Viral load and genomic integration of Human Papillomavirus type 16 in oral pre-malignant and malignant disorders

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INTRODUCTION AND PURPOSE

Due to epidemiological and molecular data, high-risk (HR) Human Papillomaviruses (HPV) have been proposed to be etiological agents in malignant transformation of oropharyngeal squamous cell carcinoma (SCC). A continuous increase in HPV-associated oropharyngeal cancer has been observed during the past 20 years, in particular after 2000; HPV16 would be responsible for at least 90% of HPV-related SCC [1].

Oropharyngeal SCC most commonly arises from the lingual and palatine tonsils with a prevalence of HPV of 60–90% in recent studies. Differently, HPV prevalence is estimated to be of 20-30% in SCC of other sites (larynx, sinonasal tract and nasopharynx) and 6–20% in the oral cavity (lip, floor of the mouth, hard palate, gum, other oral cavity sites, anterior two/third of the tongue [2].

The prevalence of oral HPV infection has significantly increased in the last decade, raising public health concerns. However, most HPV infections are transient and do not lead to cancer, so that detection of HPV DNA in pre-neoplastic and neoplastic tissues does not qualify the tumour as being HPV-associated. Our recent study detected unexpectedly high rates of HPV infection in oral potentially malignant disorders (OPMD), with elevated HR HPV loads [3]. To distinguish HPV-driven carcinogenesis from transient infections, the use of other molecular markers would be helpful. It is well acknowledged that integration of HPV DNA into the host cell genome is a critical (but not essential) step in progression to cervical cancer [4]. Integration breakpoints often occur in the HPV early gene E2, which leads to enhanced E6 and E7 oncogene expression and increased cell proliferation. In oropharyngeal HPV-related SCCs, the viral genome seemed to be often, but not always, integrated into that of the host [5-6]. HPV integration status in oral cavity SCCs has been scarcely studied given the lower HPV prevalence.

To gain insight into this issue, we sought to determine DNA loads and viral integration status in HPV 16 positive lesions of the oral cavity, OPMDs and oral carcinomas.

METHODS

This study was conducted on 80 patients enrolled at the Odontostomatologic Clinics, Umberto I University hospital, Rome, during the period January 2013 to October 2015. All patients were informed about the procedure and signed an informed consent form. Patients had benign oral lesions (e.g. fibromas, oral traumatic ulcerative lesions), Papillomatosis, OPMD (e.g. leukoplakia, lichen planus) and oral carcinomas. Poor oral hygiene was considered an exclusion criteria. Patients were referred for biopsy on the basis of clinical suspicion, without knowing results of the HPV DNA test.

Oral cells were collected using a Cytobrush with a light pressure and rotating on lesions, to obtain a large number of transepithelial cells. The brushing samples were then suspended in 1 ml PBS and then centrifuged at low speed, within a few hours. The cell pellets then underwent total DNA extraction using a QIAamp Blood and Tissue kit (Qiagen) and the quality of extracted DNA was tested amplifying the housekeeping gene GAPDH by means of real-time PCR (rtPCR) fluorogenic assays. HPV type-specific primers and TaqMan probes targeting E6 region for the more common low-risk (HPV 6, 11) and HR (16, 18, 31, 33, 53, 58) HPV genotypes were used and copy numbers measured in samples by means of HPV plasmid external curves [3]. Total DNA concentration was calculated by OD260 reading and viral load was determined as HPV copy number per nanogram of total DNA in each sample [3].

HPV16 positive DNA were subjected also to a rtPCR targeting E2 region, using primers and probe validated for cervical samples [7]. The integration status was determined by measuring the E2 to E6 ratio. If only the episomal form were present, the E2/E6 ratios would be close to 1, and if only the integrated form were present, the E2/E6 ratios would be close to zero; if the integrated form and episomal form were mixed, the E2/E6 ratios would be intermediate [7]. We considered samples with E2/E6 ratios below 0.5 to be integrated, at least partially.

The chi-square test (2-sided) was used in the statistical analysis of different groups. The non-parametric tests, Mann-Whitney U for pairwise comparisons, and Kruskal-Wallis for more than two groups, were used for analyzing viral load values of HPV16 infections. Statistical tests were considered significant if the p value was 0.05 or less. Data analysis was carried out with SPSS v.17.0 for Windows.

RESULTS

HPV detection

A total of 80 oral samples were obtained from the Odontostomatologic Clinics: in Table 1, demographic and clinical data are reported, stratified according to HPV results. Out of forty HPV positive cases, HPV16 resulted to be the most frequent genotype (20 cases, one in coinfections with HPV 31).

	HPV-positive	HPV-negative
Mean age, years (range)	54.8 (18-85)	56.8 (21-82)
Gender (Male)	22/40 (55%)	16/40 (40%)
HR HPV detected (%)[c]	34/40 (85%)	NA
LESION	HPV-positive	HPV-negative
Papillomatosis	16/28 (57.1%)	12/28 (42.9%)
Other oral lesions	7/18 (38.9%)	11/18 (61.1%)
Leukoplakia	3/10 (30%)	7/10 (70%)
Lichen planus	10/14 (71.4%)	4/14 (28.6%).
Oral carcinoma	4/10 (40%)	6/10 (60%)

Viral load in HPV 16-positive samples

Median values of HPV16 DNA loads, reported in table 2, do not significantly differ among different group of lesions, with a tendency of oral carcinomas to have lower viral loads (Kruskal-Wallis test; p=0.208).

LESION	Median values (range) of HPV16 load (copies/ng total DNA)
Non-OPMD lesions	$1.2x10^5$ ($1.2x10^3$-$1.3x10^6$)
Leukoplakia	$6.5x10^4$ ($2.7x10^3$-$1.9x10^5$)
Lichen planus	$4.5x10^4$ ($4.3x10^3$-$1.5x10^5$)
Oral carcinoma	$3x10^3$ ($7.3x10^1$-$1.1x10^4$)

Viral integration and biopsy results in HPV 16-positive samples

HPV 16 DNA resulted integrated in the cell genome at least partially (E2/E6 ratio < 0.5) in 8/21 cases, with a tendency (Mann-Whitney test; p=0.183) of integrated samples to have less HPV E6 DNA copies with respect to the non-integrated.

Papillomatosis: Among the HPV positive lesions, six were HPV 16 of which only one had integrated viral copies. Of 18 patients that underwent biopsy, all nine HPV-positive lesions were reported as dysplastic keratosis or squamocellular papillomatosis that could be considered premalignant lesions; in particular, the lesion harboring integrated HPV-16 was a squamocellular Papillomatosis. Differently, among the nine HPV-negative lesions, four had a squamocellular papillomatosis report whereas the others had different reports (not dysplastic keratosis, inflammatory keratosis) that are not considered premalignant lesions.

Other oral lesions (i.e. traumatic or non-traumatic ulcers, fibromas, a burning sensation of the oral mucosa): Among the HPV positive lesions, two were LR HPVs, four were HPV 16-positive of which three (75%) had integrated HPV 16 copies. Of these group of lesions, six traumatic and non-traumatic ulcers (all HPV-negative) were referred for biopsy: their reports were suggestive of inflammatory or non- inflammatory keratosis.

Leukoplachia: Among the three HPV positive, two were HPV 16 in a not-integrated form. Of these lesions, six (all HPV-negative) were referred for biopsy: one had report of dysplastic keratosis whereas the others were not considered premalignant lesions.

Lichen planus: Among ten HPV positive, six were HPV 16 of which only one (an Atrophic-erosive Lichen not referred for biopsy) had integrated HPV 16 copies. Seven lesions (three were HPV-positive) were referred for biopsy and their reports were suggestive of inflammatory or non- inflammatory keratosis.

Carcinoma: 10 cases of which 4 were HPV positive (40%). Among them, two were positive to HPV 16 in single infection and one HPV 16 in co-infection with HPV 31; the fourth case was a co-infection of HPV 33 and 58. Interestingly, all three HPV16-positive carcinoma cases had integrated viral genomes. Biopsy referral were available of eight (four HPV-negative): seven were confirmed malignant lesions.

CONCLUSIONS

Our previous study suggested that HPV at elevated viral loads could be associated with an increased risk of viral persistence, a key factor in carcinogenesis [3]. However, viral load measured at a single time-point is a poor predictor of the HPV infection risk to develop cancer; therefore, additional risk markers are needed for screening purposes. In this preliminary report of an ongoing study, we determined HPV16 integration status in relation to viral loads and clinical diagnosis.

In this study group, many papillomatosis should be considered at potential risk of transformation, considering both HPV16 integration and biopsy results of squamocellular papillomatosis.

Of note is the rate of HPV16 integration in non-OPMD lesions (3/4; 75%); two of them were non-traumatic ulcers and the third was a rounded lesion considered benign. These three lesions did not raise a clinical suspicious for transforming potential and were not referred for biopsy.

HPV 16 DNA integration was far less frequent (1/6 cases; 16.7%) in HPV 16-positive lichen planus, despite the overall high HPV positivity. Lichen planus is a chronic inflammatory autoimmune disease that is considered an OPMD with a low rate of transformation [8]. It's possible that the frequent ulceration makes this lesions susceptible to HPV infection but its contribution to the risk of oral cancer is still uncertain.

In all three carcinomas of the oral cavity positive to HPV 16, viral DNA was integrated, at least partially, in the cell genome thus suggesting a role in oral cancer development. A recent paper examined HPV integration status in 11 oral cavity SCCs: 7/8 HPV 16-positive SCC tissue had viral DNA integrated with different viral and cellular breakpoints [6].

Overall, from these preliminary results, HPV 16 genome integration appears to be a relatively frequent occurrence in oral lesions in some cases without a proliferative appearance. Prospective studies are needed to establish whether HPV integration may represent a useful marker to identify lesions to be taken for histopathological diagnosis.

References

1. Dalianis T. Human papillomavirus and oropharyngeal cancer, the epidemics, and significance of additional clinical biomarkers for prediction of response to therapy. Int J Oncol 2014;44:1799-805.

2. Gillison ML, Alemany L, Snijders PJ, Chaturvedi A, Steinberg BM, Schwartz S, Castellsagué X. Human papillomavirus and diseases of the upper airway: head and neck cancer and respiratory papillomatosis. Vaccine. 2012;30 Suppl 5:F34-54.

3. Pierangeli A, Cannella F, Scagnolari C, Gentile M, Sciandra I, Antonelli G, Ciolfi C, Russo C, Palaia G, Romeo U, Polimeni A. Frequent detection of high human papillomavirus DNA loads in oral potentially malignant disorders. Clin Microbiol Infect. 2016;22:95.e9-95.e15.

4. Wentzensen N, Vinokurova S, von Knebel Doeberitz M (2004) Systematic review of genomic integration sites of human papillomavirus genomes in epithelial dysplasia and invasive cancer of the female lower genital tract. Cancer Res 64:3878–3884

5. Lace MJ, Anson JR, Klussmann JP, Wang DH, Smith EM, Haugen TH, Turek LP. Human papillomavirus type 16 (HPV-16) genomes integrated in head and neck cancers and in HPV-16-immortalized human keratinocyte clones express chimeric virus-cell mRNAs similar to those found in cervical cancers. J Virol. 2011;85:1645-54.

6. Parfenov M, Sekhar Pedamallu C, Gehlenborg N, Freeman S, et al Characterization of HPV and host genome interactions in primary head and neck cancers PNAS 2014;111:15544-15549.

7. Fujii T, Masumoto N, Saito M, Hirao N, Niimi S, Mukai M, Ono A, Hayashi S, Kubushiro K, Sakai E, Tsukazaki K, Nozawa S. Comparison between in situ hybridization and real-time PCR technique as a means of detecting the integrated form of human papillomavirus 16 in cervical neoplasia. Diagn Mol Pathol. 2005;14:103-8.

8. Fitzpatrick SG, Hirsch SA, Gordon SC. The malignant transformation of oral lichen planus and oral lichenoid lesions: a systematic review. J Am Dent Assoc 2014;145:45-56.