Assessment of p16 expression and HPV infection in adenoid cystic carcinoma of the lacrimal gland

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Purpose: Adenoid cystic carcinoma (ACC) in the lacrimal gland is a rare malignancy. P16 is encoded by the CDKN2A gene, which is recognized as a tumor suppressor due to its inactivation in many types of tumors. However, p16 overexpression is also linked to adverse tumor parameters. These contradictory observations have also been confirmed in ACCs in the salivary glands. Furthermore, evidence of human papilloma virus (HPV) infection is found in a proportion of ACCs in the salivary glands. P16 is often overexpressed in HPV-related squamous cell carcinoma in parallel. To our knowledge, the role of p16 and HPV in ACCs in the lacrimal gland is still unknown.

Methods: Twenty-one ACCs in the lacrimal gland and ten matched healthy lacrimal glands were studied. P16 was detected with immunohistochemistry (IHC), and HPV was detected with in situ hybridization (ISH) and PCR in all cases. Other cell cycle proteins were also detected with IHC, including cyclin D1 and Ki67. The methylation status of the p16 promoter was detected with methylation-specific PCR (MSP) to further investigate the regulation of p16 expression.

Results: The expression rates of p16 (47.6%, 10/21), cyclin D1 (100%, 21/21), and Ki67 (52.4%, 11/21) were increased in ACCs compared to healthy lacrimal glands (negative). The results showed p16 expression was limited to the inner ductal epithelial cells in the majority of the tubular and cribriform patterns. In solid ACCs, p16 was uniformly positive. HPV was negative in all 21 cases with ISH and PCR. P16 overexpression was associated with cyclin D1 overexpression (p=0.013). Only 13 cases were tested successfully with MSP. The expression rate of p16 methylation was 23.1% (3/13) of the ACCs. Compared with primary ACCs, recurrent ACCs showed higher p16, cyclin D1, and Ki67 expression (p=0.011, p=0.026, p=0.049, respectively).

Conclusions: In summary, p16 overexpression was cell-type dependent in ACCs in the lacrimal gland, while HPV infection was negative. P16 overexpression was unrelated to HPV infection. The mechanism of p16 overexpression needs to be further investigated in ACCs in the lacrimal gland.

Adenoid cystic carcinoma (ACC) is an uncommon malignancy that arises mostly in the salivary glands, which also occurs in other exocrine glands, including lacrimal glands. ACCs in the lacrimal gland account for 1.6% of all orbital tumors [1,2]. ACC is classified into three histologic subtypes (tubular, cribriform, and solid), and ACCs can have more than one subtype, which makes grading difficult. Low- to intermediate-grade ACCs have predominantly tubular and cribriform patterns, with indolent behavior; whereas high-grade ACCs have a predominantly solid pattern, with more aggressive behavior [3]. Standard treatment for ACCs is surgery, followed by radiotherapy. Due to the complex orbital anatomy, and infiltration to the perineural and periosteum, complete surgical resection often is impossible, which results in frequent local recurrence and a poor outcome. Given the rarity of ACCs in the lacrimal gland, very little has been published on this disease in terms of molecular biology.

P16 is encoded by the CDKN2A gene (Gene ID: 1029, OMIM: 600160), which is recognized as a tumor suppressor due to its inactivation in many types of tumors [4,5]. Previous studies have demonstrated that mutation in, deletion in, and methylation of CDKN2A play roles in a proportion of ACCs in the salivary glands [6-11]. However, p16 overexpression is also linked to ACCs in the salivary glands [11]. Inactivation of CDKN2A and p16 overexpression are linked to ACCs in the salivary glands. A similar contradictory observation has also been found in breast cancer [12]. However, there are no data about CDKN2A inactivation or p16 expression in ACCs in the lacrimal gland.

It is well-known that p16 is upregulated in human papilloma virus (HPV)-associated carcinomas, including those of some parts of the head and neck such that p16 is often used as a surrogate marker for the presence of HPV. Previous
studies have addressed the presence of HPV DNA and p16 overexpression in squamous cell carcinomas of the lacrimal sac [13-15]. Evidence of HPV infection has also been found in a proportion of ACCs in the salivary glands [16], but the opposite result was found in another study [17].

Up to now, the role of p16 and HPV infection is still unknown in ACCs in the lacrimal gland. The goal of this study was to test this hypothesis. We also detected cyclin D1 expression and the methylation status of the p16 promoter to investigate the regulation of p16 expression. Finally, we investigated the association of these molecular markers with the recurrence of ACCs in the lacrimal gland.

METHODS

Patient samples: A series of 20 formalin-fixed paraffin-embedded (FFPE) ACCs in the lacrimal gland and one accessory lacrimal gland were retrieved from the Department of Pathology, Tianjin Eye Hospital, Tianjin, China (2003 to 2015). In ten cases, matched healthy lacrimal glands were obtained as the control. Our study was conducted in accordance with the Helsinki Declaration and the ARVO statement. Written informed consent was obtained. The cases were reviewed by two pathologists. Approval for the study was obtained from the Tianjin Eye Hospital ethics committee. Written informed consent was obtained.

In situ hybridization: In situ hybridization (ISH) was performed using biotinylated Pan Human Papillomavirus probes (Thermo Fisher Scientific, Amsterdam, The Netherlands) against the conserved HPV region, following the manufacturer’s instructions. These probes are able to detect HPV types 6, 11, 16, 18, 31, and 33. Positive control tissue from a condyloma acuminatum case previously showed to be positive with ISH was run in parallel, while the surrounding healthy squamous epithelium served as an internal negative control. The in situ hybridization studies were scored as positive or negative.

DNA extraction and HPV detection: Briefly, paraffin sections were deparaffinized with Xylene, followed by Proteinase K (TaKaRa, Dalian, China) digestion. Genomic DNA was isolated with TaKaRa MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa, Dalian, China). Genomic DNA was isolated from FFPE tissues with standard methods, and the histological examination before DNA extraction estimated the tumor cell content of the tumor tissue samples to be 75–90%.

As a positive control for the quality of the template DNA, the housekeeping gene TBXAS1 (Gene ID: 6916, OMIM: 274180) was amplified. HPV DNA was assessed in all samples using a modified general primer HPV PCR system (MGP) [18]. The five forward and five reverse MGP primers amplify 158–168 nt of the human papillomavirus L1 gene (Gene ID: 25479185) depending on the HPV type (Table 1). This methodology is able to detect HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. PCRs were performed with 1 μl template DNA in 25 μl final reaction volume. HPV multiplex PCR was performed with the TaKaRa Ex Taq PCR Kit (TaKaRa, Dalian, China), according to the manufacturer’s instructions. PCR amplification was performed with denaturation at 95 °C for 10 min followed by 5 cycles of 95 °C for 30 s, 42 °C for 30 s, and 72 °C for 45 s and then 45 cycles of 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 45 s, with a final step at 72 °C for 10 min. The PCR products underwent electrophoresis on 2% agarose gels and were visualized under ultraviolet (UV) illumination after ethidium bromide staining. The primers for MGP and TBXAS1 are shown in Table 2.

Immunohistochemistry: All tumor samples were fixed in 4% buffered formalin, processed, and embedded in paraffin according to routine procedures. P16 (Abcam, Cambridge, MA), cyclin D1 (Abcam), and Ki67 (Abcam) were detected in all cases on serial sections (4 µm) with immunohistochemical staining. The Envision horseradish peroxidase system was performed following the protocol supplied by the manufacturer, and DAB was used as the substrate. Antibodies, the Envision system, and DAB were produced by Gene Tech (Shanghai, China). Two sections and ten high-power fields (400X) of view per section were scored for the statistical analyses. Sections were counterstained with hematoxylin. Interpretation of the staining was done with semiquantitative scoring by at least two investigators as follows: 0: <1% of cells positive; 1+: 1–25% of cells positive; 2+: 26–50% of cells positive; 3+: 51–75% of cells positive; and 4+: 76–100% of cells positive.

Methylation-specific PCR: The bisulfite-treated DNA was amplified with PCR using the following conditions: denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30s, annealing temperature 60 °C for 30s, 72 °C for 30s, and a final elongation at 72 °C for 10 min. The p16 primers were chosen from the literature [19] (Table 1).

Statistical analysis: Data were analyzed with the SPSS statistical package. Comparison between variables was performed using the Mann–Whitney U rank test. Correlation between variables was performed using the Spearman rank correlation. All statistical tests were two-sided, and a p value of 0.05 or less was considered statistically significant.

RESULTS

Clinical data of the patients are summarized in Table 2. Among the 21 patients, nine cases were recurrent ACCs, and 12 cases were primary cases. The male to female ratio
Table 1. Primer sequences for HPV-PCR and p16-MSP.

| Primer   | Direction | Sequence, 5′-3′                          |
|----------|-----------|----------------------------------------|
| MGPA     | Forward   | ACGTTGGATGTTTGTACTGTTGGGATACTAC        |
| MGPB     | Forward   | ACGTTGGATGTTTGTACTGTTGGGATACTAC        |
| MGPC     | Forward   | ACGTTGGATGTTTGTACTGTTGGGATACTAC        |
| MGPD     | Forward   | ACGTTGGATGTTTGTACTGTTGGGATACTAC        |
| MGPI     | Forward   | ACGTTGGATGTTTGTACTGTTGGGATACTAC        |
| MGPG     | Reverse   | ACGTTGGATGTTTGTACTGTTGGGATACTAC        |
| MGPH     | Reverse   | ACGTTGGATGTTTGTACTGTTGGGATACTAC        |
| MGPI1    | Reverse   | ACGTTGGATGTTTGTACTGTTGGGATACTAC        |
| MGPJ     | Reverse   | ACGTTGGATGTTTGTACTGTTGGGATACTAC        |
| MGP18    | Reverse   | ACGTTGGATGTTTGTACTGTTGGGATACTAC        |
| TBXASI   | Forward   | GCCCGACATTGTGCAAGTCC                    |
| TBXASI   | Reverse   | GCGGGGTCGCGGAGGTTGTC                  |
| p16-M    | Forward   | TTATTAGAGGTTGGGCGGATCGC               |
| p16-M    | Reverse   | GACCCCCCAACCGCAAGTCC                  |
| p16-U    | Forward   | TTATTAGAGGTTGGGCGGATCGC               |
| p16-U    | Reverse   | CAACCCCCAACCACCAACCATAA               |

Table 2. Clinical characteristics of patients and outcomes.

| Case | Gender | Age | Outcome | Follow-up(months) |
|------|--------|-----|---------|-------------------|
| 1    | F      | 16  | -       | -                 |
| 2    | M      | 15  | -       | -                 |
| 3    | F      | 35  | -       | -                 |
| 4    | M      | 42  | AWD     | 97                |
| 5    | F      | 38  | DOD     | 30                |
| 6    | M      | 38  | DOD     | 57                |
| 7    | M      | 42  | DOD     | 61                |
| 8    | F      | 53  | DOD     | 74                |
| 9    | F      | 29  | AWD     | 41                |
| 10   | M      | 45  | AWD     | 66                |
| 11   | F      | 36  | AWD     | 135               |
| 12   | F      | 47  | AWD     | 77                |
| 13   | M      | 32  | -       | -                 |
| 14   | M      | 44  | AWD     | 70                |
| 15   | F      | 32  | -       | -                 |
| 16   | F      | 69  | AWD     | 60                |
| 17   | F      | 48  | AWOD    | 61                |
| 18   | F      | 47  | AWD     | 25                |
| 19   | M      | 43  | AWOD    | 77                |
| 20   | F      | 66  | AWOD    | 11                |
| 21   | M      | 26  | -       | -                 |

M male, F female, - not available, AWD alive with disease, AWOD alive without disease, DOD dead of disease

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was 1:1.3. The mean age at diagnosis was 40.1 years (range, 15–69 years). Follow-up data for 15 patients were available. The median follow-up time was 62.8 months (range, 11–135 months). The 5-year survival rate was 66.7% (8/12).

The distribution of the tumor histologic subtypes was as follows (Table 3): predominantly tubular, seven patients; cribriform, seven patients; and solid, seven patients. Among the 21 patients, 14 cases were low- to intermediate-grade ACCs, and seven cases were high-grade ACCs.

HPV-ISH was performed in 21 ACCs and one condyloma acuminatum. Strong nuclear staining for HPV was evident in the HPV-infected condyloma acuminatum used as positive control. In contrast, none of the ACCs showed HPV staining (Figure 1, Table 3).

As an independent test for HPV in ACCs, PCR was performed for HPV DNA using an MGP general primer HPV PCR system. The HPV PCR product was evident in the condyloma acuminatum used as positive control. However, HPV DNA was negative in 21 tested cases with PCR (Figure 2, Table 3).

P16, cyclin D1, and Ki67 were not expressed in the healthy lacrimal glands. The expression rates for p16 (47.6%, 10/21), cyclin D1 (100%, 21/21), and Ki67 (52.4%, 11/21) were increased in the ACCs compared to the healthy lacrimal glands (Table 3).

P16 expression was limited to the inner ductal cells and was negative in the myoepithelial cells in the tubular and cribriform patterns of the ACCs. Diffuse expression of p16 was found in solid patterns (Figure 3, Table 3). However, the p16 staining index in most cribriform patterns was less than 1%, scored as “0” with semiquantitative scoring.

Cyclin D1 was overexpressed in the ACCs (Figure 4, Table 3). Furthermore, p16 overexpression was associated with cyclin D1 overexpression (p=0.013). All cases had a low Ki67 staining index of less than 25%, scored as “+” or “0” (Figure 5, Table 3), which indicated the ACCs had low proliferation activity.

Compared with primary ACCs, recurrent ACCs showed higher p16, cyclin D1 and Ki67 expression (p=0.011, p=0.026, p=0.049, respectively). Only 13 cases were tested successfully.

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**Table 3. Immunophenotype, p16 Methylation Status, ISH and PCR Results.**

| Case | Predominant histologic subtype | Primary/recurrent | p16 | cyclin D1 | Ki67 | p16 Methylation status | HPV ISH | HPV PCR |
|------|--------------------------------|------------------|-----|-----------|------|------------------------|--------|---------|
| 1    | cribriform                     | P                | 0   | 1+        | 0    | -                      | Neg    | Neg     |
| 2    | cribriform                     | P                | 0   | 1+        | 0    | -                      | Neg    | Neg     |
| 3    | tubular                        | P                | 0   | 1+        | 0    | M                      | Neg    | Neg     |
| 4    | cribriform                     | P                | 0   | 1+        | 0    | U                      | Neg    | Neg     |
| 5    | tubular                        | P                | 0   | 1+        | 0    | M                      | Neg    | Neg     |
| 6    | solid                          | P                | 1+  | 3+        | 1+   | -                      | Neg    | Neg     |
| 7    | tubular                        | P                | 2+  | 2+        | 1+   | U                      | Neg    | Neg     |
| 8    | cribriform                     | P                | 1+  | 0         | 0    | -                      | Neg    | Neg     |
| 9    | solid                          | R                | 2+  | 3+        | 1+   | U                      | Neg    | Neg     |
| 10   | solid                          | R                | 2+  | 3+        | 1+   | U                      | Neg    | Neg     |
| 11   | solid                          | R                | 1+  | 2+        | 1+   | U                      | Neg    | Neg     |
| 12   | tubular                        | R                | 2+  | 2+        | 0    | U                      | Neg    | Neg     |
| 13   | tubular                        | R                | 2+  | 2+        | 1+   | -                      | Neg    | Neg     |
| 14   | tubular                        | R                | 2+  | 2+        | 0    | -                      | Neg    | Neg     |
| 15   | solid                          | R                | 1+  | 3+        | 1+   | U                      | Neg    | Neg     |
| 16   | solid                          | R                | 0   | 1+        | 1+   | -                      | Neg    | Neg     |
| 17   | cribriform                     | P                | 0   | 2+        | 0    | U                      | Neg    | Neg     |
| 18   | tubular                        | P                | 0   | 2+        | 1+   | M                      | Neg    | Neg     |
| 19   | cribriform                     | P                | 0   | 1+        | 0    | U                      | Neg    | Neg     |
| 20   | solid                          | R                | 0   | 1+        | 1+   | -                      | Neg    | Neg     |
| 21   | cribriform                     | P                | 1+  | 2+        | 1+   | U                      | Neg    | Neg     |

P, primary; R, recurrent; -, not available; M, methylated; U, unmethylated; Neg, negative.
by MSP. The expression rate of p16 methylation was detected in 23.1% (3/13) ACCs, and all cases with p16 methylation showed negative expression of the p16 (Figure 6, Table 3).

**DISCUSSION**

The demographics and survival of patients with ACCs differ by primary tumor site. The mean age at diagnosis in this study was 40.1 years, which was similar to that in other studies [20,21]. However, ACCs in other sites occurred predominantly among individuals aged >55 years [21]. ACCs in the lacrimal gland were associated with younger age at presentation. In this study, ACCs in the lacrimal gland were more common in women than in men, which is similar to most studies [21]. Because some patient follow-up data were unavailable, the 5-year survival rate (66.7%, 8/12) is less reliable in the present study. In other studies, the 5-year survival rate is 55.6% to 88.7% [21,22].

P16 is recognized as tumor suppressor due to its inactivation in many types of tumors. However, p16 overexpression is also present in several tumors [5], including the ACCs in the lacrimal gland in this study. The regulation of p16 expression is complex. Genetic or epigenetic inactivation of the CDKN2A gene by homozygous deletion and methylation decrease p16 expression, while oncogenes and DNA damage response can induce p16 overexpression [4].

The reason for p16 overexpression in HPV-related tumors is well elucidated [5]. HPV infection may affect the p16/cyclin D1/retinoblastoma protein (Rb) pathway and induce cellular replication. Cyclin D1 is a regulatory component of the cyclin D1/CDK4 complex that phosphorylates Rb. Phosphorylation of Rb allows dissociation of the transcription factor E2F from the Rb/E2F complex and the subsequent transcription of E2F target genes that are responsible for the progression through the G1 phase. P16 can interact with CDK4 and inhibit its ability to interact with cyclin D1 [13]. The HPV E7 protein induces the disassembly of the RB/E2F complex thus disrupting Rb activity, which releases p16 from its negative feedback control, causing p16 overexpression to inhibit uncontrolled cellular replication [14]. In summary, p16 is overexpressed in HPV-related tumors in an unsuccessful attempt to stop cell proliferation.

In the present study, HPV infection was not detected in 21 cases of ACCs in the lacrimal gland with ISH and PCR. The results indicated that ACCs in the lacrimal gland are not associated with HPV infection, and HPV infection was not the reason for p16 overexpression. HPV is positive in ACCs in the uterine cervix and a subset of head and neck neoplasms, such

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**Figure 1.** ISH assay for HPV in ACCs and the positive control. A: Human papilloma virus (HPV) was positive in the condyloma acuminatum (positive control, black arrow). B: Tubular pattern (red curve: inner ductal epithelial cells). C: Cribriform pattern. D: Solid pattern: HPV was negative in three histologic subtypes of adenoid cystic carcinomas (ACCs). Scale bars = 50 μm.

**Figure 2.** Detection of HPV PCR products with agarose gel electrophoresis in representative examples. The housekeeping gene was positive in all samples; human papilloma virus (HPV) was positive in the positive control but negative in the adenoid cystic carcinomas (ACCs). TBXAS1: Housekeeping gene PCR product, 100 bp; MGP: Modified general primer HPV PCR product, 158–168 bp; M: Molecular weight ladder; 1: Positive control, condyloma acuminatum; 2–9: Representative examples of ACCs.
as squamous cell carcinomas of the lacrimal sac and HPV-related carcinoma with adenoid cystic-like features in the sinonasal tract [13-15, 23-25]. However, these neoplasms are different with ACCs in the lacrimal gland. The uterine cervix, lacrimal sac, and sinonasal tract have an open environment, which is vulnerable to HPV infection. However, the lacrimal gland has a relatively closed environment, and thus, reversed infection of HPV is not easy. The relatively closed anatomy of the lacrimal gland may be the reason for negative HPV in ACCs in the lacrimal gland.

Figure 3. Expression of p16 in ACCs in the lacrimal gland. A: Healthy lacrimal glands. B: Tubular pattern. C: Cribriform pattern. D: Solid pattern. P16 expression was positive in the inner ductal epithelial cells and solid cell nests. Red curves: inner ductal epithelial cells; red arrows: myoepithelial cell; black arrows: positive staining of p16. Scale bars = 50 μm.

Figure 4. Expression of cyclin D1 in ACCs in the lacrimal gland. A: Healthy lacrimal glands. B: Tubular pattern. C: Cribriform pattern. D: Solid pattern. Cyclin D1 expression was positive in all cases. Red curves: inner ductal epithelial cells; black arrows: positive staining of cyclin D1. Scale bars = 50 μm.
Promoter methylation is a common mechanism of inactivation of tumor suppressor genes in many tumors. However, little is known about its role in the development of ACCs in the lacrimal gland. In this study, p16 methylation was found in three of 13 ACCs in the lacrimal gland (23.1%), all of which showed negative expression of the p16 protein. A similar result was also found in ACCs in the head and neck (not including the lacrimal gland) [10,11]. However, not all the unmethylated samples showed p16 expression in the present study and others [4]. Except DNA methylation, in other molecular events, such as mutation, homozygous deletion may contribute to downregulation of p16. Studies have showed mutations in p16 are infrequent in ACCs in the salivary glands [26,27]. However, there are no data about mutations or deletions in p16 in lacrimal gland carcinomas, including ACCs in the lacrimal gland. Further studies are needed to detect a mutation in p16 in ACCs in the lacrimal gland.

It has been shown that loss of Rb generates oncogenic stress that could lead to the activation of p16 expression. Cyclin D1 binds to CDK4, and consequently, phosphorylation and inactivation of Rb occur [13]. In the present study, cyclin D1 was overexpressed in ACCs in the lacrimal gland, which is similar to other tumors [28-31]. Furthermore, ACCs in the lacrimal gland with p16 positive showed stronger staining of cyclin D1, which indicated p16 overexpression might be induced by the activation of cyclin D1. A mutation in Rb or LOH can also inactivate Rb function. A previous study showed loss of heterozygosity and microsatellite alterations in the Rb (Gene ID: 5925, OMIM: 614041) gene were found in 44% of ACCs in the salivary glands [32]. More studies about inactivation of the p16/cyclin D1/RB pathway in ACCs in the lacrimal gland are needed to support this hypothesis, such as...
overexpression of cyclin D1 in the ACC cell line and tests for mutations in Rb or p53.

This study and previous studies all showed most ACCs have a low Ki67 staining index of less than 25%, which indicates ACCs have low proliferation activity [33-35]. ACCs have an indolent course, and 75% of patients are still alive after 5 years. The low Ki67 staining index is consistent with the indolent course. This study showed cyclin D1 upregulation was not correlated with the Ki67 staining index. Several previous studies showed cyclin D1 was not correlated to patients’ clinical outcome or cell proliferation in ACCs in the salivary glands, breast cancers, and lung cancers [28-31]. These results suggest that cyclin D1 overexpression alone does not induce a marked increase in the proliferation activity of ACC cells.

Cell-type-dependent expression of biomarkers (such as c-kit and EGFR) has been found in ACCs in the salivary glands, which might be important to the clinical and therapeutic stratification of ACCs [36]. ACCs are uniquely formed of dual epithelial and myoepithelial cells in the conventional tubular and cribriform patterns. The cribriform pattern shows nests of cells with microcystic spaces, and the tubular pattern consists of tubules with central luminal lined by inner luminal cells and outer myoepithelial cells. The myoepithelial cell is lost in the solid form transformation. The histogenetic differences between myoepithelial and epithelial cells may influence the location of biomarkers. This study also found p16 was limited to the inner ductal cells and was negative in the myoepithelial cells in the tubular and cribriform patterns of the ACCs. Diffuse expression of p16 was found in solid patterns. However, the p16 staining index in the cribriform patterns was less than 1%, scored as “0” with semiquantitative scoring, which might be due to the fewer ductal cells in cribriform patterns.

Recurrence is common in ACCs in the lacrimal gland. However, little is known about biomarker changes during the recurrence of ACCs. We found recurrent ACCs have higher expression of p16, cyclin D1, and Ki67. Dysregulation of the p16/cyclin D1/Rb pathway has been found in the recurrence of various human tumors [37,38], including ACCs in the lacrimal gland in this study. De Souza [39] found strong staining for p16 and cyclin D1 in recurrent salivary pleomorphic adenoma (PA), while weak or negative in PA, which suggested that these proteins might be involved in the recurrence of PA. As mentioned above, the elevated expression of cyclin D1 and p16 was associated with loss of Rb function, which can alter the invasive properties of the tumor [40]. We speculated that dysregulation of the Rb cell cycle control pathway might contribute to the recurrence of ACCs.

In conclusion, p16 was overexpressed in a proportion of ACCs in the lacrimal gland, while HPV infection was not found with ISH and PCR. P16 overexpression is unrelated to HPV infection. The mechanism of p16 overexpression needs to be further investigated in ACCs in the lacrimal gland.

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