Retrospective Analysis of Clinicopathological Characteristics of Lacrimal Gland Pleomorphic Adenoma and Mechanism of Tumorigenesis by the Imbalance Between Apoptosis and Proliferation

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Background: Lacrimal gland pleomorphic adenoma (LGPA) is the most common clinically benign epithelial tumor of the lacrimal gland and is predominantly comprised of epithelial cells and interstitial components. At present, the exact pathogenesis of LGPA remains unclear. Previous research has indicated that the occurrence of LGPA may be related to excessive cell proliferation.

Material/Methods: This study observed the clinicopathological characteristics of LGPA and investigated the tumorigenesis mechanism of cell over-proliferation caused by the imbalance between apoptosis and proliferation. A total of 27 cases were collected from the Department of Ophthalmology of the Affiliated Hospital of Chengde Medical University and the Third Medical Center of Chinese PLA General Hospital from April 2017 to November 2019. Hematoxylin-eosin (HE) staining and immunohistochemical staining were used to observe the pathological characteristics and analyze the expression of bcl-2 and bax in the lacrimal gland.

Results: Compared with normal lacrimal gland tissues, LGPA tumor tissues had obvious changes in pathological morphology. The expression of bcl-2 in LGPA lesion tissues was dramatically higher ($P<0.001$), the expression of bax was not significantly different between groups ($P=0.25$), but the ratio of bcl-2/bax was significantly higher in tumor tissues ($P=0.01$).

Conclusions: We found that the lacrimal gland tumor tissues had obvious excessive proliferation in pathomorphology, which revealed the necessity of complete surgical removal of the capsule from the perspective of pathological morphology and provided a theoretical basis for the hypothesis that the imbalance between apoptosis and proliferation could lead to cell hyperproliferation.

Keywords: Adenoma, Pleomorphic • Cell Proliferation • Lacrimal Apparatus

Abbreviations: LGPA – lacrimal gland pleomorphic adenoma

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Background

Lacrimal gland pleomorphic adenoma (LGPA), also known as benign mixed lacrimal gland tumor, is the most common type of benign epithelial tumor of the lacrimal gland and accounts for approximately 50% of benign epithelial tumors of the lacrimal gland [1,2]. The main clinical manifestations of the disease include progressive and painless monocular protrusion, displacement, swelling or sagging of the upper eyelid, with or without binocular diplopia, and a palpable lacrimal gland mass located above the orbit [3]. Since the clinical symptoms and imaging findings are non-specific, definite diagnosis of LGPA is dependent on the histologic and immunohistochemical confirmation [4]. According to Lai et al, fine-needle aspiration biopsy is an effective approach for confirmation of diagnosis and guidance of therapy because no correlation between the procedure and recurrence or malignant transformation of LGPA has been observed [5]. Despite recent developments in conservative therapy, the first choice for the treatment of LGPA is surgery [6]. Wang reported that complete and timely surgical resection was required if the tumor was suspected or diagnosed as LGPA, because it is sufficient to minimize the risk of recurrence and can effectively arrest tumor development [7]. However, Behshad et al [8] suggested the tumor might seed into the other tissues when the tumor envelope containing the pleomorphic adenoma was compromised due to attempted biopsy or incomplete surgical excision, which could lead to recurrence and potential malignant transformation over time. Harrison et al, in a retrospective study, found that the recurrence rate after surgical resection was still up to 32%, and there was a high tendency to malignancy after relapse, which seriously affected patient quality of life and resulted in a poor prognosis [9].

Various hypotheses of LGPA tumorigenesis have been proposed in previous studies, including abnormal gene expression, disordered cell cycle regulation, invasive tumor growth, and abnormal cell proliferation [10-12]. Among them, the hypothesis of excessive cell proliferation has increasingly become the focus of occurrence and development of LGPA. Liao et al [13] found that survivin, a member of the inhibitor of apoptosis family, affected abnormal cell proliferation of LGPA. Nevertheless, the relationship between the abnormal cell proliferation and the balance of apoptosis and proliferation in LGPA needs further investigation.

Thus, we started from the balance between cell apoptosis and proliferation to verify the hypothesis. The objective of our study was to observe the pathological features of lacrimal gland lesions and the expression of bcl-2 and bax in lacrimal gland tissues and to preliminarily explore its possible pathogenesis.

Material and Methods

Patient Selection

All patients were admitted to the Department of Ophthalmology of the Affiliated Hospital of Chengde Medical College and the Third Medical Center of the PLA General Hospital from April 2017 to November 2019. Among them, 19 patients with 19 eyes of LGPA patients were selected into an experimental group and 8 patients with normal lacrimal glands were selected into a control group. The 2 groups were compared in terms of age, sex, and eye type, and the differences were not statistically significant (P>0.05).

This study was approved by the Ethics Committee of the Affiliated Hospital of Chengde Medical College and the Third Medical Center of the PLA General Hospital (approval number: LL057). All patients provided signed informed consent to participate in this study.

Inclusion criteria: the patients in the experimental group were first diagnosed as LGPA by pathological examination and their lacrimal gland tumors were surgically removed; the diseased tissues of the control group had undergone orbital content removal and were confirmed by imaging and intraoperative and postoperative pathological examinations to ensure that the lacrimal gland tissues were normal and the diseased tissues might have primary disease.

Exclusion criteria: (1) preoperative chemotherapy, radiotherapy or immunotherapy; (2) pathological diagnosis including inflammatory lesions, lymphoma, adenoid cystic carcinoma and other lacrimal gland tumors.

Methods

HE staining was used to observe the morphological changes and pathological characteristics of the 2 groups of lacrimal gland tissue specimens. We cut the 2 groups of wax blocks into slices with a thickness of 2 μm. The slices were incubated at 63°C, dewaxed by xylene, dehydrated with concentration-gradient alcohol, washed with water and high-definition contrast solution, and stained with hematoxylin. Slices were then differentiated, washed with water and 85% and 95% alcohol, stained with eosin, dehydrated with absolute alcohol, and then sealed. Under a light microscope (×100, ×200), the pathomorphological features of the lacrimal glands in the 2 groups were observed.

Immunohistochemical staining was performed to detect the expression of bcl-2 and bax in the lacrimal gland tissues of the 2 groups. The main reagents used were: primary antibody rabbit-anti-human bcl-2 monoclonal antibody [anti-bcl-2 antibody...
(E17), number: ab32124, 1: 150 dilution] and rabbit anti-human bax monoclonal antibody [anti-bax antibody (E63), number: ab32503, 1: 150 dilution] produced by Abcam. The two-step kit is a universal SP kit (number: SP-9000) produced by Hebei Benyuan Biotechnology Co. Ltd.

Positive standard used was the expression of bax in human lung cancer tissue provided by Hebei Benyuan Biotechnology Company, which is recognized as the positive standard of bax, and the expression of bcl-2 in human B cell lymphoma tissue is recognized as the positive standard of bcl-2.

For the negative control, PBS buffer was used instead of the corresponding primary antibody to make a negative standard.

We cut the wax blocks of the 2 groups into 2 slices with the thickness of 2 μm and oven-baked them at 63°C, followed by dewaxing with xylene and dehydration with concentration-gradient alcohol. Endogenous peroxidase activity was eliminated using 3% H₂O₂. Samples were incubated at room temperature, we repaired antigen with microwave and sodium citrate buffer solution, followed by incubation at room temperature with 5-10% normal goat serum blocking solution. We added primary antibody (negative control with PBS buffer) dropwise and placed the solution in a 4°C refrigerator overnight. After washing in PBS buffer and dropwise addition of biotinylated goat anti-rabbit secondary antibody, we incubated the solution at room temperature, washed with PBS buffer and dropwise added horseradish enzyme-labeled streptavidin working solution. Incubation at room temperature was followed by washing with PBS buffer and developing color with DAB, and we stopped color development after staining. Samples were counterstained and mounted, and we observed the expression of bcl-2 and bax in the lacrimal glands of the 2 groups under a light microscope (×200, ×400).

Image-pro Plus 6.0 software was used to calculate the positive expression area/HP of the lacrimal gland tissues of the 2 groups. Two groups of specimens were randomly selected under the microscope (×400), and the average expression area of each field was statistically calculated.

Judgment criteria: According to the standards of bcl-2 and bax expression sites, bcl-2 was stained with cytoplasm as yellow or brown, and bax is stained in the same way after DAB color development.

Statistical Methods

SPSS 22.0 software was used for statistical analysis. The ages of the 2 groups of patients followed a normal distribution, the data are expressed as mean±sd, and the 2 groups were compared using the independent-samples t test. The comparison of gender and eye type uses the chi-squared ($\chi^2$) test (Fishers exact test). The expression of bcl-2 and bax was performed using tests of normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene’s test). When both of them conformed to normal distribution, the $t$ test was used for the homogeneity of variance and the approximate $t$ value was used for the heterogeneity of variance, then the correlation was tested by Pearson correlation analysis and the bcl-2/bax ratio was compared by independent-samples $t$ test. $P$ value<0.05 was considered statistically significant.

Results

HE Staining Results of Lacrimal Gland Tissue in the 2 Groups

Under a light microscope, it could be easily seen that the lacrimal glands in the control group were mainly composed of acini and ducts, the acini were mainly composed of serous gland cells and that the ductal epithelial cells were neatly arranged to form lumens of various sizes, with scattered lymphocytes and blood vessels. The basic lesions in the experimental group were the mixture of glandular epithelial cells, myoepithelial cells, and interstitium. Epithelial cells were arranged into glandular tubes, strips, and clumps. The size and shape of glandular ducts were different. The inner layer was cuboid or columnar cells, and the outer layer was spindle-shaped epithelial cells, which were distributed in a sheet-like or papillary nest-like way, and gradually transitioned into ovals or astrocytes. Mucinous components, hyaline-like tissues, cartilage-like tissues and scattered lymphocytes were visible in the interstitium. At the same time, it was found that the margins of the completely resected tumor were coated by the eosin-stained envelope with acellular structure, the thickness of the envelope was different, and there were signs of lymphocyte infiltration. Some glandular epithelial and myoepithelial cells grew outwards and invaded the outside of the capsule with clusters in a “sprouting” manner to differing degrees (Figure 1).

Results of Immunohistochemical Staining of Lacrimal Gland Tissue in the 2 Groups

Immunohistochemical staining results showed the positive expression products of bcl-2 and bax were brown-yellow particles, mainly located in the cytoplasm of glandular epithelial and myoepithelial cells in normal lacrimal and diseased tissues. The expression of bcl-2 in the experimental group was obviously higher than that of the control group, and the difference between them was statistically significant ($P<0.001$) (Figure 2). The expression of bax in the experimental group was compared with that of the control group, but the difference between them was not statistically significant ($P=0.25$) (Figure 3).
The bcl-2/bax ratio of the experimental group was significantly higher than the bcl-2/bax ratio of the control group, and the difference between them was statistically significant ($P=0.01$). We found no correlation between the expression of bcl-2 and bax ($r=0.06$, $P=0.77$). The detailed demographic and clinicopathological information of the two groups were listed in Table 1.

**Discussion**

Pleomorphic adenoma is a benign mixed tumor caused by abnormal proliferation of various tissue components such as glandular epithelial and myoepithelial cells, which mostly occurs in the parotid gland, submandibular gland, and lacrimal gland [14]. Because of the high biological behavior similarity to salivary gland polymorphic adenoma, LGPA is also classified as a borderline tumor [15]. Along with the continuous development in imaging and surgical technology, the rate of inadvertent biopsy of LGPA has dramatically reduced, and the early diagnosis and surgical resection have improved the prognosis of LGPA patients [7,16]. However, in a large longitudinal study, it was reported that the patients with complete follow-up had a 5-year recurrence rate of 3-32% and had the propensity to transform into carcinoma ex pleomorphic adenoma (ca-ex-PA) after recurrence. After malignant transformation occurs, the prognosis is poor, with a median survival of 3 years [2,9,17]. Therefore, the correlation between the biological behavior of LGPA and clinical outcome is an issue that deserves much attention.

It is generally believed that the pathological characteristics of tumors are closely related to the processes of tumor-initiation...
and progression and prognosis of disease [18]. Ahn et al performed a systematic observation and summarized the pathological features of biopsied lacrimal gland masses, providing a theoretical basis for identification, diagnosis, and management of lacrimal gland-occupying lesions [19]. Nevertheless, the biological behavior of LGPA has not been discussed on the basis of pathological analysis. In this study, we aimed to establish the relationship and found that the epithelial cells were arranged neatly and no pathological change was observed in the control group, while the tumor cellular density, morphology, and components of the stroma (such as mucinous components, hyaline-like tissues, and cartilage-like tissues) were significantly altered in the experimental group. The pathological findings mainly resulted from the complex mixtures of different cell types, which were in agreement with MRI findings reported in a previous study [7]. In addition, the envelopes of LGPA were very thin (only tens of micrometers thick or even less, under a light microscope), let alone on visual observation (naked eye), which illustrated that in the diagnosis and treatment process of these patients, the grossly invisible envelopes were extremely easy to break and make the tumor cells spread to the surrounding normal tissues even if performed gently. These findings add to a growing body of evidence suggesting the importance of keeping an intact envelope during the process of clinical diagnosis and surgery. So, from this point of view, biopsy should not be recommended for those patients who are highly suspected of LGPA, which may contribute to reducing the risk of tumor recurrence and metastasis. These findings are consistent with prior studies [16,17,20]. We also observed that some glandular epithelial and myoepithelial cells grew outwards and invaded the outside of the envelope with clusters in a “sprouting” manner of varying degrees, and the tumor cells were also visible outside of the envelope, although microscopic examination showed the envelope was complete.

Figure 2. The positive expression products of bcl-2 are brown-yellow particles, mainly located in the cytoplasm of glandular epithelial and myoepithelial cells, as indicated by the arrow. The expression of bcl-2 in the experimental group (A) was 14928.76 pixels and 3975.40 pixels in the control group (B) (immunohistochemical staining ×400).

Figure 3. The positive expression products of bax are brown-yellow particles, mainly located in the cytoplasm of glandular epithelial and myoepithelial cells, as indicated by the arrow. The expression of bax in the experimental group (A) was 8364.82 pixels and 5149.36 pixels in the control group (B) (immunohistochemical staining ×400).
Table 1. Characteristics of patients and summary of the experimental and control groups. P value refers to comparisons between 2 groups. OS means oculus sinister, and OD means oculus dexter.

| Parameter | Control group | Experimental group | Value |
|-----------|---------------|-------------------|-------|
| Age (years) | 46.25±25.59 | 50.37±8.73 | t=-0.44 p=0.67 |
| Sex | Male: Female | 4: 4 | 12: 7 | χ²=0.40 p=0.68 |
| Eye type | OS: OD | 5: 3 | 11: 8 | χ²=0.05 p=1.00 |
| Bcl-2 (pixels) | 4886.89±4405.22 | 16292.26±7442.26 | t=-4.02 p<0.001 |
| Bax (pixels) | 14645.90±20314.03 | 5512.73±5273.52 | t=1.25 p=0.25 |
| Bcl-2/bax | 0.71±0.62 | 9.39±13.01 | t=2.90 p=0.01 |

From a pathological perspective, this intriguing finding adds a new dimension to our understanding of LGPA and helps to explain why LGPA easily relapses after surgical resection and is prone to malignant transformation after recurrence.

In addition, we further investigated the exact pathogenesis of LGPA. Several reports have shown that the factors regulating cell survival, apoptosis, growth arrest, and cell turnover rates are important contributors to the development, progression, and regression of cell growth, and the dysregulation can result in loss of control over proliferation and lead to tumor development [13,21,22]. However, little attention has been paid to the impact of cell apoptosis and proliferation on LGPA. The present study started from the imbalance between apoptosis and proliferation to explore the pathogenesis of LGPA. Bcl-2 family proteins play a key role in regulating the balance, among them, bcl-2 is a representative anti-apoptotic factor, which can block the release of cytochrome C, maintain mitochondrial membrane integrity, and promote cell proliferation, while bax, a classic pro-apoptotic factor, can cause mitochondrial membrane damage and accelerate cell apoptosis [23,24]. Our results suggest that the expression level of bcl-2 and the bcl-2/bax ratio in the lacrimal gland tissues of patients with LGPA were significantly elevated, while no difference was disclosed in the expression of bax compared with normal lacrimal gland tissues. The trend of bcl-2 and bcl-2/bax ratio was consistent with that of salivary gland pleomorphic adenoma, so it was speculated that the occurrence of LGPA was also closely related to cell over-proliferation caused by imbalance between apoptosis and proliferation [25,26]. When the balance in cells was upset, apoptosis was inhibited and cell proliferation was stimulated, leading to a number of uncontrollable cellular changes that culminated in tumor formation. Surprisingly, this study also found that there was no correlation between bcl-2 and bax, which suggests that the disruption of the balance might be mainly due to the increased expression of bcl-2, and the change of bax was a non-essential condition. When the expression of bcl-2 was upregulated and the bcl-2/bax ratio increased, bcl-2 formed a heterodimer with bax that decreased the sensitivity of lacrimal gland histiocytes to apoptosis-stimulating factors, and mitochondrial permeability transition pore opening was prolonged or remained in an “off” state, which induced a decrease in mitochondrial membrane permeability. In this case, cytochrome C in the mitochondrial intermembrane space could not bind to apoptotic protease activating factor 1 in the cytoplasm to form the apoptosome. Therefore, the activation pathway of cysteinyl aspartate specific proteinase-3 (caspase-3) was blocked, cell apoptosis of lacrimal tissues was inhibited, and the over-proliferation of tumor cells was induced, which all contributed to LGPA tumorigenesis.

Unfortunately, the cause and mechanism of the overexpression of bcl-2 in the lacrimal gland tissues of patients with LGPA have not been clearly reported in the literature. Ashkenazi et al [27] found that the bcl-2 gene was located on chromosome 18q21 in diffuse large B cell lymphoma, multiple myeloma, and chronic lymphocytic leukemia, and participates in the process of chromosome translocation t (14; 18) (q32; q21), which leads to the overexpression of bcl-2 protein because the gene is located at the immunoglobulin heavy chain of chromosome 14q32. Further genomic chromosome detection and molecule studies in biology and other aspects are needed to confirm whether it occurs with the same chromosomal translocation in the development of LGPA to strike the balance.

Our study has certain limitations. First, due to the small size and non-homogeneity of the sample, there may be bias in observing the positive expression, so we need to expand the sample size in further research. Due to limitations arising from cost and availability, the investigations of related factors were conducted to utilize immunohistochemical methods rather than the more specific approaches such as in situ hybridization or polymerase chain reaction. This study still provides not only novel insights into the biological behavior of LGPA, but also a promising starting point for further investigations into another effective management methods other than surgical excision.

Conclusions

We observed that LGPA had a pathological change in the mixture of glandular epithelial cells, myoepithelial cells, and...
interstitial components, which might be related to the excessive proliferation of cells caused by the imbalance between apoptosis and proliferation. Further and more detailed clinical and experimental studies in a wider and deeper range are needed to verify our results.

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of Interest

None.