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Short communication

Both mucosal and cutaneous papillomaviruses are in the oral cavity but only alpha genus seems to be associated with cancer

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ABSTRACT

Background: Human papillomaviruses are associated with invasive cancers in the cervical, anogenital, and oropharyngeal areas. Persistent HPV infections, particularly with high-risk HPV such as HPV 16, are involved in the carcinogenesis of a subset of oropharyngeal cancers. The majority of published studies on HPV prevalence in these tumors concentrated on identifying high-risk mucosal types.
Objectives: To determine the HPV type specific prevalence in different samples collected from the oral cavity of three groups of patients: (A) healthy ($n = 25$); (B) non-malignant lesions ($n = 47$); and (C) cancers ($n = 78$).
Study design: To evaluate the prevalence of HPV genotypes in the oral cavity, samples were analyzed by PCR with: MY09/MY11 followed by GP5+/GP6+, CP65/CP70 followed by CP66/CP69, and FAP59/FAP64 primers. The presence of viral transcripts was ascertained by RT-PCR with specific primers for the E7 region.
Results: Mucosal HPV types were associated with the presence of cancers. This trend was statistically significant if the analysis was performed for HPV 16 ($p = 0.04$), which is the most prevalent type detected in oropharyngeal cancers. Conversely, cutaneous HPVs were associated with non-malignant lesions ($p = 0.007$). The multiple correspondence analysis confirmed these data. Viral transcripts of only mucosal HPVs were detected in non-malignant lesions and cancers.
Conclusions: Different types of HPVs infect the oral epithelium, but only the mucosal types, particularly HPV 16, are clearly associated with tumors. The discovery that cutaneous HPVs are associated with potential malignant oral disorders brings other data to understand the significance of their presence in the oral cavity.

1. Background

Human papillomaviruses (HPVs) are implicated in the etiology of invasive cancers in the cervical, anogenital, and oropharyngeal areas.[1,2] HPV types are clustered in different genera; in particular, alpha-papillomavirus (α-HPV) mostly isolated from genital lesions, also known as mucosal HPV, and beta- (β-HPV) or gamma-papillomavirus (γ-HPV) isolated from skin lesions, also called cutaneous HPV.[3,4] Persistent high-risk HPV infections, particularly by HPV 16, are associated with a subset of squamous cell carcinoma of the head and neck (HNSCC).[2,5,6] The majority of published studies on the prevalence of HPV DNA in HNSCC concentrated on identifying high-risk mucosal types.[5–7] Only few reports indicated the presence of cutaneous HPV in these cancers and recently, different types of cutaneous HPVs were detected in oral samples.[8,9]

2. Objectives

The present study aimed to determine the differences between the prevalence of mucosal or cutaneous HPV types in oral cavity specimens.

3. Study design

3.1. Patients, sample collection and nucleic acids extraction

This study was approved by the San Carlo Nancy Hospital IDI Ethics Committee and informed written consent was obtained for

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Table 1
Clinical/histological diagnosis and drinker/smoker status.

Groups	*n*	Gender		Age ± SD Median (range)	Drinkers (*n*)	Smokers (*n*)	Clinical/histological diagnosis (*n*)	Localization (*n*)
		Male	Female					
A: healthy patients	25	10	15	40.76 ± 6.50 43 (25–61)	Yes (12) No (9) na (4)	Yes (18) No (3) na (4)	Healthy	Oral cavity (25)
B: non-malignant lesions	47	23	24	53.27 ± 11.20 54 (23–82)	Yes (11) No (23) na (13)	Yes (16) No (18) na (13)	Leukoplakia (26) Papilloma (10) Erythroleukoplakia (5) Lichen (5) Verrucous papilloma (1)	Oral cavity (21) Oropharynx (26)
C: cancers	78	56	22	63.39 ± 12.71 66 (33–84)	Yes (45) No (33)	Yes (48) No (30)	SCC (75); In situ ca. (2) Verrucous ca. (1)	Oral cavity (60) Oropharynx (10) Tonsil (8)

na, not available.

all participants. Subjects were assigned to three patient groups. Group A ($n = 25$), subjects attending outpatients for dental disorders with normal oral epithelium; group B ($n = 47$), patients with leukoplakia, or erythroleukoplakia, or lichen, or papilloma, that can be considered as non-malignant lesions[10]; and group C ($n = 78$), patients with different oropharyngeal cancers. Oral rinse with 0.4% sucrose solution, and oral mouth swabs were collected from all patients in groups A and B whereas biopsies from neoplastic lesions were collected in group C. DNA and RNA from exfoliated cell pellets and fresh biopsies were extracted by QIAamp DNA Mini Kit and RNeasy Plus Mini Kit, respectively, according to the manufacturer instructions.

3.2. DNA PCR amplification

All the samples were prepared to obtain the same concentration of 200 ng of DNA in 5 μL. HPV DNA was detected by following primers: MY09/MY11 followed by GP5+/GP6+, for α-HPV; CP65/CP70 followed by CP66/CP69 for β–γ-HPV; and FAP59/FAP64 for α–β–γ-HPV.[11–14] Amplification of β-globin gene served as control for the input DNA. PCR was performed with Platinum TaqDNA polymerase (Invitrogen, Milan Italy) in the presence of 1.5 or 3 mM $MgCl_2$ for MY and FAP or GP and CP primers, respectively.

In each amplification positive controls consisted of DNA from HPV16 positive CaSki cell line and from clinical samples already tested positive for cutaneous HPV[15] whereas negative controls were samples with no DNA added. Typing of the HPV-positive samples was performed by automated sequencing (Biogen GeneLab, Italy) and sequence analyses by BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

3.3. Viral transcripts

Same amount of RNA was reverse transcribed using E7 antisense primers and Omniscript Reverse Transcriptase (Qiagen) for 1 h at 42 °C. All the assays were performed both with and without reverse transcriptase to exclude the presence of contaminating DNA. CaSki and SiHa cell lines or clinical samples already tested positive for specific cutaneous HPV were positive controls.[16,17]

The synthesized cDNA underwent PCR analysis with Platinum TaqDNA polymerase (Invitrogen, Milan, Italy) and specific primer for the E7 gene. The E7 primers utilized were already described[18–20] or designed by the Beacon Design program (BioRad, Milan, Italy).

3.4. Statistical analysis

Pearson Chi-square test, χ^2 test for trend and Fisher Exact tests were used to evaluate the prevalence of the different HPV types among the groups. All p values were reported as 2-sided and p values less than 0.05 denoted statistically significant association. Multiple correspondence analysis (MCA) was used to identify specific profiles and presented as a graphic representation of the statistical relationships between distinct features, whose position in the graphic is exclusively informative.[21] SPSS software (SPSS version 18.0, SPSS Inc., Chicago, IL, USA) and MedCalc1 (10.0.1) statistical programs were used for all analyses.

4. Results and discussion

Data regarding clinical/histological diagnoses and other patient features are reported in Table 1. The median age was 43, 54 and 66 years for groups A, B and C respectively. Smokers and drinkers were distributed in a similar manner among the groups. However healthy patients had a high proportion of drinkers and smokers but these differences are not statistically significant (p 0.48 and 0.22, respectively) if we consider the trend (linear associations among the variants). PCR analyses revealed the presence of α- and β-HPVs and the absence of γ-HPVs or other HPVs. Only slight differences in HPV prevalence with no statistical significance were demonstrated in males versus females. Oral rinses and washes gave same results in term of HPV prevalence with more than 90% concordance (data not shown). Similar results were already reported by Smith et al.[22] indicating the utility of oral exfoliated cells for HPV detection. In our comparison analyses oral wash results were utilized.

The distribution of the different types among the different patient groups is shown in Fig. 1. In group A, only 2 samples (8%) showed the presence of mucosal HPVs whereas 10 samples (25%) tested positive for cutaneous HPV. In group B 6 samples (12.7%) were positive for mucosal HPV and 24 samples (51%) tested positive for cutaneous types having the highest prevalence for HPV 20 (10 samples). In contrast, only 16 samples (20.5%) tested positive for the cutaneous HPV among the cancers samples (group C), where 17 samples (21.8%) tested positive for mucosal HPV having the highest prevalence for type 16 (12 samples). Multiple infections were detected in 7 samples (4,6%). Only one multiple infection showed simultaneous presence of mucosal and cutaneous HPV. Thus, a high number of cutaneous HPV circulates in the oral cavity representing about 66% of the total number of HPV types found. Bottalico et al.[9] reported that in the HIV-negative population β-HPV types were the most frequently detected and represented 64% of the total

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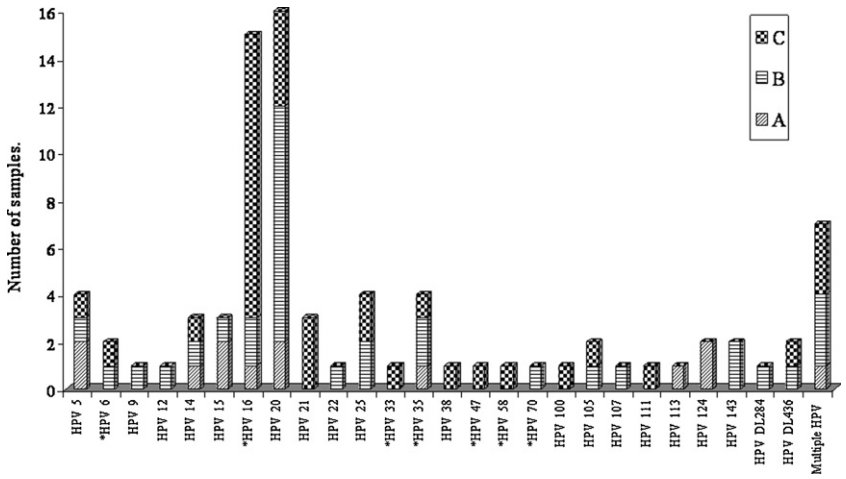

Fig. 1. Distribution of HPV types among oropharyngeal samples. The *y*-axis represents the number of positive samples for each type and the *x*-axis illustrates the different HPV types detected as described in the text. The column motives denote different patient's groups: (A) healthy patients; (B) non-malignant lesions; (C) cancers. Mucosal HPVs are marked with asterisk.

HPV types detected, followed by 28% of α-HPV types and 8% of γ-HPV types. In addition, Lindel et al.[23] demonstrated the presence of HPV DNA in 18 (35%) out of 51 tumors with high prevalence of cutaneous types. However, Koskinen et al.[24] failed to show any cutaneous HPV DNA in tumors from 61 HNSCC patients. In our study different general-primer-mediated nested PCRs were employed as in the Bottalico et al.[9] report. Moreover in a recent review there are indications that these methodologies are useful in detecting both cutaneous and mucosal HPV.[25] Koskinen et al.[24] did not employ nested PCR and this could explain the lack of cutaneous HPV detection. It is well known that β-HPVs are usually present in a very low copy-number and that it may be only detected by nested PCR.

Fig. 2 shows that mucosal types were associated with the presence of cancers with a significant trend reaching statistical significance only in cases where the HPV 16 was detected ($p = 0.04$). This result is consistent with many reports indicating that in H/N tumors, HPV 16 appears to be the most prevalent.[26,27] Conversely, cutaneous HPVs were associated with non-malignant lesions, having a statistical positive trend ($p = 0.007$) and a clear decrease in patients with neoplasia (group C). Associations between features are represented graphically in the MCA, that is a qualitative statistical method of analysis, exploring associations between categorical variables, and in our case we assumed an equal weight of all the variables.[21] The position of the different variables indicated that cancers were in the same space position as the presence of mucosal HPV, absence of cutaneous HPV, and localization in the tonsils. Vice-versa cutaneous HPVs were in the opposite space location together with the non-malignant lesions (Fig. 3). Thus, these data suggest that cutaneous and mucosal HPVs seem to be differently associated with H/N tumors. Literature data report a close association between the presence of viral transcripts and the biological activity/significance of HPV in H/N cancers.[20,28,29] RT-PCR with primers specific for the detected HPV types were performed according to well established procedures[20,25] in order to ascertain the presence of viral transcripts; an example of these analyses is reported in Fig. 4. Viral transcripts were detected in cancers and few

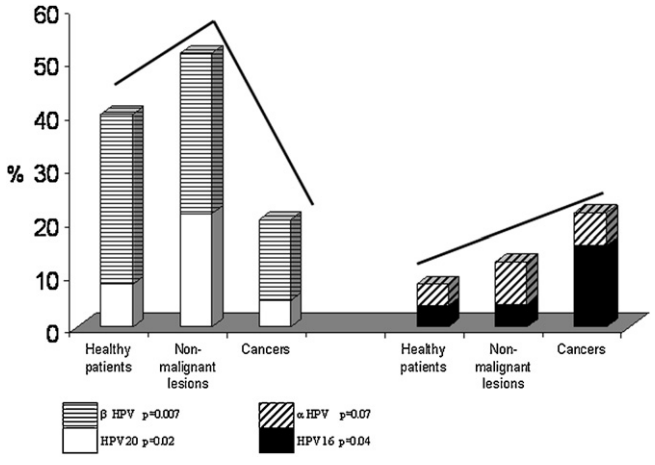

Fig. 2. HPV trends in H/N carcinogenesis. Sample analyses for the presence of HPV and the statistical calculation of *p* for the trends were performed as in methods. Data for the most frequent HPV types are also reported. The *y*-axis represents the percentage of positive samples. Lines over the bars represent a superimposed line graph with the same data to give a better graphic representation of the trend.

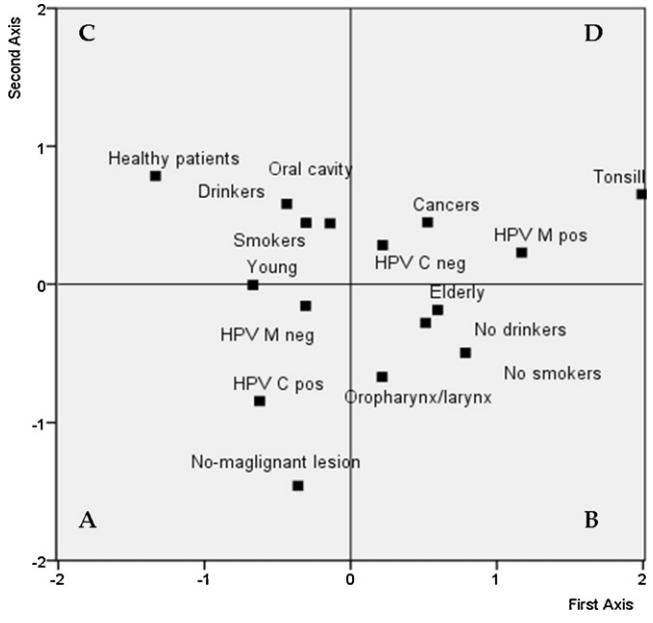

Fig. 3. Multiple correspondence analysis (MCA). MCA shows the association among the indicated variables distributed in 4 quadrants. Quadrant A indicates a correlation of cutaneous HPV with non-malignant lesions and quadrant D the association of cancers with the presence of mucosal HPV, in particular in the tonsils. Quadrants B and C include features that do not correlate well with tumor status. The numbers on the axes represent a score indicating the contribution of each feature to overall variability.

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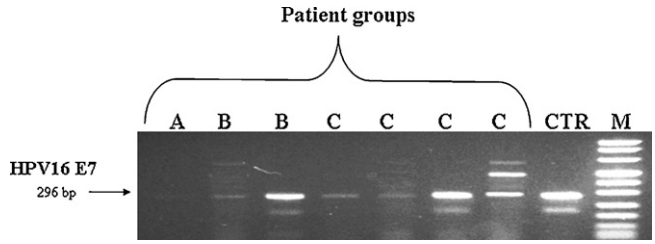

Fig. 4. Viral transcripts. Total RNA was extracted and c-DNA was retro-transcribed as described in methods. An example of an agarose gel of amplified products for HPV16 E7 region in different patient groups is showed. Positive control (CTR) was RNA extracted from HPV 16 positive CaSki cells. M, molecular weight marker VIII (Roche, Milan, Italy).

non-malignant lesions positive for mucosal HPV, whereas cutaneous viral transcripts were not found. Altogether data from the mRNA analysis suggest that not only the cutaneous and mucosal HPVs differ in trend toward carcinogenesis, but also their biological activity is detectable for mucosal viruses only, particularly HPV16. However lack of cutaneous HPV transcripts could reflect differences in the primer sensitivity rather than in the levels of viral transcription. This hypothesis seems unlikely as our primers for cutaneous HPV showed comparable sensitivity to the mucosal ones. Indeed they are able to detect viral transcripts in skin cancers [16,17] from starting quantity of RNA equal to that utilized to detect mucosal HPV in SiHa cells (data not shown).

However, the clear association between cutaneous types and non-malignant lesions, even in the absence of detectable levels of viral transcripts, gives rise to another fascinating hypothesis regarding cutaneous HPV viruses in the oral cavity.[9] Weissenborn et al. demonstrated that in skin precancerous lesions the viral load was much higher compared to the tumor tissue, suggesting a possible role in the early stage of transformation in association with co-carcinogens such as UV irradiation.[30] Here, the close association of cutaneous HPV with non-malignant lesions may indicate that this priming action toward tumors could take place in correlation with different carcinogens (i.e. chemical compound in smokers/drinkers patients). Only studies carried out on a larger number of patients will be able to bring conclusive responses to this hypothesis.

In conclusion, we demonstrated that mucosal and cutaneous HPVs may infect the epithelium of the upper aerodigestive tract, but only HPV 16 is clearly associated with cancers (especially those of tonsillar origin) as previously reported.[29,31] The discovery that cutaneous HPVs are associated with non-malignant lesions brings other data to understand the significance of their presence in the oral cavity.

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Competing interest

The authors declare that they have no conflict of interest.

Ethical approval

Ethical approval was given by committee of San Carlo Nancy Hospital IDI.

Judgement's ref. number was not released by the committee when the project started.

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