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Presence of DNA of Human Papillomavirus 16 but No Other Types in Tumor-Free Tonsillar Tissue

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According to PCR, the prevalences of human papillomavirus (HPV) DNA were 6.3% (13 of 206) in tonsillitis or hypertrophic tonsillar tissues and 0.6% (1 of 174) in exfoliated cells from normal tonsils. HPV-16 was the only type detected in tonsillar tissues, but it did not appear to lead to L1 antibody production.

Of the squamous cell carcinomas of the head and neck, human papillomavirus (HPV) DNA has been repeatedly reported in tonsillar carcinoma, suggesting a role for HPV in tonsillar carcinogenesis (5, 9, 10, 13, 22). The reason for the strong association of HPV with tonsils remains unclear. Previous data on the presence of HPV DNA in tumor-free tonsils are based on small series of experiments (24). A significantly increased risk for squamous cell carcinomas of the head and neck is associated with HPV-16 seropositivity (11), but serum antibodies against HPV-16 proteins have been detected in healthy individuals as well (6). Our aim was to study HPV in tumor-free tonsils and to correlate these findings with the seroprevalence of HPV antibodies.

We enrolled 212 patients operated on in 2001 and 2002 because of tonsillitis ($n = 135$) or tonsillar hypertrophy ($n = 77$) at Helsinki University Central Hospital (Table 1). After tonsillectomy, a piece of tissue was immediately frozen at −70°C. A 100-μm cryo-section per tonsil per patient was subjected to DNA extraction. Five-micrometer sections from both ends of the tissue were toluidine blue stained to verify that the tissue section consisted of at least 30% epithelial cells. After every tenth sample, only optimal cutting temperature compound without tonsillar tissue was cryo-sectioned as a negative control for the DNA extraction; none of these sections showed positive β-globin or HPV by PCR. Serum samples were drawn on the day of surgery.

Tonsillar exfoliated cells from 189 control subjects (age and sex matched to patients) with normal tonsils were collected bilaterally from tonsillar surfaces with a Cytobrush Plus Cell Collector (Medscand Medical). The brushed samples were stored in phosphate-buffered saline at −70°C. Serum samples were collected on the same day.

DNA was extracted from tonsillar tissue with the DNA mini kit (QIAGEN) and from exfoliated cells with the MagNA Pure LC DNA isolation kit I (Roche). In order to assess the quality of the DNA submitted to HPV PCR, we applied β-globin PCR (15) and found that 97% (206 of 212) of DNA samples from tonsillar tissue and 92% (174 of 189) of DNA samples from tonsillar exfoliated cells were positive for β-globin.

HPV DNA was identified by nested PCR with consensus primers MY09/11 (14) and GP5+/6+ (3). The genotype was determined by direct sequencing of the approximately 150-bp amplicons with an ABI PRISM Dye Terminator and analyzed on an ABI 377 DNA sequencer (Applied Biosystems) and on the basis of >90% homology with HPV sequences deposited in GenBank with BLAST software.

Serum antibodies against HPV-16 L1, E6, and E7 were analyzed by a newly developed method. The HPV proteins were expressed as fusion proteins with N-terminal glutathione *S*-transferase (GST) as described previously (16, 17). Fluorescence color-coded polystyrene beads (xMAP microspheres; Luminex) were derivatized with glutathione, and for each antigen, a distinct color code class of beads was loaded with the antigen by incubation with lysate from bacteria overexpressing the respective GST fusion protein. Washed antigen-loaded beads were mixed and incubated with human serum, diluted 1:100 in phosphate-buffered saline with 2-mg/ml lysate protein from bacteria overexpressing GST as a blocking reagent. Unbound proteins were removed by washing. The beads were subsequently incubated with biotinylated donkey anti-human immunoglobulin G (IgG) plus IgM (H+L) (Dianova), washed, and incubated with *R*-phycoerythrin-labeled streptavidin. Bead color code fluorescence identifying the bead class and phycoerythrin fluorescence as a marker of bound human Ig were analyzed in a Luminex 100 analyzer, with results given as median fluorescence intensity (MFI) of a minimum of 100 beads analyzed per class/antigen. Specific reactivity was calculated as the difference between the MFI of antigen-loaded beads and the MFI of GST-loaded beads, which defined the background. Predefined cutoff values for seropositivity of 300 MFI for HPV-16 L1 and E7 and 600 MFI for HPV-16 E6 were used.

Of the 206 patients whose tonsillar DNA was positive for β-globin, 6.3% had detectable HPV DNA in their tonsillar tissue. The histological morphology of these 13 HPV-positive samples showed no features (koilocytes, dyskeratotic cells, or parakeratosis) consistent with HPV infection (data not shown). Sequencing revealed that all were HPV-16 and that

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TABLE 1. DNA and seroprevalence of HPV in patients with tonsillitis or tonsillar hypertrophy and exfoliated control subjects with normal tonsils

Patient or control group (*n*)	Age (yr)		Prevalence of HPV-16 DNA (%)[a]	No. (%) with seroprevalence to HPV-16		
	Mean	Range		L1	E6	E7
Patients with tonsillitis or tinsillar hypertrophy tonsils						
Preschool children (26)	4.6	1.5–6	3 (11.5)	2 (7.7)	0 (0.0)	0 (0.0)
School children (47)	10.4	7–16	3 (6.5)	0 (0.0)	1 (2.1)	0 (0.0)
Younger adults (57)	21.0	17–25	5 (9.3)	9 (15.8)	4 (7.0)	0 (0.0)
Midlife adults (57)	32.0	26–40	2 (3.6)	13 (22.8)	1 (1.8)	0 (0.0)
Older adults (25)	51.9	41–72	0 (0.0)	1 (0.4)	0 (0.0)	0 (0.0)
Total (212)	23.2	1.5–72	13 (6.3)	25 (11.8)	6 (2.8)	0 (0.0)
Control subjects with normal tonsils						
Preschool children (26)	4.8	2–6	0 (0.0)	0 (0.0)	1 (3.8)	2 (7.7)
School children (36)	10.1	7–15	0 (0.0)	1 (2.8)	1 (2.8)	0 (0.0)
Younger adults (27)	22.4	18–25	0 (0.0)	2 (7.4)	2 (7.4)	1 (3.7)
Midlife adults (59)	32.4	26–40	0 (0.0)	9 (15.3)	0 (0.0)	0 (0.0)
Older adults (41)	53.4	41–74	1 (2.4)	4 (9.6)	0 (0.0)	0 (0.0)
Total (189)	27.5	2–74	1 (0.6)	16 (8.5)	4 (2.1)	3 (1.6)

[a] β-Globin-negative samples were excluded.

the sequences amplified from different patients were not identical (data not shown). The preschool group showed the highest prevalence of HPV DNA in tonsillar tissue (Table 1) but not significantly. No HPV DNA was detectable in the older adults.

Among the 174 β-globin-positive DNA samples from tonsillar exfoliated cells of control subjects, only 1 (from a male 46 years of age) harbored HPV DNA, typed as HPV-58 (Table 1). Thus, the presence of HPV-DNA in tonsillar tissue was significantly higher than that in tonsillar exfoliated cells ($P = 0.002$, Fisher's two-tailed exact test).

Of the 212 patients, 29 had serum antibodies against at least one HPV-16 protein (L1, E6, or E7) and 13 harbored HPV DNA in their tonsillar tissues. Double positivity for HPV-16 DNA and serum antibodies appeared in two of these patients; both were adult males and had antibodies against HPV-16 L1 (all coefficients <0.10, all $P > 0.4$ [Spearman correlation coefficients]). No concordance thus existed between the presence of HPV-16 DNA in tonsillar tissue and seropositivity.

Of the patients, 6.3% harbored HPV-16 DNA but no other types in their tonsillar tissue. The detection of HPV DNA in tumor-free tonsils has been reported in only a few studies (4, 19, 23, 25, 26), and others failed to detect the viral DNA in tonsillitis samples (2, 8, 12, 21). The overall frequency of HPV DNA in normal tonsillar mucosa or benign tonsillar lesions is 8.5% (17 of 200, compiled from several studies), consisting of 70% with HPV-16 and 30% with HPV-6/11, as summarized in a recent review (24). We observed a tendency of HPV-16 DNA to be more frequent in preschool children than in the other age groups. It is not yet known whether children acquire this HPV-16 DNA in their tonsils by vertical transmission from their mothers; additional sources such as breast milk, siblings via kissing, or exposure to contaminated fomites are feasible (24).

HPV DNA was seldom detectable in normal tonsillar exfoliated cells (Table 1), suggesting that HPV DNA either locates in the crypt epithelial cells or normal tonsils do not harbor HPV DNA. Earlier data on this are controversial. One study showed less HPV DNA in oral exfoliated cells than in biopsies (7), but another reported that 57.9% of the patients ($n = 190$) with cancer of the oral cavity or oropharynx were positive for HPV in both tumor tissue and oral exfoliated cells (20). Reliability of the exfoliated cells for analysis of HPV DNA by PCR needs to be evaluated further regarding the sampling methods (cytobrush or mouth rinse), tumor or tumor-free samples, and specific anatomic sites of the head and neck.

We found no correlation between the presence of HPV DNA in tonsillar tissue and seropositivity to the HPV-16 L1. This may be because these tumor-free tonsils harbored too few copies of viral DNA to induce an antibody response (1, 18) or the infection was transient.

In conclusion, we found only HPV-16 DNA, but not other types, in tumor-free tonsillar tissue, and this did not correlate with seropositivity against HPV proteins. A decrease in HPV DNA in tumor-free tonsillar tissue was evident with advancing age. Brushed exfoliated cells from normal tonsils seldom revealed HPV DNA.

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