that IgG immune complex could cause the release of complement C3a, C5a and C5b-9 [17].

Previous studies used transcriptomics to preliminarily determine the involvement of the complement system in the pathogenesis of LGBLEL[10]. Transcriptomics analyzes organisms at the transcriptional level, including the regulation and function of genes at different stages of disease, while proteomics is a

comprehensive and in-depth study of the proteome of disease and is used to screen key protein biomarkers. Proteins are the real executors in the occurrence and development of diseases and can reflect the characteristics of diseases most directly. Therefore, proteomics can reflect the dynamic changes of protein levels in the body under the pathological state by detecting the proteomic expression of tissue samples at a specific time. Proteomics can more intuitively reflect the molecular mechanism of disease onset and provide new ideas for disease prevention, diagnosis and treatment. Therefore, based on previous studies, the current study applied proteomics analysis to examine the role of the complement system in the mechanism of LGBLEL. To our knowledge, this is the first study to use proteomics to analyze the complement system in LGBLEL.

Unlike orbital CH, the complement system signal pathway was differentially expressed in LGBLEL tissue and appeared to be involved in the pathogenesis of LGBLEL. Furthermore, according to the proteomic analysis, the classical pathway of the complement system and the lectin pathway were down-regulated in LGBLEL, while the bypass pathway was up-regulated. All three pathways play a key role in LGBLEL pathogenesis via activating C3 and then C5, C6, C7, C8 and C9, suggesting that C3 may be a key target for the treatment of LBGLEL.

In order to confirm whether the complement system is involved in the pathogenesis of LGBLEL, we verified the key genes and proteins (C1qA, C3, C5 and C9) related to the complement system via RT-PCR, immunohistochemical staining and western blotting. The results showed that the mRNA and protein expression levels of C1qA, C3, C5 and C9 were significantly higher in LGBLEL than in orbital CH, suggesting that the complement system is involved in the pathogenesis of LGBLEL. C1qA is an important factor in the classical signaling pathway of the complement system, while the bypass pathway can directly activate C3. The high expressions of C1qA, C3, C5 and C9 suggest that both the classical pathway and the bypass pathway play important roles in LGBLEL.

In this study, the proteomic analysis suggests that the activation of C3 in the complement system may be associated with the B cell receptor signaling pathway through the activation of CR2 by C3b, C4b and C3d. Studies have shown that the complement system plays a role in the differentiation and function of B cells and T cells; thus, the complement system, B cell receptor signaling pathway and T cell receptor signaling pathway may be interrelated to jointly promote the occurrence of disease [18, 19]. Previous studies have suggested that the B cell receptor signaling pathway and T cell receptor signaling pathway play important roles in the pathogenesis of LGBLEL; however, the mechanism between the complement system signaling pathway and other signaling pathways needs to be further studied [1,20].

This study had some limitations and the role of the mannosebinding lectin pathway in the pathogenesis of LGBLEL was not verified. Due to the difficulty in obtaining normal lacrimal gland tissue, orbital CH was used as the control group, in line with previous studies [10]. In conclusion, the results of this and of previous studies indicate that both the classical pathway of the complement system and the bypass alternative pathway are involved in the pathogenesis of LGBLEL.

# Abbreviations

LGBLEL: lacrimal gland benign lymphoepithelial lesions

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

# Declarations

### 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

Rui Liu, None; Jing Li, 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

Rui Liu and Jing Li 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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# Table

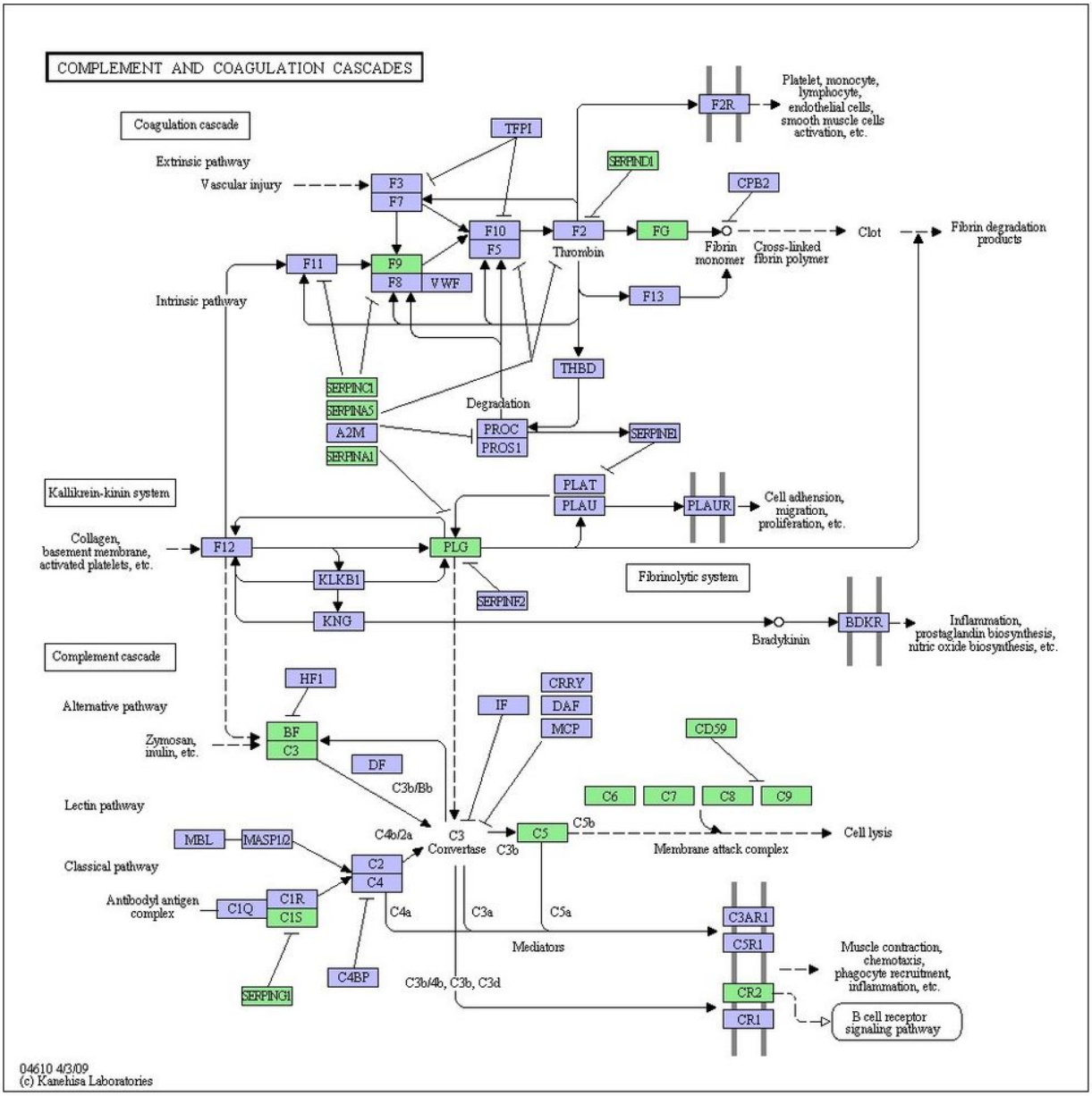
Table 1
Sequences of real-time PCR primers

| Gene | Primer Sequence 5’ -3’ |
|---|---|
| GAPDH | F: GCCTTCCGTGTCCCCACTGC |
| | R: GGCTGGTGGTCCAGGGGTCT |
| C3 | F: CCAGTTTCGAGGTCATAGTGG |
| | R: CCGTCCAGCAGTACCTTCC |
| C5 | F: CGCTTGACCAGTTGGTAGG |
| | R: CTTCCCTGGCCTGATTTTC |
| C9 | F: ATGGTCACAATGCGATCCTT |
| | R: CTCCGCAGTCATTGTCACC |
| C1qA | F: TGGTGACCGAGGACTTGTG |
| | R: GCTGGTCCTTGATGTTTCCT |

# Figures

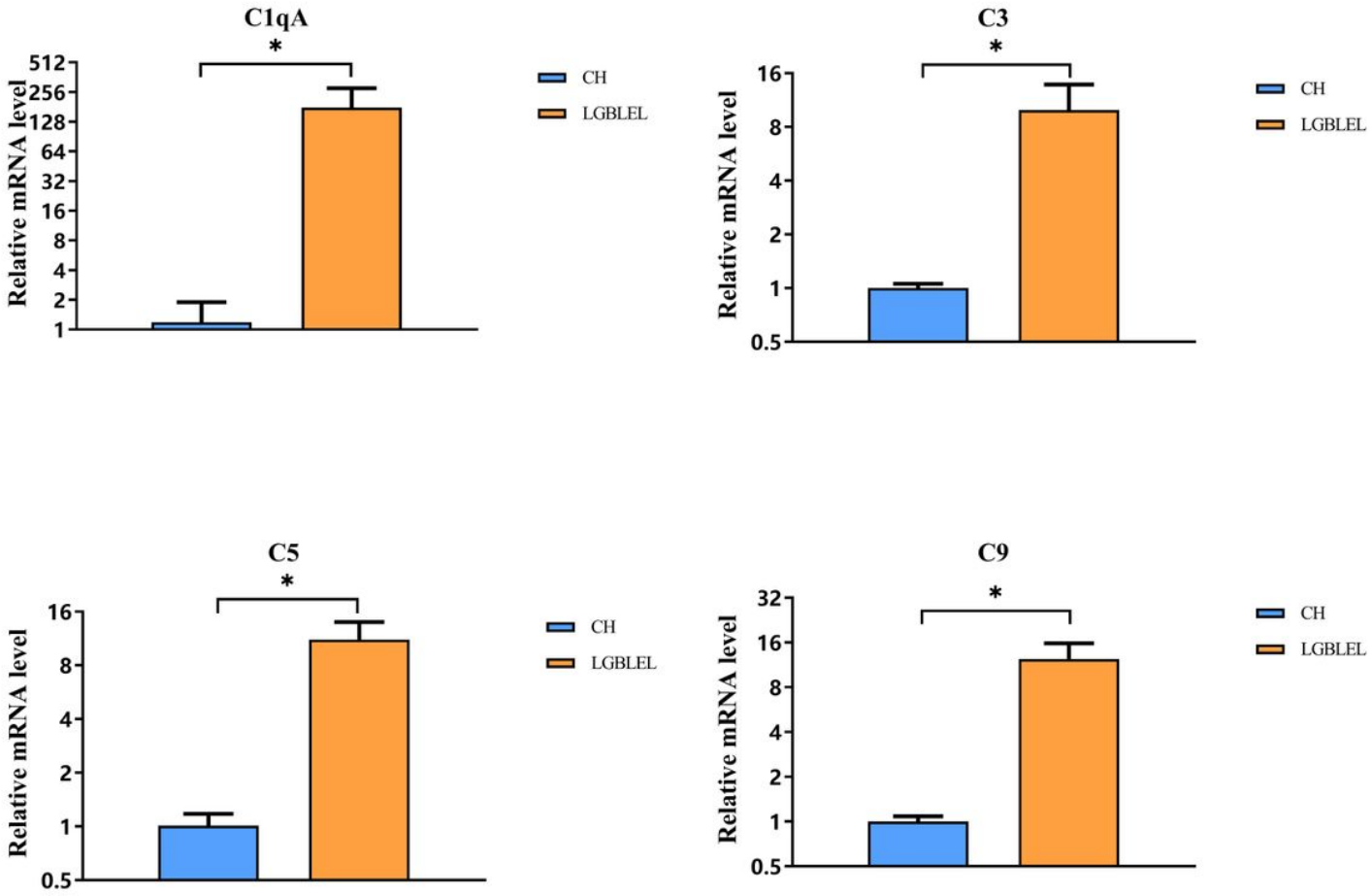
| | Term | Count | P-Value | Gene |
|---|---|---|---|---|
| A | hsa04610: Complement and coagulation cascades | 23 | 1.07E-19 | KNG1, C7, C9, CR2, CFB, C3, C6, C5, F9, SERPING1, C1S, PLG, C8G, C8A, FGG, FGA, FGB, SERPINA5, CD59, SERPINC1, CFH, SERPINA1, SERPIND1 |

B



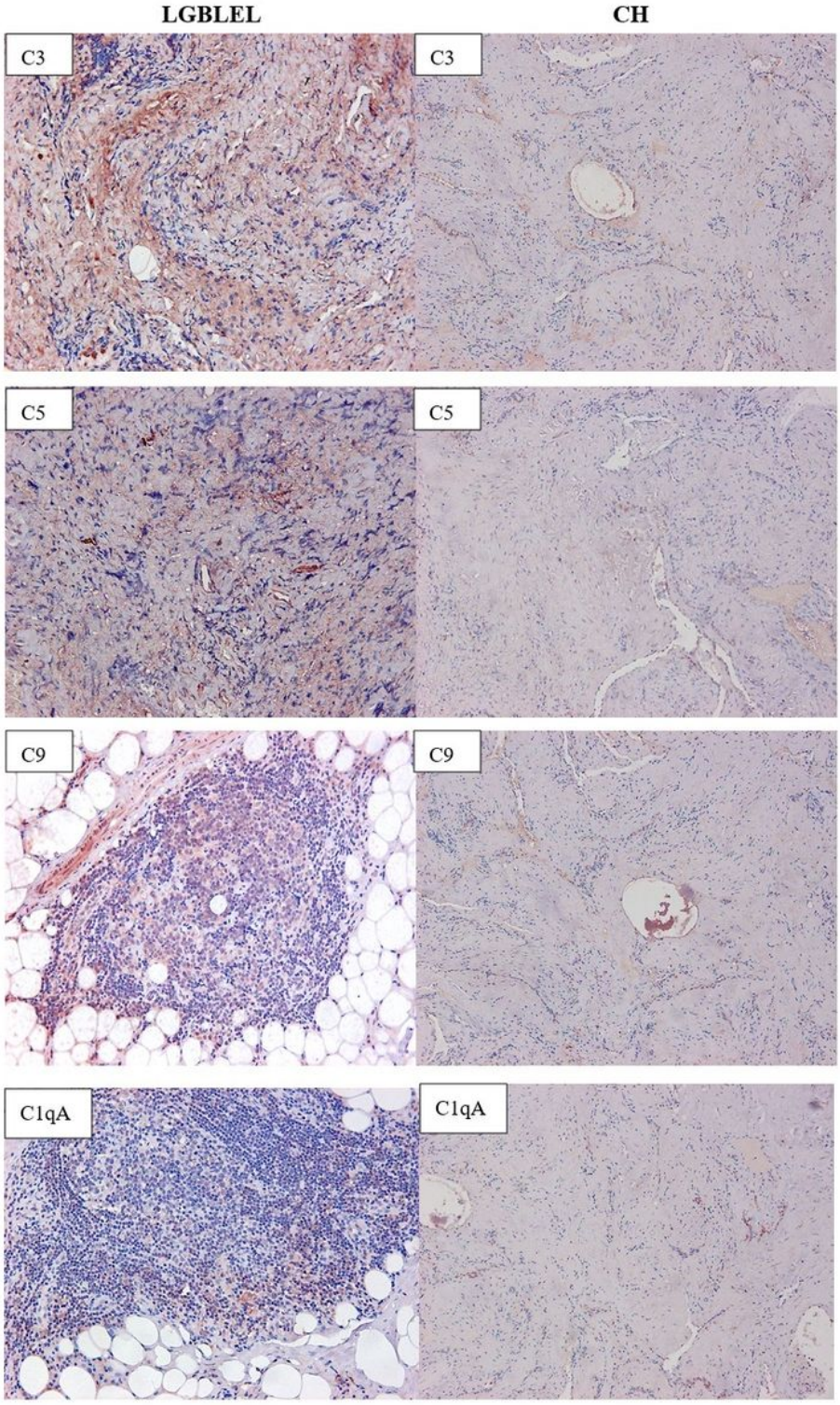

## Figure 1

Results of proteomic analysis. A: Compared with orbital cavernous hemangioma, the complement system signaling pathway was differentially expressed in LGBLEL tissues (P<0.0001). B: The signal pathway of the complement system and the expression of related proteins. Green represented up-regulated and purple represented down-regulated.



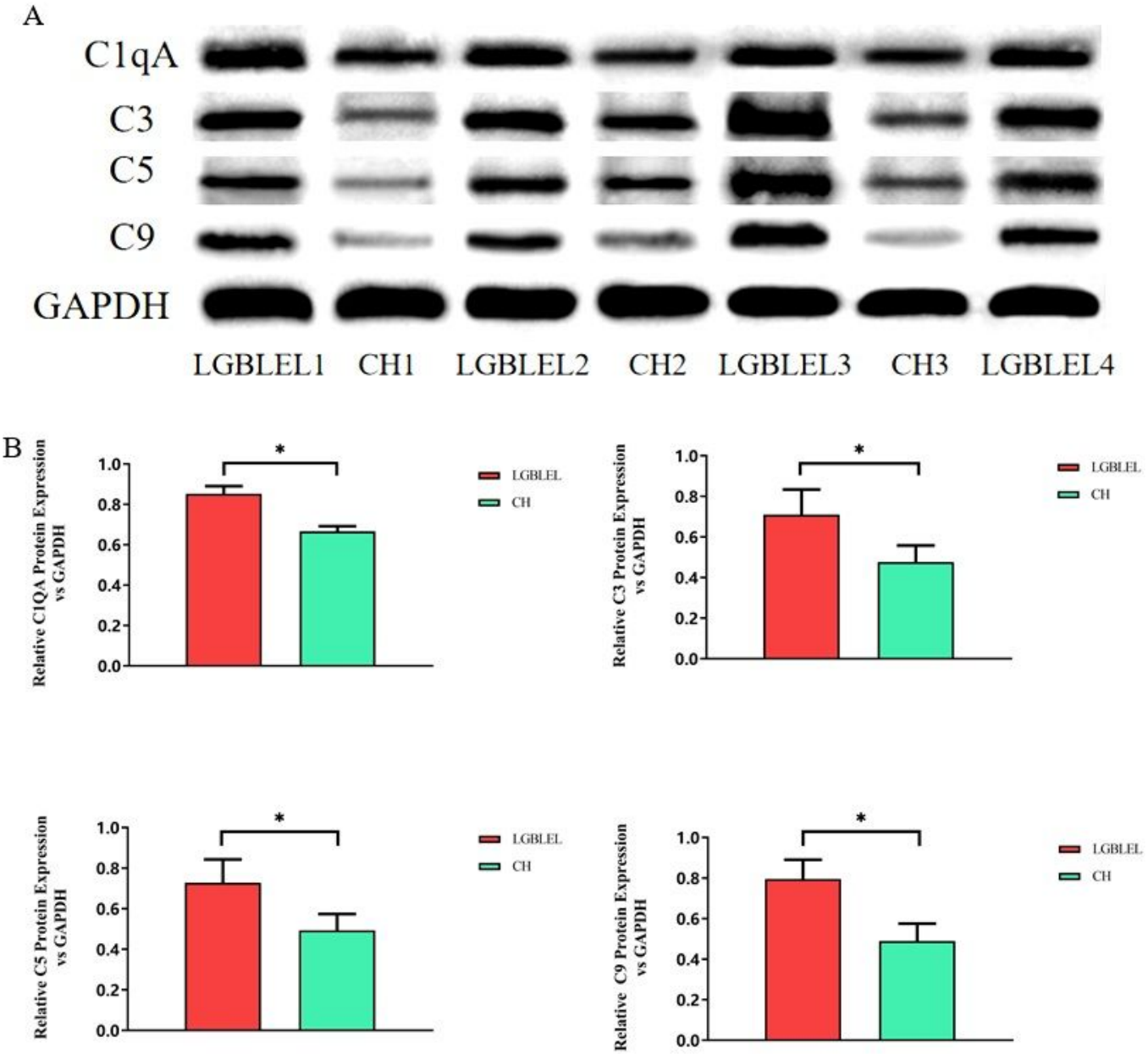

## Figure 2

mRNA expression levels of important genes in the complement system signaling pathway. Compared with orbital cavernoid hemangioma, mRNA expression levels of C1qA, C3, C5 and C9 in LGBLEL tissues were significantly increased (P<0.0001). "*" means statistically significant.




## Figure 3

Immunohistochemical staining results of important proteins in the complement system signaling pathway. The results showed that C1qA, C3, C5 and C9 proteins were brownish yellow in LGBLEL tissues (SP method, magnification, ×200), and the expression levels of these proteins were higher than those in orbital cavernous hemangioma (SP method, ×100).




## Figure 4

Detection results of proteins associated with the complement system signaling pathway. A: Western blotting showed that the expression of related proteins in the complement system signaling pathway of LGBLEL was higher than that in orbital cavernous hemangioma. B: The protein contents of C1qA, C3, C5 and C9 were significantly higher than those of orbital cavernous hemangioma (P=0.0008; P = 0.0375; P = 0.0306; P = 0.0073).