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HEAD AND NECK

Detection of high-risk human papillomavirus in the tonsils of galilee region adults and young adults undergoing tonsillectomy

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Abstract

Introduction The presence of high-risk HPV in non-malignant tonsil tissues from patients who underwent tonsillectomy in the Galilee area might explain the low incidence of HPV-related oropharyngeal malignancy in the country. The aim of this retrospective study was to study the prevalence of human papillomavirus (HPV) in non-malignant tonsillectomy specimens of adults in the Galilee area.

Materials and methods We conducted a retrospective analysis of all tonsil samples in our medical center. Tonsils from patients over 20 years of age who underwent tonsillectomy for the indication of recurrent tonsillitis and sleep apneas were eligible. Samples of formalin-fixed paraffin-embedded (FFPE) tonsillar tissue were tested for the presence of HPV DNA using polymerase chain reaction and by p16 immunohistochemistry.

Results Of the 71 tonsil samples, age range 20–65, none were positive for HPV DNA. Fifty-two FFPE specimens of tonsil with HPV-positive cancer tested positive by the same method served as positive controls.

Conclusions HPV DNA is rare in non-malignant tonsil tissues of young adults and adults who underwent tonsillectomy in the Galilee area. Further research should be done in larger cohorts.

Keywords Human papillomavirus · Tonsillectomy · Prevalence

Abbreviations

HPV	Human papillomavirus
FFPE	Formalin-fixed paraffin-embedded
SCCHN	Squamous cell carcinomas of the head and neck
OPSCC	Oropharyngeal squamous cell carcinoma
PCR	Polymerase chain reaction

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Introduction

The human papillomavirus (HPV) is a small double circular-stranded DNA that belongs to the Papillomaviridae family [1]. The virus has more than 90 distinct types, which are classified according to their relationship with benign and malignant lesions into low (HPV6, and HPV11) and high HPV (HPV16 and HPV18) risk, respectively [2]. Infection with sexually transmitted human papillomavirus is the cause of virtually all cervical cancers; in the past decade molecular evidence has provided support of the pathogenesis of a subgroup of squamous cell carcinomas of the head and neck (HNSCC) [3].

Cancers involving the head and neck represent a major health care concern, being the sixth most prevalent type of cancer worldwide [4, 5]. Most (90%) head and neck cancers are squamous cell carcinomas [4]. Oropharyngeal squamous

cell carcinoma (OPSCC) is predominantly seen in the tonsils and the base of the tongue [6]. It is well known that tobacco and alcohol consumption constitute a major risk factor for HNSCC, but over the last 10–15 years HPV has played an important etiologic role as well. It has a distinct biology, natural history, epidemiology, prognosis, and a separate approach to treatment [4, 5].

Unlike in other head and neck tumors, patients with oropharyngeal carcinomas positive to HPV tend to be younger (< 60 years), with no history of smoking and drinking alcohol [4–7], and with a high socioeconomic status and level of education [4].

HPV-positive OPSCC has male predominance with a threefold higher incidence in males than females [4–6] and a significantly higher survival rate than HPV-negative OPSCC, believed to be the result of enhanced sensitivity to radiation and chemotherapy [7]. HPV16 is the dominant serotype among the HPV types causing OPSCC, and it constitutes 95% of the cases [5–8].

Currently, there is great interest in HPV's role in head and neck cancer, especially oropharyngeal cancer. The incidence of the latter has increased significantly in many areas, including the USA and Europe [9], while data in Israel and the Galilee area are lacking.

Although the virus is known to play an important role in OPSCC the natural biology remains unclear. It is also not clear whether other HPV types that infect the tonsils are associated with malignancy. Also unclear is whether detection of active infection releasing virions in tonsils is possible, or if there exists a premalignant phase in the tonsils as in the cervix (cervical intraepithelial neoplasia), which can become invasive and metastatic when accumulating mutations [5].

To date, no published study has examined the prevalence of HPV in tonsils of young adults in Israel. The Galilee population is very diverse and testing for the prevalence of the virus depending on age, sex, and religion can provide more information about the disease and its risk groups. This research explored the characteristics of the HPV population and overall prevalence in young adults in the Galilee.

Methods

Design

This study was carried out at our medical center as a retrospective analytical study.

Study population

In total, we found 71 young adults and adults between the ages of 20 and 65 who underwent tonsillectomy due to chronic/recurrent tonsillitis and sleep apneas between the years 2005 and 2015. Seventy-one patients agreed to participate in the study, four refused to participate, and five patients were lost to follow-up. We decided to include patients over the age of 20 to increase the likelihood of them being sexually active.

FFPE archival biopsy material from these patients was gathered. A standardized socio-demographic questionnaire was used to evaluate the presence or absence of risk factors (patient's age, education, HPV vaccination, and gender, and parents' education).

Exclusion criteria were: those who refused to participate in this trial, those who had been vaccinated against HPV prior to the surgery, and those who had technical difficulties performing any of the laboratory tests.

Detection of high-risk HPV

We analyzed FFPE sections of tonsils. Initially, a representative section from paraffin block was stained with H&E and analyzed under a light microscope to ensure the presence of epithelium in the section. Paraffin sections were cut from FFPE blocks and deparaffinized to isolate the tissue. The samples were tested for the presence of high-risk HPV genotype using the Xpert® HPV Readiness kit (GeneXpert, Cepheid AB, Sunnyvale, CA). Xpert includes reagents for the simultaneous detection of 13 hrHPV types (HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68) and 1 possible hrHPV type (HPV66), a human reference gene (HMBS [hydroxymethylbilane synthase]), and an internal Probe Check Control (PCC). The 14 targeted HPV types are detected in five fluorescent channels: fluorescent channel 1 (HPV16), fluorescent channel 2 (HPV18 and -45) ("HPV18/45"), fluorescent channel 3 (HPV31, -33, -35, -52, and -58), fluorescent channel 4 (HPV51 and HPV59), and fluorescent channel 5 (HPV39, -56, -66, and -68). The human reference gene (fluorescent channel 6) verifies specimen adequacy. The PCC verifies reagent rehydration, PCR tube filling in the cartridge, probe integrity, and dye stability. In total, the assay utilizes six fluorescent channels for the detection of individual types of HPV, groups of HPV, and the human reference gene. Each fluorescent channel has its own cutoff parameters for target detection/validity. If sufficient signal is detected by the human reference gene (i.e., if the sample has sufficient cellularity), the assay results are reported as an overall "positive" if any type of targeted HPV is detected, but, additionally, HPV16 and HPV18/45 and, collectively, the other high-risk HPV types detected by the assay are reported specifically as "positive" or "negative." Fifty-two FFPE samples with squamous cell carcinoma that stained positive for P16 were used as a

positive control. As explained above, the Xpert kit has its own internal control to make sure good-quality DNA was extracted from the FFPE samples.

P16 immunohistochemistry

Immunohistochemistry was carried out for p16 representative section cut from FFPE sections of tonsils using a monoclonal antibody P16 anti mouse IgG clone JC8 (Santa-Cruz Biotechnology, Dallas, TX) according to standard protocol. The staining pattern for P16 was compared to known p16 expression OPSCC tissues, as positive controls and sections of normal tonsils which tested negative for the presence of HPV DNA using polymerase chain reaction and by IHC, as negative controls (Fig. 1).

Sample size calculation

According to a previous study [8] the rate of HPV in patients who underwent tonsillectomy is 0–12.5%. Most reports refer to pediatric populations and rarely to adult populations. Assuming that the HPV rate is 6% for 50 subjects, two-sided confidence interval for proportion is 95% CI (2% and 16%). Assuming a lower HPV rate of 0% [2–6] for 50 subjects, two-sided confidence interval for proportion is 95% CI (0–7.9%).

Results

Patients' characteristics are summarized in Table 1. Of the 71 adults, more than half were female (37/71), median age was 24 (range 20–58 years), mean age was 27.

The religious sectors represented in our cohort were: 37% Jewish, 35% Muslim, 21% Druze, 6% Christian, and 1% Bedouin.

The educational level of the patients during the study: three completed elementary school (4%). 37 completed high school (52%), 7 are earning their bachelor's degree (10%), 21 have completed a bachelor's degree (30%), 2 have a master's degree (3%), and 1 has a higher degree (1%).

The educational level of the patients' mothers: three attended but did not complete elementary school (4%), 22 completed elementary school (31%), 37 completed high school (52%), 1 is earning his bachelor's degree (2%),5 have a bachelor's degree (7%), 3 have a master's degree (4%).

The educational level of the patients' fathers: three attended but did not complete elementary school (4%), 19 completed elementary school (28%), 33 completed high school (48%), 11 have a bachelor's degree (16%), 2 have a master's degree (3%), and 1 has a higher degree (1%); 2 fathers are missing information due to lack of contact with their child.

None of our specimens tested positive for HPV. In other words, we found that HPV prevalence in young adults who underwent tonsillectomy due to chronic tonsillitis is zero, regardless of their age, religion, or gender. On the other hand, all of the positive control tissues tested positive for high-risk HPV using the same technique.

It should be noted that two patients were vaccinated for HPV after they underwent tonsillectomy.

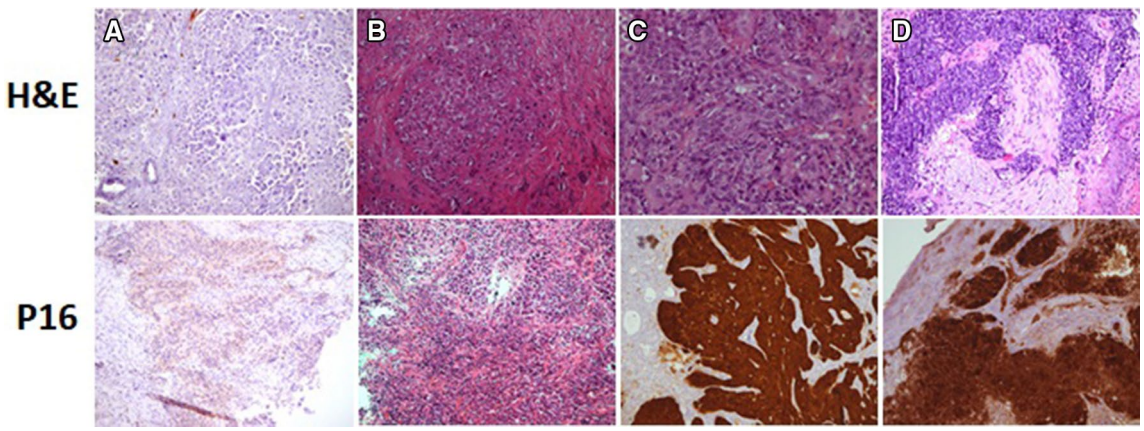

Fig. 1 Immunohistochemical staining of positive and negative controls. Expression of H&E on top panel and P16 on bottom panel. Negative controls images of tongue tissue SCCA (**a**), and of tongue tissue with poorly differentiated SCCA (**b**). Positive controls images of rear palate tissue with poorly differentiated SCCA (**c**), and of tongue tissue with poorly differentiated SCCA (**d**). *H&E* hematoxylin and eosin, *SCCA* squamous cell carcinoma

Table 1 Demographic characteristics

Patient characteristics[a]	Number	%
All patients	71	100
Sex		
Female	37	52
Male	34	48
Age		
Median (range)	24 (20–58)	
Mean ± standard deviation	27 ± 7.2	
Religion		
Jewish	26	37
Muslim	25	35
Druze	15	21
Christian	4	6
Bedouin	1	1
Education		
Patient education		
Completed elementary school	3	4
Completed high school	37	52
Bachelor's degree	21	30
During bachelor's degree	7	10
Master's degree	2	3
Higher degree	1	1
Maternal education		
Attended elementary school	3	4
Completed elementary school	22	31
Completed high school	37	52
During bachelor's degree	1	2
Bachelor's degree	5	7
Master's degree	3	4
Paternal education		
Attended elementary school	3	4
Completed elementary school	19	28
Completed high school	33	48
Bachelor's degree	11	16
Master's degree	2	3
Higher degree	1	1
Lack of information[b]	2	–

[a]During the study

[b]Were excluded from the percentage

Discussion

In recent years HPV has played an important etiologic role in the development of OPSCC, whereas smoking and alcohol consumption that constituted a key role until recently are declining. The main explanation for this is the changing sexual behavior in recent years: the age of sexual initiation has decreased, there is a higher number of lifetime sex partners, and more men and women perform oral sex, especially young people [10].

Our research population included 71 young adults and adults aged 20–65 who underwent tonsillectomy due to chronic/recurrent tonsillitis and sleep apneas in our medical center between the years 2005 and 2015. The diverse population of the area is represented in our cohort.

In our research, all 71 samples of tonsils tested negative for high-risk HPV DNA, while all of the positive control tissues tested positive for high-risk HPV using the same technique. It should be noted that we tested all samples available in our archives for this age group. Tonsillectomy is a surgery that is done mainly in a much younger age group, primarily due to obstructive sleep apnea. For this reason, the incidence of the virus may be biased, and actually may be higher than what we obtained from our research. Nevertheless, sample size calculations suggested that differences in subgroups could be demonstrated and this number should be sufficient.

The epithelium overlaying the lymphatic tissue in the tonsil crypts is of squamous cell type. There are several microbial organisms that can infect the tonsils (Epstein–Barr virus, adenovirus, etc.) and during the past 10 years increasing evidence suggests that HPV can also infect the tonsils [11]. Therefore, to avoid study bias and to confirm the reticulated crypt epithelia from which the SCC arises, we verified the presence of epithelium in each sample by light microscopy prior to examining for the presence of the HPV DNA using PCR (polymerase chain reaction)/p16 immunohistochemistry, Thus, we believe that our results represent a true lack of HPV in these tonsils.

In the HPV infection in men (HIM) cohort study, which tested oral rinse-and-gargle samples from 4072 men aged 18–70 years in Brazil, Mexico, and the USA, oral HPV infection was uncommon in healthy men (4.4% of men acquired incident oral HPV infection and 1.7% oral oncogenic HPV) and most were cleared within 1 year. It should be noted that unlike our method of testing the epithelial tissue, it is possible that testing oral rinse-and-gargle samples does not accurately represent the infected tonsils, and therefore they found a lesser infection rate. In addition, it was mentioned in that study that smoking and marital status affected the acquisition of oral HPV infections [12].

In recent years there have been a quite number of studies showing the increase in incidence of HPV-positive OPSCC and the prevalence of the high-risk HPV in the USA and Europe. HPV-positive OPSCC incidence increased from 1970 to 2007 in Stockholm from 23% in 1970 to 79% in 2000–2007, with dominance of serotype HPV-16. The significant increase was seen between the years 2000–2002 and 2006–2007 with 68% and 93%, respectively [13]. Recent data collected between the years 1984 and 2004 in the USA showed an increase in the prevalence of HPV oropharyngeal cancer, whereas the prevalence of HPV-negative cancer decreased [14].

A systematic review of 18 articles by Kreimer et al. showed that oral HPV was found in 4.5% of healthy individuals ($n=4441$); furthermore, HPV16 was found in only 1.3% of 3977 healthy subjects [15].

The main explanation for this is the changing sexual behavior in the past years, i.e., early sex debut, high number of lifetime sex partners, oral sex, and possibly the introduction of HPV vaccine [16, 17].

A study in the US suggests that if the current sexual trends continue, by the end of 2020 the annual number of HPV-positive oropharyngeal cancers will surpass the annual number of cervical cancer cases [14]. Data on sexual behavior in the Israeli population are lacking.

Two FDA-approved HPV prophylactic vaccines are currently available—the bivalent and quadrivalent vaccines. The quadrivalent vaccine is composed of four HPV-specific virus-like particles from the major capsid of HPV types 6, 11, 16, 18, and the bivalent vaccine is composed of two HPV types—16 and 18. The vaccine can be administered to adolescents before the age of sexual activity [4]. Israel previously approved the vaccine for women, and only recently has the vaccine been approved for men as well, because of both HPV-related OPSCC and to increase the herd immunity. The Center for Strategic and International Studies published a report in 2014, following the decision of the Japanese government to suspend its recommendation for HPV vaccination, causing a dramatic drop in vaccination rate from around 70 to 1%, due to public concerns over the vaccination's side effects [18]. Similar concerns were seen in Australia, the UK, France, and India. The misinformation related to the vaccination has an effect on a certain population in Israel as well, and especially in conservative and religious groups.

Recent research in Costa Rica suggests a high vaccine efficacy against oral HPV16/18 infection, which supports the possibility that vaccination may reduce the risk of HPV-positive OPSCC among women, with efficacy expected among men as well [19]. Yet, patients who received the HPV vaccination prior to their tonsillectomy procedure were excluded from the study.

The primary limitation of our study was the small cohort, yet in studies with a large study population the results were similar to ours. Our medical center provides health services for a population of 566 thousand. Our cohort, although it included all of the tonsils FFPE found in our archives, might be too small.

A study carried out in Brazil on 100 children between the ages of 2 and 13 who underwent tonsillectomy also did not find any samples positive for HPV DNA, although this age group is believed to be too young to practice oral sex [2].

Moreover, a cross-sectional study between 2004 and 2008 in the UK, which examined FFPE tonsil tissue in 3377 patients (mean age 23) who underwent tonsillectomy, found all of the samples examined to be negative to HPV DNA [5].

On the other hand, other European studies found a significant percent of tonsil samples positive for HPV. HPV DNA was detected using PCR with general primers GP5+/GP6+ in 8.5% in a Greek study that examined 106 samples of paraffin-embedded adenoid and/or tonsillar tissues from children with benign tonsillar and/or adenoid hyperplasia (age 2–14 years) [20]. In addition, in a study carried out in Belgium on 80 paraffin-embedded tissues from tumor-free tonsils from healthy children and adults who underwent tonsillectomy, 12.5% tonsils were positive for high-risk HPV using GP5+/6+ consensus PCR [21].

Table 2 Tonsillar HPV frequency in several countries

References	Country	Sample size	Age range	Technique	HPV frequency
[2]	Brazil	100	2–13	PCR	0%
[6]	UK	3377	0–69	PCR	0%
[12]	Brazil, Mexico, USA	4072	18–70	PCR	1.7–4.4%
[20]	Greece	106	2–14	PCR	8.5%
[21]	Belgium	80	Children and adults	PCR	12.5%
[24]	Finland	73	1–16	PCR	8%
[29]	USA	102	20–39	ISH and IHC	3.9%
[30]	USA	129	1–12	PCR	0%
[31]	China	42	3–12	PCR	0%
[32]	China	177	NA	PCR	1%
[33]	Turkey	165	5–21	PCR	7%
[34]	Russia	8	2–14	PCR	13%
[35]	USA	50	3–12	PCR	4%
Current study	Israel	45	20–26	PCR and IHC	0%

PCR polymerase chain reaction, *ISH* in situ hybridization, *IHC* immunohistochemistry

A recent study done by Amit et al. [22] demonstrated a rate of HPV-related OPSCC in Israel to be approximately threefold lower than in Western countries, which might be explained in part by the findings of our study. The Institut Català d' Oncologia, and the International Agency for Research on Cancer have published a human papillomavirus and related diseases report in December 2018 showing a fivefold lower crude incidence rate of OPSCC in Israel than the world average, further supporting our findings, albeit a small sample size [23]. The articles describing HPV frequencies found in tonsils which are described above are summarized in Table 2.

As was nicely shown elsewhere [24–28] a tonsillar HPV geographical distribution does not appear to parallel worldwide oropharyngeal cancer incidence. The authors suggested that this lack of correlation could be because pediatric patients are more easily able to eliminate a HPV infection, or prior HPV infection protects against future infection. Alternately, a correlation between childhood infection and adult cancer may exist but be difficult to discern at this time due to the currently limited literature, thus the importance of the current study and alike.

Another limitation for our study was the nature of our population. The socio-demographic questionnaire showed the diversity of our study population: men and women from a wide age range and from different religions in the population of the Galilee were included. Maternal and paternal education level was relatively moderate, as most mothers and fathers had only a high school education (52% and 48%, respectively). However, we could not find any correlation to HPV status since none of our samples tested positive.

Variability in tissue fixation is known to affect subsequent DNA extraction and is of concern particularly with our retrospective samples.

Because of the sensitive questions and the nature of the population, patients were not asked about substance abuse and about their sexual behavior. Therefore, in practice, some of them may not have had sex or had not practiced oral sex, and thus their relevance for the purpose of calculating the prevalence HPV is unclear.

Conclusions

Our results suggest that HPV DNA is rare in non-malignant tonsil tissue in the young adult age group of the Galilee population. Further research using larger sample size is needed to determine the prevalence of the high-risk HPV in this population.

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Compliance with ethical standards

Conflict of interest Reut Aviv Mordechai, Shay Steinberg, Liat Apel-Sarid, Ety Shaoul, Simona Rozen, Lubna Khoury, and Ohad Ronen declare that they have no conflict of interest.

Ethical approval and informed consent The study was approved by the institutional review board prior to its commencement (Registration number: NHR010714). Written consent was waived by the institutional review board.

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