

Human Papillomavirus Frequency in Oral and Oropharyngeal Cancer in Greece

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Abstract. *The presence of human papillomavirus (HPV) was successfully analyzed by both general and type-specific HPV PCR in 103 samples from 115 patients diagnosed with oral and oropharyngeal cancer in Greece during the years 1986-2007. Results: In total 13/103 (13%) tumours were HPV-positive and the majority of these were HPV-16-positive. Of the tonsillar cancer samples, 12/28 (43%) were HPV-positive and, notably, 1/6 (17%) collected between 1992-1998 and 11/22 (50%) collected between 2000-2007 were HPV-positive. Of the tongue cancer samples, 1/38 (3%) were HPV-positive, while none of the 41 oral cavity cancer samples was HPV-positive. Conclusion: Almost half of all the Greek tonsillar cancer patients had HPV in their tumours, with HPV-16 as the dominant type, and a tendency towards an increase in the proportion of HPV tumours was observed when comparing the percentage of HPV-positive tumours collected between 1992-1998 with those collected between 2000-2007.*

Worldwide, cancer in the oral cavity and pharynx is approximately the 12th most common cancer (1). However, the frequency of these malignancies varies considerably

between different countries and different geographic regions (2). Oral and pharyngeal cancer is a major cause of morbidity, with a yearly incidence worldwide of roughly 300,000. Major risk factors for developing oral and pharyngeal carcinomas are tobacco usage and alcohol consumption (3). However, during the past two decades, studies have accumulated showing that high-risk types of human papillomavirus (HPV) may also be a risk factor for the development of head and neck cancer (3-6). Furthermore, it has been reported that patients with HPV-positive head and neck cancer have a better prognosis than those with HPV-negative head and neck cancer (7, 8) and this has also been shown to be the case in tonsillar squamous cell carcinoma (4). Notably, HPV-positive tumours occur more often in younger patients without the classical risk factors, and sexual behaviour has been suggested to be a risk factor in this patient group (9). In concordance, in a recent study of tonsillar cancer incidence during the past 32 years in the Stockholm area (Sweden), we reported that the median age was 10 years less for patients with HPV-positive tonsillar cancer compared to that of patients with HPV-negative tonsillar cancer. It was also shown in the same study that between 1970 and 2002 there was a parallel 3-fold increase in the incidence of tonsillar cancer, as well as of the proportion of HPV-positive tonsillar cancer (from 23% in the 1970s to 68% during 2000-2002). The increase of tonsillar cancer was observed despite a decrease in smoking incidence in Sweden (10). These findings suggest that HPV may in fact be an important causative agent for a substantial proportion of tonsillar cancers (10, 11). The aim of the present study was to examine the presence of HPV in samples, including cancer of the tongue, the oral cavity and tonsillar cancer, collected from Greece, a country which has very high adult tobacco usage (12).

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Patients and Methods

Patient samples. In this study, 115 paraffin-embedded tumour samples from Metaxa Cancer Hospital, Piraeus, Greece, collected from patients diagnosed with oral and oropharyngeal cancer between the 1986 and 2007 were included. This research was conducted according to ethical permissions 1455/23-01-07 and 129/07/8-2-2007 from Metaxa Cancer Hospital and 2005/431-31/4 from Karolinska Institutet. Thirty-one patients had been diagnosed with tonsillar cancer (mean age 62 years, median age 63 years and range 42-82), 38 patients with tongue cancer (mean age 64 years, median age 67 years and range 28-91) and 46 patients with oral cavity cancer, excluding tongue cancer, (mean age 61 years, median 63 years and range 36-86).

Detection of HPV DNA by PCR with general primers. The DNA was extracted according to a standard phenol extraction protocol, or according to the manufacturer's instruction for the High Pure RNA Paraffin Kit (Roche Molecular Biochemicals, Mannheim, Germany) with exclusion of the DNase treatment as described previously by Hammarstedt *et al.* (10). To verify the presence of PCR amplifiable DNA (13), an S14 PCR was run, which amplifies the human ribosomal S14 gene (14). A combination of HPV consensus primers, GP5+/6+ and CPI/CPIIG, was used for the detection of HPV DNA in general, in order to minimize false-negative samples due to deletions and mutations in the viral genome coding for the E1 or L1 proteins. HPV plasmids (HPV-6, -11, -16 and -33) were used as positive controls. The GP5+/6+ protocol recognizes at least 27 HPV types and the primers are located in the conserved L1 region (15). Negative controls without any DNA were also set up to detect DNA contamination. The PCR mix consisted of 100-200 ng sample DNA, 10xPCR buffer (5 µl) (Applied Biosystems, Foster City, CA, USA), dNTP (1.25 mM/dNTP), MgCl₂ (25 mM), primers (10 pmol each), BSA (10 µg/µl) and 5 U/µl Taq-polymerase (AmpliTaq DNA polymerase, Applied Biosystems) for each sample. A 40-cycle amplification was run in a thermo-cycler (PerkinElmer, Norwalk, CT, USA). The PCR program started with a denaturation (4 min), then 40 cycles with denaturation at 94°C (1 min) followed by primer binding at 44°C (1 min) and then Taq polymerase activity at 71°C (2 min). The amplified products were then detected on a 2.5-3.0% agarose gel and the samples were visualized with UV light. Products with a band at 130-150 bp were considered as positive. The CPI/IIG protocol amplifies a 188 bp DNA fragment with primers located in the E1 open-reading frame and recognizes at least 29 different HPV types (16). The PCR procedure was carried out as described above.

HPV-16 type-specific PCR. To determine the proportion of HPV-16 positive tonsillar tumours an HPV-16 type-specific PCR with primers specific for HPV 16 (17) was set up. This PCR was used to type all the HPV-positive tumours and also run in all the HPV-negative tonsillar cancer samples. The HPV-16 type specific PCR-mix consisted of 100-200 ng sample DNA, 10x PCR buffer, 1.25 mM/dNTP, 25 mM MgCl₂, 10 pmol/µl of each primer and Taq Polymerase Gold (5 U/µl). The PCR program was run in a thermo-cycler (PerkinElmer) with denaturation at 95°C (4 min) and then forty-cycle amplification with 30 s denaturation (95°C), 30 s annealing (55°C) and finally 60 s elongation (72°C). The PCR products were treated as described above.

Statistical analysis. Fisher's exact test (two-tailed) was used for comparison of HPV-positive tonsillar cancer incidence 1992-1998 and 2000-2007.

Table I. Presence of HPV DNA in pretreatment tonsillar, tongue and oral cavity squamous cell carcinoma biopsies.

Cancer origin	Number of cases	Excluded ¹	HPV DNA -positive ²	HPV-16 -positive ³
Tonsil	31	3	12 (43%)	9 (75%)
Tongue	38	7	1 (3%)	0
Oral cavity	46	2	0	0
Total	115	12	13 (13%)	9 (69%)

¹Blocks excluded due to unamplifiable DNA, or for technical reasons.

²(%) denotes the fraction of HPV-positive samples of the non-excluded cases in the specific category.

³(%) denotes the fraction of HPV-16-positive cases of HPV-positive samples in each specific category.

Table II. Presence of HPV DNA in pretreatment tonsillar squamous cell carcinoma biopsies obtained between 1992-2007.

Calendar years	HPV DNA-positive
1992-1998	1/6 (17%)
2000-2007	11/22 (50%)

Results

The presence of HPV DNA was investigated in 115 pretreatment tumour biopsies and twelve samples were excluded from further analysis, nine due to undetectable DNA and three due to S14 contamination. The overall prevalence of HPV DNA detected in the samples was 13% (13 out of 103; Table I). Out of the 13 HPV-positive tumours, 12 were detected with the general GP5+/6+ primers and one additional tumour was shown to be positive to HPV DNA by HPV-16 type-specific PCR, while no extra HPV-positive tumours were found when using the CpI/CpII primers. HPV was detected in 12/28 (43%) of the tonsillar carcinomas and 1/31 (3%) of the tongue carcinomas. None of the other oral cavity samples was HPV-positive. The high-risk HPV-16 type was predominantly represented in the HPV-positive cases, 9/13 (69%), and more specifically in 9/12 (75%) of the HPV-positive tonsillar cancer cases. In the tonsillar cancer samples, HPV was more commonly found in the samples from patients (11/22; 50%) diagnosed 2000-2007 as compared to samples taken from patients (1/6; 17%) diagnosed 1992-1998, however this difference was not significant (Table II).

The patients were between 28 and 91 years of age (median 64 years and average 62 years), however information about the patient's age was unavailable or unverified in 6 cases (4 tonsillar cancer patients and 2 tongue cancer patients). Examining the available data, in the tonsillar cancer group, the

Table III. Presence or absence of HPV DNA in evaluated tonsillar cancer samples distributed in patients by age group.

Age group (years)	Patients with HPV-positive tumours	Patients with HPV-negative tumours
40-49	2	1
50-59	2	5
60-69	5	4
70-79	2	3
80-89	0	1
Unknown	1	2

patients with HPV-positive cancer had an age range of 42-70 years (mean=60, median=63), while the patients with HPV-negative tumours had an age range of 45-82 years (mean=63, median=62; Table III). Although the patients with HPV-positive tumours were 1.6 years younger on average than the patients with HPV-negative tumours, there was no significant age difference between the two groups.

Discussion

In the tonsillar cancer samples examined, 43% of the tumours were HPV-positive, while only 3% of all the tongue cancer samples and none of the other oral cavity carcinomas were HPV-positive. The fact that HPV was most commonly observed in tonsillar cancer from Greece is similar to previous reports from other parts of Europe and the USA (3, 8). Moreover, 50% of the tonsillar cancer samples obtained during the time period 2000-2007 were shown to be HPV-positive as compared to only 17% HPV-positive tonsillar cancer samples obtained during the time period 1992-1998, possibly indicating that in Greece the proportion of HPV-positive tonsillar cancer had increased. However, the number of samples analyzed from the earlier period was too small for this increase to be statistically significant. The higher proportion (50%) of HPV-positive tonsillar cancer observed during the time period 2000-2007 in the Greek material was lower than that (68%) observed in the Stockholm area during a similar time period (2000-2002) (10). Nevertheless, in agreement with the observation in Sweden that the proportion of HPV-positive tonsillar cancer is increasing, the tendency seems to be the same in Greece, although as mentioned above the data should be considered with caution, since the number of Greek tonsillar samples was limited.

In addition, as reported by others, in the tonsillar carcinomas HPV-16 (75%) was also the dominant subtype in the present study (3, 10). However, in contrast to other reports (9, 10), the average and median age did not differ significantly between patients with HPV-positive and -negative tumours. This could, however, have been due to the limited number of patients (9, 10).

In summary, in agreement with other reports on oral and oropharyngeal cancer (3), HPV was most commonly found in the tonsillar cancer (43%) samples in the present study. Moreover, the incidence of HPV-positive cancer showed a tendency to increase from the period of 1992-1998 (17%) to the period of 2000-2007 (50%), which was similar to the observations in Sweden (10) and the USA (11).

In conclusion, HPV is an important risk factor for tonsillar cancer in Greece, and, possibly as elsewhere, the incidence of HPV-positive cancer in Greece is also increasing.

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