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Mapping of Human Papilloma Virus, p16, and Epstein-Barr Virus in Non-Malignant Tonsillar Disease

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Objectives: Due to their location in the entrance of the aero-digestive tract, tonsils are steadily exposed to viruses. Human papilloma virus (HPV) and Epstein-Barr virus (EBV) are two potentially oncogenic viruses that tonsils encounter. The incidence of HPV positive tonsillar cancer is on the rise and it is unknown when infection with HPV occurs.

Aim: To investigate if tonsils are infected with HPV and EBV, to study the co-expression of HPV and its surrogate marker p16, and to evaluate the number of EBV positive cells in benign tonsillar disease.

Materials and Methods: Tonsils from 40 patients in a university hospital were removed due to hypertrophy, chronic or recurrent infection. These were analyzed for presence of HPV, its surrogate marker p16, and EBV. HPV was studied using PapilloCheck (a PCR method), while p16 was identified in epithelial and lymphoid tissue with immunohistochemistry and EBV using EBER-ISH (Epstein-Barr encoding region–in situ hybridization).

Results: HPV was not detected, and p16 was present at low numbers in all epithelial samples as well as in 92.5% of the lymphoid tonsillar samples. At least one EBER-positive cell was seen in 65% of cases. Larger numbers of EBER-expressing cells were only seen in two cases.

Conclusion: These findings demonstrate that EBV and HPV infect tonsils independently, but further studies are warranted to confirm their infectious relationship.

Key Words: Human papillomavirus, Epstein-Barr virus, non-malignant tonsillar disease, EBER-ISH, PapilloCheck, immunohistochemistry.

Level of Evidence: Cross-sectional study

INTRODUCTION

At least seven viruses are associated with human cancer: hepatitis B virus, hepatitis C virus, human herpes virus 8, Merkel cell polyoma virus, human lymphotropic virus type 1, human papilloma virus (HPV), and Epstein Barr virus (EBV).[1] In the head and neck region, the last two are well documented oncoviruses.

HPV is a small, non-enveloped, double-stranded, encapsulated DNA virus.[2] More than 150 different HPV types have been discovered (https://pave.niaid.nih.gov/#home and elsewhere[3]). Some are classified as low-risk (eg, HPV type 6 and 11) and associated with the etiology of primarily benign diseases such as recurrent respiratory papillomatosis.[4] High-risk HPV (eg, HPV type 16, 18) has a documented impact on cancer development.[5,6]

It is well known that HPV is transmitted sexually,[7] however, alternative pathways such as vertical (mother-to-child) transmission and transmission via surfaces and fomites have been reported.[8]

Despite the fact that tobacco and alcohol use is decreasing in many countries, and these are causative agents of tonsillar cancer, the incidence of tonsillar cancer is on the rise.[9] HPV has been held responsible for the increase.[9–11] In general, patients with HPV-positive tonsillar cancer have higher rates of survival than patients with the HPV-negative counterpart.[12,13] However, there is a subgroup of HPV-positive head and neck cancer, in which HPV is integrated in the genome instead of being located episomally. In this particular group, survival rates have been shown to be as poor as in HPV-negative cancer.[14] This demonstrates the importance of identifying HPV-positive head and neck cancer cases and assessing whether HPV is integrated or located episomally.

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Treatment strategies should be tailored depending on HPV status[15–17] and location of HPV with respect to the genome.[14]

P16 is a tumor suppressor protein, however, in tongue base[18,19] as well as tonsillar cancer[20] it is used as a surrogate marker for HPV infection.[9] The HPV oncoprotein E7 binds to and inhibits the retinoblastoma protein pRb which leads to release of transcription factor E2F which in return leads to progression to S phase in the cell cycle. As cells divide, levels of p16 rise in an attempt to control cell division in HPV infected cells which cannot succeed as its mechanism of action is being surpassed.[21,22]

p16 can also be overexpressed due to other reasons, such as inactivation of the retinoblastoma protein.[23] Thus, we wanted to know if there is abundance of p16 in benign tonsillar disease and if it correlates with the presence of HPV.[21]

EBV is a gamma herpesvirus which infects most individuals in their childhood or adolescence via the saliva. EBV is associated with cellular hyperproliferative disorders such as infectious mononucleosis, B cell and solid tumors.[24] EBV gives rise to a persistent latent infection of the host in the memory B cells, explained by the germinal center model (GCM).[25] In the germinal center, EBV biology mirrors B cell biology, and EBV-infected memory B cells downregulate virus gene expression to avoid immune recognition.

Non-malignant tonsillar diseases are characterized by hypertrophy and/or acute or chronic infection. The role of HPV in the development of these diseases has been studied extensively with divergent results (0–12.5% of cases showing HPV infection).[26–30] The connection between HPV, EBV, and p16 in benign tonsillar tissue is not well understood. In a study by Jiang et al., HPV/EBV co-infection rates in tonsillar carcinoma and cancer of the base of tongue were reported as 25% and 70%, respectively. Fifteen percent of non-malignant tonsils showed presence of EBV whereas HPV could not be detected (using in situ hybridization).[31] It has also been shown that HPV could be a cofactor facilitating persistence of EBV in the oral cavity.[32] If HPV was found in pediatric tonsillar disease it could support the possibility of vertical transmission of the virus.

The purpose of this study was to examine the expression of HPV, EBV, and p16 in a cohort of patients with non-malignant tonsils. Our aims were to determine if HPV/EBV co-infection is common in benign tonsillar disease and to assess the co-occurrence of expression of HPV with p16 in non-malignant tonsils.

MATERIALS AND METHODS

This cross-sectional study included resected tissue from patients diagnosed with hypertrophy of and/or infections in the tonsils. The study was approved by the Regional Ethical Review Board of Umeå University (Umeå, Sweden) and the BiobankNorth (County Council of Västerbotten, Sweden; approval nos. Dnr *2018-01-29*; 2017-277-31 M (2015-323-32 M, 2012-379-32 M, 2010-277-31 M) and *2018-03-19;* 471-13-008, respectively) and Regional Ethical Review Board of Uppsala University Dnr 2013/387/2.

Materials

Nineteen left and 21 right tonsils from 40 patients (19 females and 21 males, mean age 16.9 years, range 3–58 years) were randomly included in the study after operative intervention at Uppsala University hospital during 2014. Twenty patients were operated due to hypertrophy of the tonsils, 14 were preoperatively diagnosed with chronic and 6 with recurrent tonsillitis (Table I). Ten of the patients underwent tonsillotomy (partial removal of tonsils) whereas 30 underwent tonsillectomy (total removal of tonsillar tissue).

Specimens were frozen in OCT Cryomount (Histolab, Sweden) and stored at –80°C. Unfrozen tissue has been used in previous projects[33–35] at Uppsala University Hospital.

Two members of the research group (KOL, KN) dissected representative portions, including epithelium and lymphoid tissue/germinal center, from the specimens for HPV analysis with PapilloCheck. Slides for p16 and EBER analysis were obtained through equal setting from selective parts of the remaining tonsillar tissue and fixed in formaldehyde.

Methods

HPV detection and PCR

Five 10-μM sections were cut from each tonsillar specimen. The microtome and all other used instruments were cleaned using 70% alcohol, and a fresh blade was installed before each specimen was cut. Then, the sections were placed in 2.0 mL Eppendorf tubes and centrifuged shortly.

The QIAamp DNA FFPE Tissue Kit (QIAgen, Hilden, Germany) was used for DNA extraction. On the first day, tonsillar sections were incubated with a solution of Proteinase K in lysis buffer (ATL) at 56°C. Day 2, the tonsillar tissue was first incubated for 60 minutes at 90°C, before a solution of buffer ATL and 99.5% ethanol was added. The tissue was spun briefly and transferred to the QIAamp MinElute column, washed two times, and centrifuged at 13 000 rpm for 3 minutes. Using 100 μl elution buffer (ATE) DNA was removed from the column.

Both quantity and quality of DNA were assessed prior to analysis of specimens with PapilloCheck (Greiner BioOneGmbH, Frickenhausen, Germany). Quantity was measured with a spectrophotometer and quality analyzed based on DNA purity by spectrophotometric analysis of A260/280 ratio. We evaluated the ability to amplify the housekeeping ß-globin genes of three different amplicon lengths (536 bp, 268 bp, and 100 bp) in order to assess the degree of DNA integrity/fragmentation.

A length of about 350 bp is needed in order to allow HPV typing with PCR. Endogenous ß-globin DNA was amplified by general PCR thermal cycling (M3000, Thermo Fisher Scientific, Waltham, MA, USA), starting with 3-min incubation at 95°C, and then 40 cycles at 95°C for 30 s, 72°C for 30 s, and 55°C for 1 min.

The PapilloCheck (Greiner BioOneGmbH, Frickenhausen, Germany) is a PCR method that identifies 13 high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), five possibly oncogenic HPV types (HPV 53, 66, 70, 73, and 82), and six low-risk HPV types (HPV 6, 11, 40, 42, 43, and 44). PapilloCheck uses multiplex PCR with fluorescent primers to amplify DNA fragments (350 nucleotides) within the E1 open reading frame of the HPV genome. As a positive control, amplification of an internal HPV template in the PCR master-mix, appears on the chip as a signal on the PCR control spot. To assess cellularity, an internal PCR control is used, which targets a region within the human ADAT-1 gene (adenosine deaminase tRNA specific 1). All samples were tested following instructions from the manufacturer (Greiner BioOneGmbH, Frickenhausen, Germany).

p16 immunohistochemistry and scoring system

A Ventana staining machine (BenchMark ULTRA; Ventana Medical Systems, Tuscon, AZ, USA) was used for detecting p16. Slides were pretreated in Tris-EDTA (10 mM Tris-HCl, 1 mM disodium EDTA; pH 8.0), thereafter an antibody against p16

TABLE I.
Patient Characteristics.

	Patient Characteristics Side					Operation	
	Left	Right	Sex	Age	Diagnosis	Tonsillectomy	Tonsillotomy
1	X		Male	4	Tonsillar hypertrophy	X	
2		X	Female	3	Chronic tonsillitis	X	
3	X		Male	17	Chronic tonsillitis	X	
4		X	Female	6	Recurrent tonsillitis		X
5		X	Female	4	Tonsillar hypertrophy		X
6	X		Male	6	Tonsillar hypertrophy		X
7	X		Male	6	Tonsillar hypertrophy		X
8		X	Female	33	Chronic tonsillitis	X	
9	X		Female	12	Chronic tonsillitis	X	
10		X	Male	30	Tonsillar hypertrophy	X	
11	X		Male	7	Recurrent tonsillitis	X	
12		X	Female	25	Chronic tonsillitis	X	
13		X	Male	21	Chronic tonsillitis	X	
14		X	Male	9	Tonsillar hypertrophy	X	
15	X		Male	28	Tonsillar hypertrophy	X	
16		X	Male	6	Tonsillar hypertrophy		X
17		X	Male	3	Tonsillar hypertrophy		X
18	X		Female	4	Tonsillar hypertrophy	X	
19	X		Female	4	Tonsillar hypertrophy		X
20		X	Male	7	Tonsillar hypertrophy		X
21		X	Female	16	Chronic tonsillitis	X	
22		X	Male	22	Tonsillar hypertrophy	X	
23	X		Female	29	Recurrent tonsillitis	X	
24	X		Female	6	Tonsillar hypertrophy	X	
25	X		Female	12	Chronic tonsillitis		X
26		X	Male	18	Chronic tonsillitis	X	
27	X		Female	4	Tonsillar hypertrophy	X	
28	X		Male	14	Chronic tonsillitis	X	
29		X	Female	15	Tonsillar hypertrophy	X	
30		X	Female	58	Tonsillar hypertrophy	X	
31	X		Male	19	Chronic tonsillitis	X	
32		X	Male	24	Tonsillar hypertrophy	X	
33		X	Male	7	Tonsillar hypertrophy		X
34		X	Female	36	Chronic tonsillitis	X	
35	X		Male	33	Chronic tonsillitis	X	
36	X		Female	31	Tonsillar hypertrophy	X	
37		X	Male	40	Recurrent tonsillitis	X	
38	X		Male	15	Recurrent tonsillitis	X	
39		X	Female	19	Recurrent tonsillitis	X	
40	X		Female	22	Chronic tonsillitis	X	

(monoclonal mouse anti-human; cat. no. sc-56330, Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:200 was used. The Ultraview Universal DAB Detection Kit (Ventana Medical Systems) made the antibody visible to the eye. P16 staining was counted using light microscope (BX51; Olympus Corporation, Tokyo, Japan). The quickscore system was used to score the percentage of p16-positive cells as well as intensity of p16 staining.[36] Epithelium and lymphatic tissue were scored separately. The percentage of p16-positive cells was counted manually in the entire lymphatic and epithelial tissue as follows: 1 = 0–4%, 2 = 5–19%, 3 = 20–39%, 4 = 40–59%, 5 = 60–79% or 6 = 80–100%. Intensity of p16 staining was scored using a 4-grade scale: 0 = negative, 1 = weak, 2 = intermediate, and 3 = strong. The intensity of the p16 staining multiplied by the proportion of p16-positive cells generates a product named quickscore which ranges from 0–18.[36]

The histopathological slides were examined by two members of the research group independently (KN and AH). After comparison, consensus was reached.

EBER in situ hybridization—EBER-ISH

All 40 tonsil specimens were subject to investigation with EBER-ISH (Epstein-Barr encoding region–in situ hybridization). A commercially available kit was used.

Two sections were cut from each tonsil. One of the sections was incubated with RNA Positive Control Probe (800-2846, Ventana) to make sure that RNA was present in the sample. In the second section EBER1 and EBER2 (800-2842, Ventana Medical Systems, Roche Diagnostics GmbH, Mannheim Germany) were traced by the Inform EBER Probe. NBT-BCIP Detection System was used for visualization (800-092, Roche Diagnostics GmbH, Mannheim, Germany). The staining was executed in a Bench Mark Ultra (Ventana Medical Systems, Inc, Tucson, AZ, USA) following manufacturers guidelines.

EBER positive cells were calculated manually and independently by two members of the research group (AH and senior pathologist KN). Scores were compared and consensus was reached. Positivity for EBER is defined as >20% or >50%.[37] No scoring system has been described for low numbers of EBER positive cells, thus we modified Detres quickscore system[36] in order to facilitate statistical analysis. The same percentage cut-off values were used: 1 = 0–4%, 2 = 5–19%, 3 = 20–39%, 4 = 40–59%, 5 = 60–79%, 6 = 80–100%. Intensity was not graded, instead percentage values were multiplied with 0 = absence of staining or 1 = presence of staining. This generated a score ranging from 0 to 6. All cells in the slides were studied.

STATISTICS

Descriptive statistics using SPSS (version 23.0.0.2) were used. Differences between p16 score in epithelial and lymphatic tissue were calculated using the Wilcoxon signed ranks test, since normal distribution could not be assumed.

RESULTS

HPV

HPV DNA was not detected in any of our samples despite good quality and quantity of DNA (evaluated with amplificability of housekeeping gene ß-globin).

P16

P16 score in the epithelium ranged from 1 to 9 with a mean of 3.2 (Fig. 1B). Thus, p16 was found in all epithelial parts of the samples. p16 score in the lymphatic tissue ranged from 0 to 4 with a mean of 1.1 (Fig. 1A). P16 was found in 92.5% of samples. Measured p16 in epithelial tissue was higher than that in lymphatic tissue (P = .000001, Wilcoxon signed ranks test).

EBER Score

All EBER control slides were positive confirming that staining was successful.

EBER quickscores ranged from 0 to 2 (see Fig. 2) and was generally low with a mean of 0.7. At least one EBER-positive cell was found in 65% of cases. At most, 76 EBER-positive cells (sample from a 4-year-old female operated due to tonsillar hypertrophy) and ~850 EBER positive cells were found (sample from a 22-year-old male operated due to tonsillar hypertrophy). Examples of stainings are provided in Fig. 3.

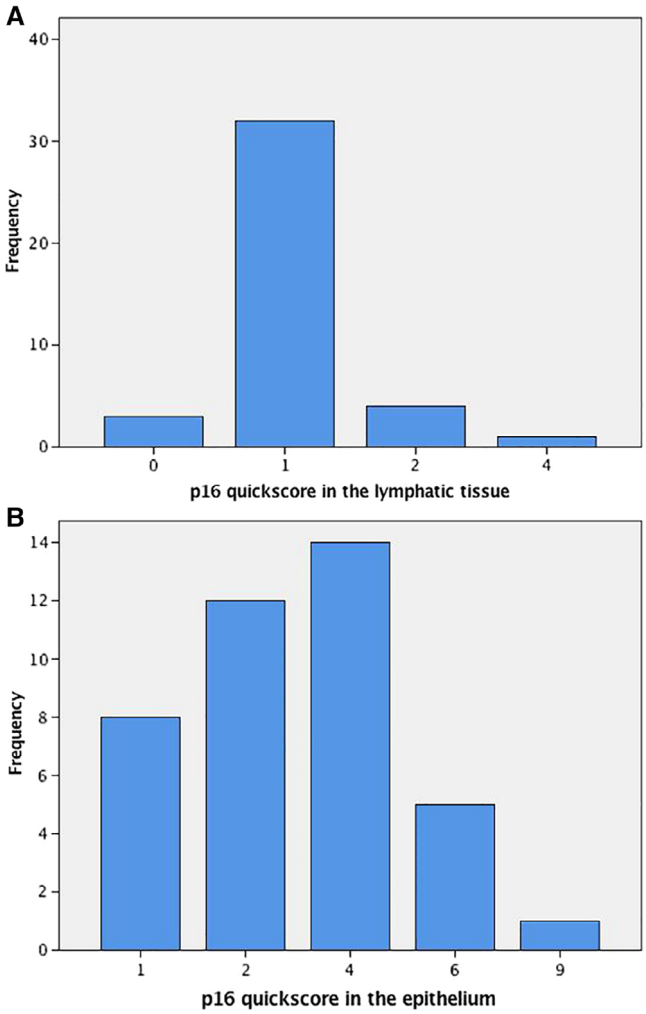

Fig. 1. Frequency of quickscores in the lymphatic tissue (1A) and in the epithelium (1B). Range 0–18.

DISCUSSION

The purpose of this study was to investigate prevalence of EBV, HPV, and its surrogate marker p16 in tonsils removed for other reasons than cancer in a cohort of 40 patients.

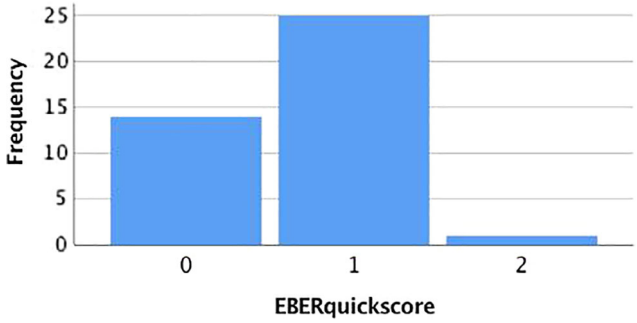

Fig. 2. Frequency of EBER modified quickscores in the tonsillar tissue. Range: 0–6.

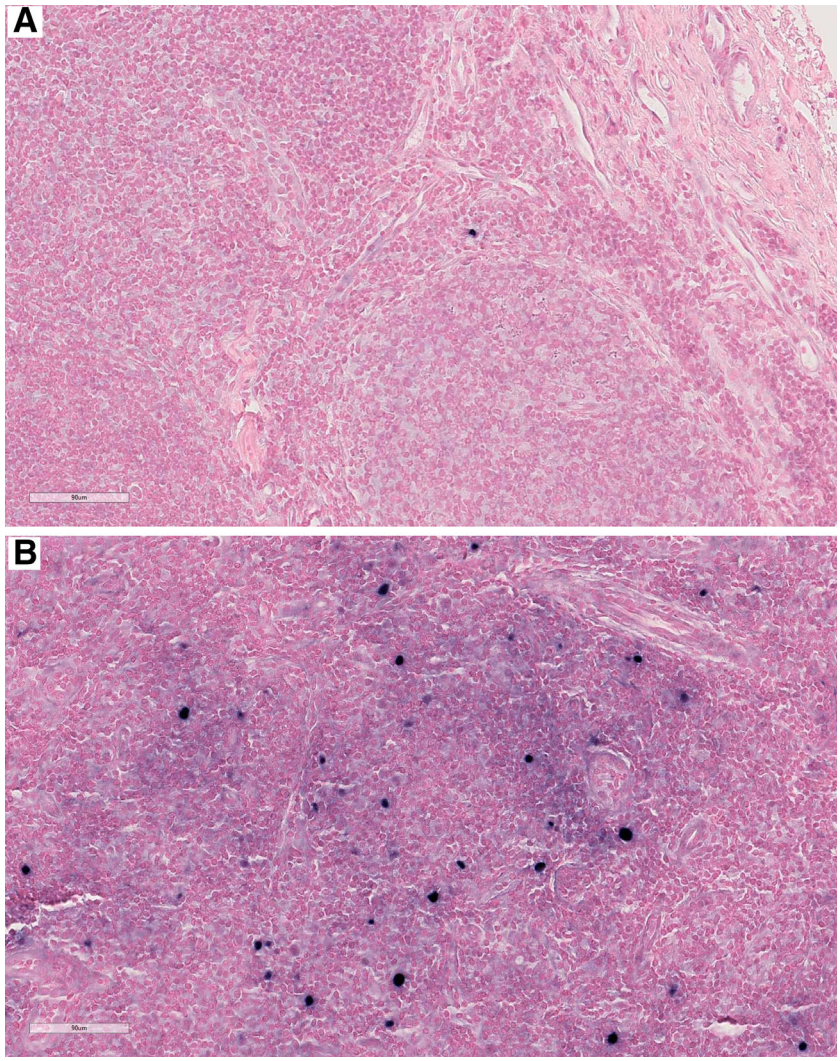

Fig. 3. Example of EBER positivity in a single cell (A) compared to EBER positivity in >800 cells (B). Segments of total slides shown for demonstration.

While HPV was analyzed using PapilloCheck, a sensitive PCR method genotyping 24 pathogenic HPV types, none of these genotypes were present in the tonsils.

Our finding that all tonsils were HPV negative fits in well with other reports, as absence or low numbers of HPV-positive cases in benign tonsillar disease have been demonstrated previously.[26,28,38–42] The largest study on benign tonsillar tissue evaluated 3377 benign tonsils without detecting any HPV.[43] These data are somewhat conflicting since a Belgian study found HPV detection rates in 12.5% (high-risk HPV) and 15% (low-risk HPV) in benign tonsillar tissue from adults and children.[30] The presence of HPV in tonsils was also confirmed in a Greek study with an HPV DNA prevalence of 9.4% in pediatric hypertrophic tonsillar tissue.[27] After showing that HPV-positive oropharyngeal cancer is on the rise,[44] Ernster et al. compared prevalence of HPV 16 and 18 in benign tonsillar disease for two separate time periods, 1979–1982 and 1997–2001 in Colorado. Tested viruses could not be found.[45] A Swedish study from 2017 showed prevalence of HPV in mouth-washes in 10.3% of cases, nevertheless HPV could not be found in the tonsillar tissue acquired during tonsillectomy on the same patients.[46]

A possible explanation for the different outcomes could be differences in detection methods. However, above mentioned studies all used sensitive PCR methods rendering this explanation unlikely. Another explanation by Rusan et al. is that most studies only examine one tonsil, whereas they showed in their study that HPV 6 was detected in one tonsil, but not the other, which could lead to an underestimation of HPV prevalence.[38]

Despite absence of HPV in our tonsillar specimens, p16 was present in the epithelium in all cases and in the lymphoid tissue in 92.5% of cases. However, intensity and number of positive cells were generally low when comparing to inverted sinonasal papilloma[47] and oropharyngeal cancer.[19] These low numbers of p16 seen probably resemble normal p16 expression in a regulated cell cycle, where p16 functions according to design. In a normal cell, p16 bound to Cyclin Dependent Kinases (CDKs) is capable of

inhibiting Cyclin D from binding the CDKs. Subsequently, retinoblastoma protein remains unphosphorylated and as such bound to transcription factor E2F which implies that no cell division takes place.[21]

Respiratory viruses were found in about 70% of investigated tonsillar specimens in 2012 in a larger cohort in which our studied tonsils were included.[48] Considering p16's role as a marker for cellular senescence,[49] our observed p16 levels could also result from viruses/bacteria involved in the pathogenesis of benign tonsillar disease "stressing" tonsillar cells into senescence. Cut-off values for p16 positivity vary in the literature, but according to a systematic review from Grønhøy Larsen et al., higher sensitivity to predict HPV presence is reached when using a p16 cut-off of ≥70%. Only one specimen in this study showed p16 staining >20%, indicating that other mechanisms than HPV are involved in the slight increase seen.

Few studies on presence of both the surrogate marker p16 and HPV DNA in benign tonsillar disease are available, and scoring systems for p16 immunohistochemistry differ. Therefore, these kinds of results need to be interpreted with caution. A study from 2010 found benign tonsillar tissue to be p16 positive in 28%, though HPV DNA could only be found in 1% of cases.[40] The abovementioned Belgian study analyzed p16 presence in 42 of 80 study subjects and found p16 positivity in every second case (HPV DNA present in 12.5%), with weak to moderate staining intensity of 10% of epithelial cells.[30] Quabius et al. showed 73% weak and 18% moderate epithelial p16 staining in their HPV DNA negative cohort.[50]

At least one EBER-positive cell was present in 65% of cases. These numbers are substantially lower than cut-off values for EBV positivity that are used in diffuse B-cell lymphoma (>20% or >50%[51–53]).

In previous studies EBV DNA was found in pediatric tonsils in 43–80% of cases[54–56] and in adult tonsillar tissue in 71% of cases.[55] All these studies used PCR to detect EBV DNA copies. In our study, EBER in situ hybridization was used since we wanted to know how many individual cells were infected with EBV. The EBER is considered as a good marker for EBV infection because it is expressed at very high levels and is therefore detectable in almost all EBV-infected cells.[57] Notably, the tonsils included in this study were previously part of a larger study where the quantitative PCR analysis revealed EBV DNA in 58 of 111 samples (52%).[33] In the present study, at least one EBER-positive cell was found in 65% of cases, findings that are in line with abovementioned EBV prevalence in the tonsils. Further, we found only two samples having quickscore >2 (Fig. 2), with 76 and about 850 EBER-positive cells, respectively. The low detection of EBER can be explained by the life cycle of EBV. The EBER gene transcription is reduced when EBV switches from latent infection to lytic replication.[58] Hence, high EBER expression might indicate that EBV has established latent infection in these two tonsil samples.

EBV and HPV are both well-known oncogenes with long duration of action. Due to these similarities, co-infection with HPV and EBV has been studied previously as well as in this study, in order to establish if the viruses occur simultaneously, which could suggest that they influence each other. Co-infection was not found in epithelial tumors of the head and neck by Atula et al.,[59] but could be seen in a study by Jiang et al. in tonsillar cancer and cancer of the base of tongue in 25% and 70% of cases, respectively.[31] In a study from 2019, Ruuskanen et al. demonstrated presence of EBV in 62%, and HPV in 14% of cases of nasopharyngeal carcinoma, without co-infection.[60] Drop et al. reported HPV/EBV co-infection in cancer of the oropharynx, larynx, and oral cavity in 28.6%, 21.4%, and 50%, respectively.[61] However, most patients in this study also had classical risk factors (smoking and alcohol abuse) for developing oropharyngeal cancer, which also could influence susceptibility to infection. We were unable to detect co-infection with HPV and EBV.

Healthy tonsillar tissue is difficult to define as well as to obtain, since tonsils are one of the first lymphoid tissues that virus and bacteria encounter on their way to infect the body. Due to the relatively small number of tonsils investigated here and the relatively young age of the study population, it is difficult to predict when HPV/EBV infections occur. Larger studies are warranted to study these matters further. However, it can be difficult to get access to benign tonsils removed at all relevant ages since tonsillar disease mostly affects children and younger adults and tonsils in Sweden are removed at a mean age of 13.3 years.[62]

CONCLUSION

In this cohort of 40 non-malignant tonsils HPV could not be found, p16 was present in low numbers in the epithelium and lymphoid tissue and EBV EBER was present in 65% of cases. However, larger numbers of EBV positive cells were only present in 2 of 40 cases.

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