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ORIGINAL ARTICLE

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Epstein-Barr virus may contribute to the pathogenesis of adult-onset recurrent respiratory papillomatosis: A preliminary study

Martin Formánek[1] | Debora Formánková[1] | Pavel Hurník[2] | Adéla Vrtková[3,4] | Pavel Komínek[1,5]

[1]Department of Otorhinolaryngology and Head and Neck Surgery, University Hospital Ostrava, Ostrava, Czech Republic

[2]Department of Pathology, University Hospital Ostrava, Ostrava, Czech Republic

[3]Department of Applied Mathematics, Faculty of Electrical Engineering and Computer Science, VSB-Technical University of Ostrava, Ostrava, Czech Republic

[4]Department of Deputy Director of Science and Research, University Hospital Ostrava, Ostrava, Czech Republic

[5]Department of Craniofacial Surgery, Faculty of Medicine, University of Ostrava, Ostrava, Czech Republic

Correspondence
Martin Formánek, Department of Otorhinolaryngology and Head and Neck Surgery, University Hospital Ostrava, 17 listopadu 1790, 708 52 Ostrava, Czech Republic
Email: martin.formanek@fno.cz

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Abstract

Objective: Human papillomavirus (HPV) causes adult-onset recurrent respiratory papillomatosis (AORRP), but AORPP prevalence is much lower than HPV prevalence. Thus, HPV infection is necessary, but not sufficient, to cause AORRP and other factors likely contribute to its pathogenesis. The present study aimed to investigate whether co-infection with herpetic viruses may contribute to the pathogenesis of AORRP.

Design: Prospective case-control study conducted from January 2018 to November 2019.

Settings: Tertiary referral centre.

Participants: Eighteen consecutive patients with AORRP and 18 adults with healthy laryngeal mucosa (control group) undergoing surgery.

Main outcome measures: Cytomegalovirus, Epstein-Barr virus (EBV), herpes simplex viruses 1 and 2, human herpesvirus 6, varicella zoster virus and HPV (including genotyping) were detected in biopsies of papilloma or healthy mucosa using real-time polymerase chain reaction and reverse line blot. Dysplasia and Ki67 levels were determined in papilloma specimens.

Results: EBV was present in 6 (33.3%) AORRP patients and no control patients ($P = .019$). Presence was not dependent on tobacco exposure ($P = .413$) or HPV genotype or concentration ($P > .999$). EBV presence was strongly related to increased cell proliferation ($P = .005$) and number of previous surgeries ($P = .039$), but not dysplasia ($P > .999$). Human herpesvirus 6 was found in 3 (16.7%) AORRP biopsies, with one false positive. No other herpetic virus was found.

Conclusions: Unlike other herpetic viruses, EBV seems to interact with HPV, enhancing cell proliferation and contributing to the pathogenesis and progression of AORRP. Further research is required to elucidate specific interactions and their role in the pathogenesis of AORRP.

1 | INTRODUCTION

Recurrent respiratory papillomatosis (RRP) is a chronic viral disease characterised by the exophytic growth of squamous cell tumours on the mucosa of the aerodigestive tract. The disease is categorised as juvenile or adult RRP according to the age of the patient at disease onset. Although it is a benign disease, RRP has an unpredictable clinical course; it tends to recur and spread, and it can undergo

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malignant conversion.[1,2] RRP is caused by human papillomavirus (HPV) types 6 and 11, which infect cells within the basal layer of the mucosa.[3,4] HPV 11 positivity is associated with worse disease course and prognosis.[5]

In contrast to the relatively low incidence of RRP, asymptomatic HPV infection is common.[6-10] The mechanism underlying the progression from HPV infection to RRP is unclear, but it seems likely that other factors contribute to pathogenesis in terms of activating or reactivating HPV. The roles of tobacco exposure and immunodeficiency have been considered, but these risk factors are present in a very small group of patients with RRP.[8,11,12] In a recent study, herpes simplex virus (HSV) type 2 (HSV-2) co-infection was significantly more frequent in biopsies from patients with adult-onset recurrent respiratory papillomatosis (AORRP) than patients with healthy laryngeal mucosa.[8] This suggests that HSV-2, and perhaps other herpetic viruses, may be one of the possible risk factors for AORRP contributing to its development in terms of the activation of latent HPV infection. However, less suitable, non-quantitative immunohistochemical detection was used and other herpetic viruses were not detected. The present study aimed to investigate the risk potential of all of the main human herpetic viruses. Cytomegalovirus (CMV), Epstein-Barr virus (EBV), HSV-1, HSV-2, human herpesvirus 6 (HHV-6) and varicella zoster virus (VZV) were quantitatively detected in biopsies from patients with AORRP and compared to patients with healthy respiratory tract mucosa. To the best of our knowledge, this is the first study to investigate these factors as risk co-factors for AORRP.

2 | METHODS

2.1 | Ethical considerations

This case-control study was approved by the Ethics Committee of the University Hospital and was performed in accordance with the Declaration of Helsinki using good clinical practice and following the applicable regulatory requirements. The study was registered at ClincialTrials.gov under the identifier NCT02592902. Written informed consent was obtained from each patient before initiating any procedure. The study was conducted from January 2018 to November 2019 at a tertiary referral hospital.

2.2 | Participants

Adult patients who had been diagnosed with RRP of the larynx were included in the study. Only patients with recurrent (at least one recurrence during the previous 2 years) and histologically confirmed squamous cell papillomas on the laryngeal mucosa were included in the study. The control group consisted of patients with a vocal cord cyst who underwent microlaryngoscopy with cyst removal and had no other pathology of the laryngeal mucosa. Healthy mucosa was obtained from the abundant part of the mucosal microflap after cyst removal. Patients with contraindications for general anaesthesia, who had laryngeal papillomas undergoing microlaryngoscopy for the first time or who did not consent to be included in the study were excluded.

2.3 | Endoscopy

The extent of the disease was evaluated perioperatively using a 4-mm rigid HD endoscope (Olympus Europa SE & Co., Hamburg, Germany) and 6 months after the surgery at the outpatient clinic using a 2.7-mm flexible HD endoscope (Olympus). The Derkay/Coltera score was used for scoring.[13]

2.4 | Virus detection

Biopsy specimens of the laryngeal papillomas and of healthy laryngeal mucosa (control group) were obtained during microlaryngoscopy. The presence of CMV, EBV, HSV-1, HSV-2, HHV-6 and VZV in the biopsies was detected by real-time polymerase chain reaction (PCR). HPV testing and genotyping was performed using single-step PCR and reverse line blot.

QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) was used for DNA isolation. The manufacturer's recommended protocol was followed. The GeneProof® CMV PCR Kit (GeneProof a.s., Brno, Czech Republic) was used for CMV detection. This kit has a specificity of 100% and sensitivity (limit of detection) of 122.594 IU/ml with 95% probability (target sequence: specific conservative DNA sequence of a single-copy gene encoding the 4 IE antigen). The GeneProof® EBV PCR Kit was used for EBV detection; it has a specificity of 100% and sensitivity (limit of detection) of 196.088 IU/ml with 95% probability (target sequence: DNA sequence of the single-copy gene encoding the nuclear antigen 1). The GeneProof® HSV-1 PCR Kit was used for HSV-1 detection; it has a specificity of 100% and sensitivity (limit of detection) of 122.124 cp/ml with 95% probability (target sequence: specific conservative DNA sequence of a single-copy gene encoding the glycoprotein B). The GeneProof® HSV-2 PCR Kit was used for HSV-2 detection; it has a specificity of 100% and sensitivity (limit of detection) of 197.215 cp/ml with 95% probability (target sequence: specific conservative DNA sequence of a single-copy gene encoding the glycoprotein B). The AmpliSens® HHV6-screen-titre-FRT PCR kit (InterLabService Ltd., Moscow, Russian Federation) was used for HHV-6 detection; it has a specificity of 100%, sensitivity 400 cp/mlv. The GeneProof® VZV PCR Kit was used for VZV detection; it has a specificity of 100% and sensitivity (limit of detection) of 113.05 cp/ml with 95% probability (target sequence: specific conservative DNA sequence of the single-copy gene ORF62). Ampliquality HPV-Type Express v.3.0 (AB ANALITICA srl., Padova, Italy) was used for HPV testing and genotyping with a diagnostic sensitivity of 99% and specificity of 98.6%. The test allows identification of 40 HPV

TABLE 1 Characteristics of the study participants

	RRP group			Control group			*P* value
Age, years	46.6 (13.5)			52.4 (12.5)			0.185*
Male	11			6			0.182**
Female	7			12			0.182**
Immunodeficiency	0			1			>0.999***
Diabetes mellitus	0			1			>0.999***
Voice professional	6			5			>0.999***
Allergy	2			1			>0.999***
Tobacco exposure	Yes	Stop	No	Yes	Stop	No	0.741**
	6	3	9	4	3	11	

Note: Data are presented as arithmetic mean (standard deviation [SD]) or absolute frequency.
RRP = recurrent respiratory papillomatosis.
*Two-sample *t* test;
**Pearson's chi-squared test
***Fisher's exact test.

genotypes: HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, 87, 89, and 90. There is potential cross-reactivity of HPV 18 with HPV 45, HPV 26 with HPV 44, HPV 51 with HPV 82 and HPV 62 with HPV 81. HPV positivity is evaluated as minor, moderate or strong.

2.5 | Histopathology

An additional biopsy specimen of each laryngeal papilloma was sent to the Department of Pathology, where histopathological examination was performed. During examination, the diagnosis of squamous cell papilloma was confirmed and the level of dysplasia and Ki67 determined. Dysplasia was evaluated as none (0), mild (1), moderate (2) or severe (3).

2.6 | Statistical analysis

The primary analysis was performed using standard tools of exploratory data analysis. For the description of numerical variables, the mean and standard deviation (SD) or the median and interquartile range (IQR) are used. Categorical variables are presented with absolute and/or relative frequencies. Observed groups were compared in terms of demographics (eg age, sex), medical history (eg history of immunodeficiency, presence of diabetes mellitus) and the presence of risk factors (eg tobacco exposure, presence of herpetic viruses and HPV). A patient was labelled a stop-smoker if he or she quit smoking at least 2 years ago. The statistical analysis was performed using R software (version 3.6.0). The following statistical tests were used: two-sample *t* test, Mann-Whitney *U* test, Pearson's chi-squared test and Fisher's exact test with a 5% significance level.

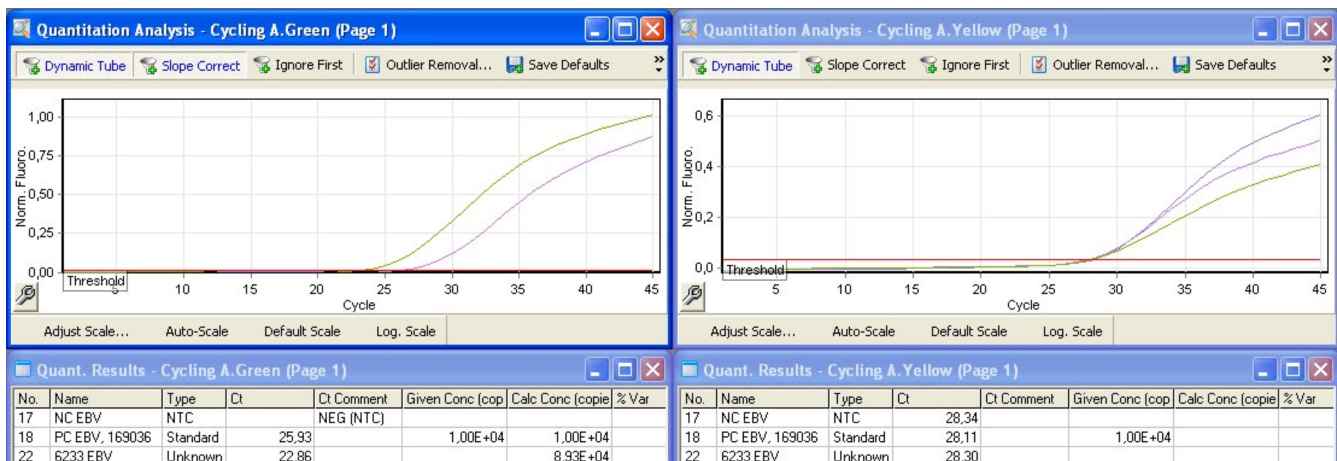

FIGURE 1 EBV positivity (light purple curve in the left graph) of viral load 89,300 copies/ml in quantitative real-time PCR. Yellow curve in the left graph represents positive control and curves in the right graph represent internal controls for test validity

TABLE 2 Number of herpetic viruses present in biopsies

	RRP group	Control group
CMV	0	0
EBV	6	0
HSV-1	0	0
HSV-2	0	0
HHV-6	3	0
VZV	0	0

Abbreviations: CMV = cytomegalovirus; EBV = Epstein-Barr virus; HSV = herpes simplex virus HHV = human herpesvirus; RRP = recurrent respiratory papillomatosis; VZV = varicella zoster virus.

TABLE 3 Human papilloma virus (HPV) and Epstein-Barr virus (EBV) presence in biopsies

HPV 6/11 positivity	EBV presence, n		EBV concentration range for EBV + patients, IU/ml
	EBV+	EBV-	
Minor	2	2	1500-11000
Moderate	1	4	350
Strong	3	6	11000-1.8 x 10^5

3 | RESULTS

A total of 36 patients were included in the study: 18 patients with laryngeal RRP and 18 patients with healthy laryngeal mucosa. We found no significant differences between the groups (Table 1). No side effect from biopsy was noted in either group. Conversely, all patients reported improvement of their voice after surgery because healthy mucosa was obtained from the abundant part of the mucosal microflap after cyst removal.

3.1 | Virus presence

HPV was found in all of the biopsies from patients with RRP. Thirteen patients were positive for HPV 6, and 5 patients for HPV 11; no other genotype was found. HPV genotype and presence were not dependent on tobacco exposure (Fisher's exact test, *P* = .567 and 0.897, respectively). HPV was found in none of the patients in the control group.

EBV was present in 33.3% of patients in the RRP group and in no patient in the control group (*P* = .019; Figure 1; Table 2). The number of EBV copies varied from 350 to 1.8 x 10^5 IU/ml. EBV presence was not dependent on tobacco exposure (Fisher's exact test, *P* = .413) or HPV genotype (Fisher's exact test, *P* > .999) or concentration (Table 3). EBV presence was strongly related to increased cell proliferation in all layers of the epithelium and to the number of surgeries performed in the last 2 years. Ki67 in all layers across the epithelium was 40.0 in EBV-positive compared to 31.0 in EBV negative patients (*P* = .005). The significantly higher Ki67 was observed in lower, middle and upper third of the laryngeal epithelium (Table 4).

HHV-6 was found in 16.7% (3/18) of biopsies in the RRP group, but in one case it was a false positivity due to the presence of non-infectious DNA integrated into the somatic genome (Figure 2). Two biopsies were positive for both EBV and HHV-6 (including the false-positive case). One biopsy was positive for HHV-6 only. No other herpetic virus was found in the biopsies of either group (Table 2).

TABLE 4 AORRP characteristics according to EBV presence

	EBV+				EBV-				*P* value
No.	6				12				—
D/C score before surgery	7.5 (5.0-16.0)				12.0 (7.5-15.0)				0.636*
D/C score before/ 6 months after surgery	0.55 (0.29-0.60)				0.64 (0.25-0.70)				0.679*
Level of dysplasia	0	1	2	3	0	1	2	3	>0.999**
	3	2	1	0	6	5	1	0	
No. of surgeries last 2 years	2.5 (1.3-3.8)				1.0 (1.0-1.0)				0.039*
Ki67 lower third of the epithelium	90.0 (90.0-93.8)				80.0 (78.8-86.3)				0.010*
Ki67 middle third of the epithelium	20.0 (16.3-23.8)				7.5 (4.5-16.3)				0.023*
Ki67 upper third of the epithelium	7.5 (3.5-10.0)				2.0 (0.0-3.5)				0.032*
Ki67 total	40.0 (37.0-41.3)				31.0 (27.4-35.0)				0.005*

Note: Data are presented as absolute frequency or median (interquartile range). D/C = Derkay/Coltera; EBV = Epstein-Barr virus; HPV = human papillomavirus.

*Mann-Whitney *U* test,

**Fisher's exact test.

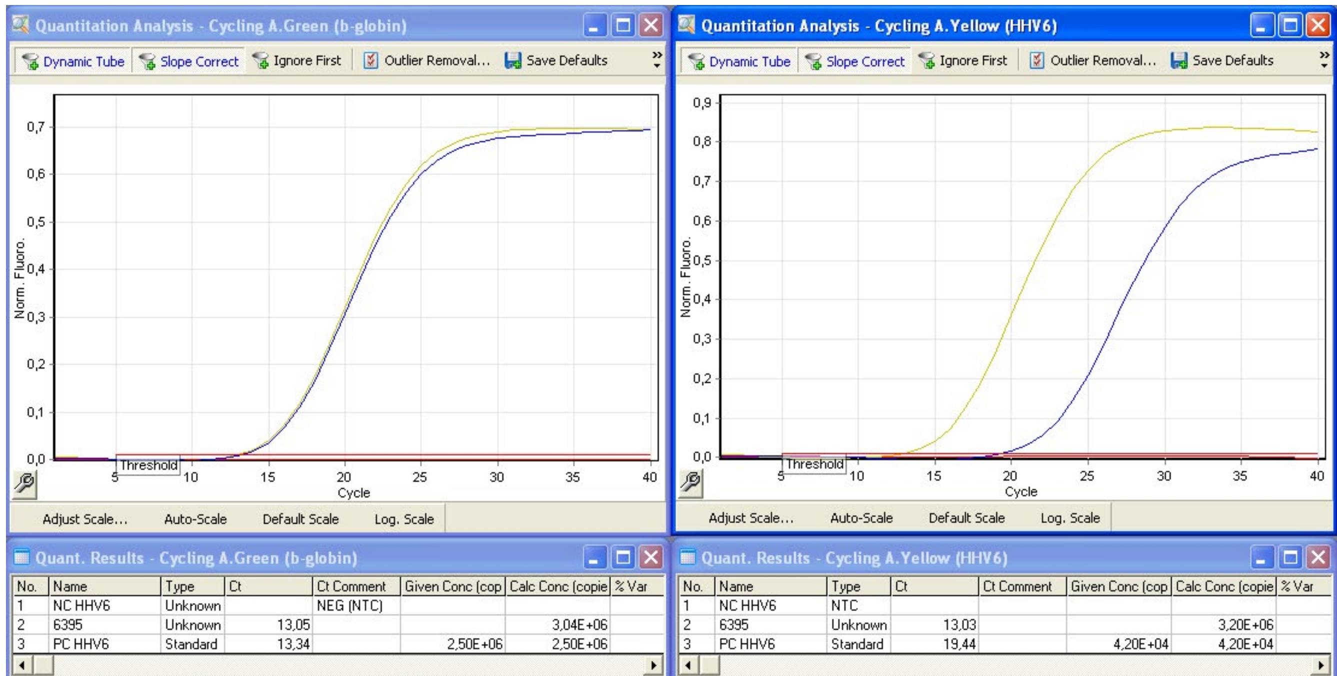

FIGURE 2 HHV-6 false positivity in quantitative real-time PCR due to the presence of non-infectious DNA integrated into the somatic genome. Yellow curve represents sample, blue curve represents positive control, and red (horizontal) curve represents negative control in both graphs. Beta-globin gene DNA (left graph) and HHV-6 DNA (right graph) ratio of approximately one to one (3.04×10^6 vs. 3.20×10^6) indicates the detection of HHV-6 DNA integrated into the somatic genome, not active HHV-6 infection

4 | DISCUSSION

In Europe, AORRP is more frequent than juvenile-onset RRP, with an incidence of 0.54 per 100,000 adults.[14] In contrast to this relatively low incidence, HPV was present in the oral mucosa of 1.9% of healthy women in a large study by Lang Kuhs et al[9] In a study by Rautava at al., HPV was present in the oral mucosa of 15.1%–24.1% of women during a 6-year follow-up.[10] Data about the asymptomatic presence of HPV in the respiratory tract of adults from large cohorts are missing. Previously, we found asymptomatic HPV in the larynx in 10% of adults, but no case in the current control group, indicating a possible cumulative presence in > 5% of healthy adults.[8] Data from children indicate asymptomatic HPV in the respiratory tract of 10%-19.6%.[6,7] Accordingly, AORRP seems to develop only in approximately 1 of 4,000 to 44,000 adults infected by HPV. Thus, HPV infection is necessary, but not sufficient, to cause AORRP, and other factors likely contribute to the pathogenesis of AORRP.

It seems that EBV may interact with HPV and contribute to the pathogenesis and progression of AORRP in up to one-third of patients. A synergistic effect between two or more viruses interacting at different stages of tumour development has been described.[15,16] Although the EBV life cycle in epithelial cells remains poorly understood, its neoplastic potential is clear, as it is the main cause of nasopharyngeal carcinoma and is associated with an increased risk of oral squamous cell carcinoma.[17-19] EBV is found in other head and neck carcinomas as well, but its exact role in pathogenesis has not yet been clarified.[20,21] However, HPV and EBV co-infection has already been shown to enhance the invasiveness of head and neck cancer cells and is associated with poor prognosis.[20-22] EBV co-infection is also supposed a co-factor in the genesis and/or progression of cervical carcinoma.[23]

HPV and EBV both infect and replicate in upper aerodigestive tract epithelia.[24,25] Their life cycles share a relationship with the stratified epithelium, where the productive phase of the HPV life cycle and lytic reactivation of EBV are both induced upon differentiation of epithelial cells.[17] Although the exact mechanisms of interaction still need to be revealed, results from the oral cavity have revealed several potential mechanisms that could be considered.[17] Findings indicate that HPV infection has the potential to stabilise EBV in latently infected oral epithelial cells and increase EBV viral yield upon differentiation of the same cells by promoting EBV lytic reactivation.[17] It could also lead to an increase in the likelihood of neoplasm and in the number of subsequent infection events. Induction of the lytic cycle in a fraction of cells can also increase the expression of viral and cellular cytokines and growth factors, such as vascular endothelial growth factor, which can enhance the proliferation of neighbouring cells.[26-33] The enhanced proliferation corresponds to the results of our study, as EBV co-infection was strongly related to a significant increase in antigen Ki67 in all layers across the epithelium. Ki67 is a protein associated with cellular proliferation and ribosomal RNA transcription, and is a clinically important proliferation marker for grading multiple types of cancers, with well-established prognostic value.[34] The enhanced proliferation was also confirmed by a significantly higher number of previous papilloma

surgeries in EBV-positive patients with AORRP. We found no significant difference in the extent of disease. However, the authors consider it a marginal parameter that can be misleading, as it is very dependent on patient compliance. Less compliant patients usually have severe findings regardless of risk factors, as more time is given for the papillomas to grow and spread. Interestingly, EBV presence did not relate to dysplastic papilloma changes. Further research is required to elucidate the specific interactions between EBV and HPV and their role in pathogenesis.

According to the current results, CMV, HSV and VZV do not seem to contribute to the onset or course of AORPP. The effect of HHV-6 remains unclear, but the virus is very common in adults, and no sample was positive solely for HHV-6 when the false-positive result was excluded.

There are several issues that could potentially influence our results and need to be discussed. First, some authors consider EBV DNA detection using PCR to be a less suitable method with higher risk of false positivity because EBV could reside in B-lymphocytes that infiltrate tumour sites.[21] However, this could be the case only for malignant tumours, in which cell invasion occurs through the basement membrane. AORRP is strictly a pathology of the epithelium, where only antigen-presenting cells are found, and their number is reduced by HPV infection.[35] If false positivity were the case, the number of EBV-positive samples would have to be much higher in both groups considering that latent EBV positivity is estimated in more than 90% of the world's population.[36]

Second, the results should not be influenced by tobacco exposure, as tobacco exposure was not often present in the study group and neither HPV presence nor EBV presence was dependent on it.

Third, in contrast to the results of our previous study, HSV-2 co-infection does not seem to be a co-factor for AORRP, as it was present in none of the biopsies. To elucidate this issue, all papilloma biopsies sent for histopathological examination were subsequently evaluated by a single pathologist for the presence of HSV using immunohistochemistry with the same methodology as in our previous study.[8] Biopsies of healthy mucosa could not be immunohistochemically evaluated because only one biopsy was obtained in each patient in the control group, which was used for PCR detection. The pathologist was blinded to the PCR results. Nine samples were slightly positive for HSV in both the nucleus and cytoplasm. False immunohistochemical HSV positivity was caused by the presence of an artificial tissue alteration (artefacts) considering that every positivity was slight and diffuse (nuclear and cytoplasmic). Artefacts could be caused by many different factors. According to the producer of HSV antibody, false-positive results may be seen because of non-immunological binding of proteins or substrate reaction products, or they may be caused by pseudoperoxidase activity, endogenous peroxidase activity, or endogenous biotin. In addition, the producer did not offer a positive control, and antibody dilution was the lowest recommended.

The main limit of our study is the sample size, which allows herpetic virus presence to be addressed as the primary aim of our study, as well as essential subgroup characteristics, but it is too small to compare all characteristics of subgroups of patients with AORPP. Additional studies with larger cohorts are necessary.

5 | CONCLUSIONS

Unlike other herpetic viruses, Epstein-Barr virus seems to interact with human papillomavirus, enhancing cell proliferation and contributing to the pathogenesis and progression of adult-onset recurrent respiratory papillomatosis. Further research is required to elucidate specific interactions and their role in the pathogenesis of adult-onset recurrent respiratory papillomatosis.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTION

All those named above are significant contributors to either design, data collection and analysis or interpretation of results. All contributed to the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ORCID

Martin Formánek https://orcid.org/0000-0002-5759-2073

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