Acta Odontologica Scandinavica, 2011; 69: 406–409

informa
healthcare

ORIGINAL ARTICLE

Low prevalence of high risk genotypes of human papilloma virus in normal oral mucosa, oral leukoplakia and verrucous carcinoma

NASROLLAH SAGHRAVANIAN[1], KIARASH GHAZVINI[2], SHAHAB BABAKOOHI[3], ALIREZA FIROOZ[3] & NOOSHIN MOHTASHAM[1]

[1]*Dental Research Center, Department of Oral & Maxillofacial Pathology, Faculty of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran,* [2]*Department of Microbiology and Virology, Quaem Hospital, Mashhad University of Medical Sciences, Mashhad, Iran, and* [3]*Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences, Tehran, Iran*

Abstract

Objective. Squamous Cell Carcinoma (SCC) is the most common malignancy of the oral cavity and Verrucous Carcinoma (VC) is its least invasive form with no metastasis potential. Leukoplakia (clinical term for hyperkeratosis and acanthosis with and without dysplasia) is the most common pre-malignant lesion of the oral cavity. Human Papilloma Virus (HPV) has been recognized as an etiologic factor for mentioned lesions. In this study the relationship between high risk types of this virus (HPV_{HR}) with VC and Leukoplakia is investigated. ***Materials and method.*** Forty-one paraffin-embedded blocks including 21 VC, 20 leukoplakias in addition to 18 normal oral mucosal tissues as the control group were studied by Polymerase Chain Reaction (PCR) to detect HPV 16, 18, 31 and 33. ***Results.*** In three specimens of VCs (14.3%) HPV_{HR} was detected. Each of these contained both HPV 16 and 18. All three positive specimens belonged to women older than 65 and were obtained from the vestibule of the mandible. Neither Leukoplakia nor normal mucosa showed HPV presence. No significant relationship between HPV and VC was found compared with the control group ($p = 0.23$). ***Conclusion.*** This study did not show relationship between HPV and leukoplakia. Although no statistically significant relationship was found between HPV and VC, but considering sample size limitation for such uncommon cases, its presence in three cases of 14 makes more investigations to overcome the controversies necessary.

Key Words: *human papilloma virus, oral leukoplakia, oral squamous cell carcinoma, verrucous carcinoma*

Introduction

Oral carcinoma including lip cancer is the 10th common cancer in men and the 13th in women [1], although it is the most common cancer in some countries. In the literature the oral carcinoma is frequently considered equivalent to squamous cell carcinoma (SCC) because the latter encompasses 90–95% of cases [2]. Oral carcinoma leads to 9000 cases of death each year in the US [3] and ~ 300,000 new cases are diagnosed in the world annually [4].

Leukoplakia is circumscribed thickening of mucosa by white patches or an irregularly growing, well-defined warty plaque. Verrucous carcinoma (VC) is a non-metastasizing variant of well-differentiated SCC characterized by an exophitic, warty, slowly growing neoplasm with pushing margins [5]. It was first described by Ackerman in 1948 [6] and is considered as the least aggressive form of SCC. Although it usually involves mucous membranes of head and neck including the oral cavity, but it can arise in other cutaneous surfaces like external genitals, anorectal region and skin of the extremities, in particular the sole of the foot [7,8].

VC comprises ~ 3% of oral SCCs with preponderance in men over 60 [9]. This lesion may be observed beside the leukoplakia, which is the most common pre-malignant oral lesion [10]. Proliferative Verrucous Leukoplakia (PVL), a specific form of leukoplakia with the highest potency for malignant transformation, should be differentiated from VC. PVL is a chronic progressive lesion which first develops as a white plaque of hyperkeratosis and finally becomes

Correspondence: Nooshin Mohtasham, Associate Professor, Dental Research Center, Department of Oral & Maxillofacial Pathology, Faculty of Dentistry, Mashhad University of Medical Sciences, Vakil-Abad Boulvard, Mashhad, Iran. Tel: +982188963804. Fax: +982188206570. E-mail: shahabmdt@gmail.com

(Received 20 July 2010; revised 20 January 2011; accepted 23 February 2011)

ISSN 0001-6357 print/ISSN 1502-3850 online
DOI: 10.3109/00016357.2011.572560

multi-focal with confluent, exophytic and proliferative features [11]. Also, regarding histopathological similarities among leukoplakia, VC and some forms of papillary SCCs, one must scrutinize sufficiently in diagnosis to differentiate each of these entities from the others because each carries a different prognosis [10,11].

A variety of factors can provoke development of aforementioned lesions, from which the Human Papilloma Virus (HPV) has attracted great attention [12]. To now, ~ 130 genotypes of HPV have been recognized and categorized as High Risk (HR) or Low Risk (LR) according to their potency to induce malignant transformation [13,14]. This double-stranded DNA virus belongs to the papillomaviridae family and, in spite of vast studies, its clear-cut association with oral cancers has not been elucidated yet as certain, as with cervix and anogenital malignancies [14]. Over-expression of E6 and E7 proteins of HPV, particularly in HR types, provokes binding of these proteins to tumour suppressor proteins, P53 and Rb (retinoblastoma), and consequently a disruption in normal cell cycle leading to creation of transformed immortal cells [15]. Up to 81% of HR genotypes have been detected in normal oral mucosa [14,16]. The investigations in association between HPV and head and neck cancers were commenced in 1960s and Syrjänen [17] in 1983 showed the HPV involvement in oral SCC. However, the subsequent studies reported that association controversially ranged from 0–100% [15]. Some authors suppose HPV as the major risk factor [16], whereas others maintain no role for it [18,19].

Different techniques are used for detection of virus genome such as Polymerase Chain Reaction (PCR), In Situ Hybridization (ISH) and Southern Blot (SB), among which the most sensitive and accepted test is PCR [14,20]. Considering the possibility of the presence of LR types of HPV in oral mucosa for which no significant role in malignant transformation is considered [19,21], in this study we investigated the presence of DNA of HR genotypes of HPV consisting of genotypes 16, 18, 31 and 33 in normal oral mucosa as a control group, leukoplakia as the most common pre-malignant lesion and verrucous carcinoma (VC) as the most indolent form of SCC with PCR. The detection of HPV genomes in pre-malignant lesions of oral cavity is a basic practical study which may have an important role in finding etiology of the disease and development of future treatments.

Materials and methods

In this case-control study, the paraffin-embedded blocks of all the patients referred to a teaching hospital with diagnosis of leukoplakia and verrucous carcinoma (VC) of oral cavity form 2002–2006 were collected from the pathology department and were re-examined by two pathologists who were unaware of the previous diagnoses. After diagnosis confirmation, 21, 20 and 18 specimens of VC, leukoplakia and normal oral mucosa were included, respectively. The VC group included 10 men (age = 59.6 ± 11) and 11 women (age = 59 ± 11.3). The leukoplakia patients were 15 men (age = 61.5 ± 15.5) and five women (age = 62.8 ± 3.7). The control group consisted of seven men (age = 59.6 ± 12.2) and 11 women (age = 55.5 ± 13.6). Ethically, in order not to take biopsy from healthy individuals, samples for the control group were taken from non-neoplastic and reactive oral lesions which possessed normal epithelium like fibrotic lesions and mucoceles. For PCR, each paraffin-embedded block was cut under the hood and resulting thin slices were put in the Ependorf microtubes. The sections were deparaffinized by xylol and, for complete tissue digestion, the Proteinase K buffer solution and Proteinase K enzyme (20 mM) were used. The DNA was extracted by Phenol Chloroform method in which the DNA is precipitated by an isoamyl alcohol. After extracting DNA strands the temperature was increased to 95°C. Gentle centrifugation was carried out in order to eliminate the existing Proteinase K enzyme. The precipitated DNA was lyophilized and solved in the TE (Tris, EDTA) solution and was stored at 4°C for the next steps of PCR. For obtaining more purified DNA, the saturated phenol method (Phenol/Chloroform ratio = 50/50) and Chloroform isoamyl alcohol with a 24/1 ratio were also used. The conventional PCR with one pair of primers was used. To pre-screen the samples for the presence of HPV, the PCR was performed in the extracted DNA with combination primers of GH2/PCO4 and PCO3/PCO4 for β-globin gene and the samples which were amplified by at least one of these primers were selected and confirmation of HPV presence was done by HPV primers (GP5+/GP6+: forward: 5'-TTTGTTACTGTGGTAGATACTAC-3' and reverse: 5' GAAAAATAAACTGTAAATCATATTC-3') which amplify conserved sequences in the HPV-L1 region [22,23].

Validity was confirmed based on molecular weight on the beginning of PCR. For typing, specific primers which target E6/E7 segments were used to detection of HPV 16, 18, 31 and 33 [24]. Final volume of 50 μl were put in 0.2 ml microtubes containing 200–700 ng of DNA, 20 mM Tris-HCl, pH 8.4, $MgCl_2$ (2.5 mM), KCl (50 mM), 1U Amplitaq Gold DNA polymerase, uracil-N-glycosilase, 200 μM deoxynucleotides (dNTPs) and biotinylated GP5+/GP6+ and β-globin primers. Hella cells DNA was used as a positive control and distilled water for negative control. Finally the electrophoresis was carried out for the solution in Agarose gel 1.5% and the product was dyed with ethidium bromide.

Results

The most common sites for VC were mandible vestibule, lower lip, buccal mucosa and gum in order of frequency and for leukoplakia were buccal mucosa, tongue, upper lip and floor of the mouth.

Only three specimens were positive (Figure 1) for HPV DNA (5%), all of which were in the VC group (3/21, 14.3%). No HPV was detected among leukoplakias or control group (Table I). No statistically significant difference was seen in HPV expression in three studied groups ($p = 0.1$). All the three HPV positive VCs contained both genotypes 16 and 18 and were obtained from the mandible vestibule from women older than 65. According to Fisher exact test, no statistically significant relationship existed between gender and age with HPV expression ($p = 0.138$ and $p = 0.124$, respectively).

Discussion

Leukoplakia as the most common oral pre-malignant lesion has clinical and histopathological similarities with VC. Literatures have pointed to a myriad of provokers contributing to the emergence of VC and leukoplakia [21,25,26] among which the epitheliotrohic virus, HPV, has been focused frequently. To now more than 130 genotypes of HPV have been discovered, a narrow proportion of which (Genotypes 1, 2, 4, 6, 7, 11, 12, 16, 18, 30, 32 and57) are present in the oral cavity [14,16]. Regarding carcinogenicity, HPVs are divided into Low Risk ones (HPV_{LR}: 6, 11, 13, 30, 34, 40, 42–44, 53 and55) and High Risk ones (HPV_{HR}: 16, 18, 31, 33, 39, 45, 51, 52, 54 and 56) [27].

HPV contribution and the prognostic role in anogenital cancers have been proved but, despite nearly three decades of study, such a contribution in oral cavity malignancies has been an issue of debate [15,28]. Conducted meta analytic studies have expressed the association of HPV and oral SCCs

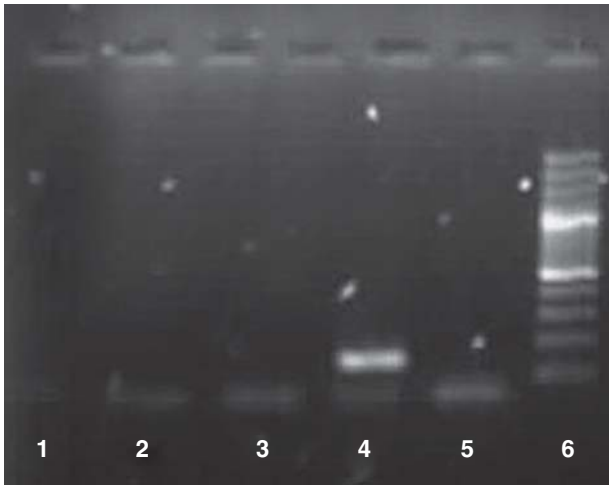

Figure 1. Results of polymerase chain reaction (PCR). Lanes showing products of PCR. Molecular size marker: Lane 6, negative: Lanes 1, 2, 3, 5; positive: Lane 4.

Table I. The prevalence of HPV infection in case and control groups according to sex.

	Control		Leukoplakia		Verrucous carcinoma	
Results	Female	Male	Female	Male	Female	Male
HPV positive	0	0	0	0	3 (both HPV 16 and 18)	0
HPV negative	10	8	5	14	10	8
Total	10	8	5	14	13	8

from 22–38% [20,29,30]. The genomes of HPV have been found interestingly in 1.5% [17] to 100% [14] of oral SCCs, a range with a controversial width. In a systematic review in head and neck SCCs, the association had been 25.9% [31].

Two similar descriptive studies in Iranian patients have been performed. In one of them HPV 16 was found in four specimens of 51 cases of oral SCC, whereas no HPV 18 was detected [32]. In another one, a total of 19 malignant and pre-malignant oral lesions, HPV 16 in five cases and HPV 33 in one case were detected with no finding of HPV 18 and 33 [33]. In the present study we investigated the likely association of HPV with VC, which is an indolent from of SCC and should be differentiated from papillary SCC on one hand and some type of leukoplakia in particular PVL on the other one. HPV and VC in this study were associated in 14.3% of cases, which is not statistically significant ($p = 0.23$).

Lubbe et al. [25] have also pointed to similar findings. Albeit, some other studies have shown stronger associations, in particular with genotypes 16 and 18 [12], which have a partial genotype similarity with our study (i.e. three HPV 16 cases). However, Fujita et al. [34] found no HPV 16 in VC.

The majority of studies in which the HPV had been associated with leukoplakia are those in which HPV had also been reported high in normal oral mucosa [14,21]. In contrast, Szarka et al. [35] did not find HPV in normal tissue as much as in leukoplakia. In our study, no association was found between leukoplakia and HPV, which is confirmed by some studies [17,36]. The controversy also extends to the normal oral mucosa. The prevalence of HPV in normal oral mucosa has been reported to be 1–60% [37]. In the present study we did not find any HPV_{HR} [17,19,32,34] in normal oral mucosa in contrast to some other studies which reported some HPV_{HR}s in normal oral mucosa in small [38] or larger [27] proportionates of samples.

Conclusion

Considering plenty of contributing factors in neoplastic transformation in the oral cavity, HPV has been focused vastly as a major culprit. The current study did not reveal a statistically significant relationship between this virus and neoplastic changes.

However, though no statistically significant relationship was found between HPV and VC, but considering sample size limitation for such uncommon cases, the presence of HPV_{HR} in three of 21 specimens and particularly co-existence of both HPV 16 and 18 genotypes necessitates more investigations about the role of HPV in promoting the oral lesions toward malignancy.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Bibliography

[1] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. GLOBOCAN 2008, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer; 2010. Available at: http://globocan.iarc.fr, accessed 23 March 2011.

[2] Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. Oral Oncol 2009;45:309-16.

[3] Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43-66.

[4] Rautava J, Luukkaa M, Heikinheimo K, et al. Squamous cell carcinomas arising from different types of oral epithelia differ in their tumor and patient characteristics and survival. Oral Oncol 2007;43:911-9.

[5] Barnes L, Eveson JW, Reichard P, et al. WHO classification of tumors. Pathology and genetics head and neck tumors. Lyon: IARC press; 2005. p 22–3.

[6] Ackerman LV. Verrucous carcinoma of the oral cavity. Surgery 1948;23:670-8.

[7] McCoy JM, Waldron CA. Verrucous carcinoma of the oral cavity. A review of forty-nine cases. Oral Surg Oral Med Oral Pathol 1981;52:623-9.

[8] Koch BB, Trask DK, Hoffman HT, et al. National survey of head and neck verrucous carcinoma: patterns of presentation, care, and outcome. Cancer 2001;92:110-20.

[9] Bouquot JE. Oral verrucous carcinoma. Incidence in two US populations. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998;86:318-24.

[10] Alkan A, Bulut E, Gunhan O, Ozden B. Oral verrucous carcinoma: a study of 12 cases. Eur J Dent 2010;4:202-7.

[11] Cerero-Lapiedra R, Baladé-Martínez D, Moreno-López LA, et al. Proliferative verrucous leukoplakia: a proposal for diagnostic criteria. Med Oral Patol Oral Cir Bucal 2010;15:839-45.

[12] Rsenquist K. Risk factors in oral and oropharyngeal squamous cell carcinoma: a population – based case –control study in southern Sweden. Swed Dent J Suppl 2005;1:1-66.

[13] zur Hausen H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst 2000;92:690-8.

[14] Koyama K, Uobe K, Tanaka A. Highly sensitive detection of HPV-DNA in paraffin section of human oral carcinomas. J Oral Pathol Med 2007;36:18-24.

[15] Campisi G, Panzarella V, Giuliani M, et al. Human papillomavirus: its identikit and controversial role in oral oncogenesis, premalignant and malignant lesion (Review). Int J Oncol 2007;30:813-23.

[16] Munger K. The role of human papillomaviruses in human cancers. Front Biosci 2002;1:641-49.

[17] Syrjänen KJ, Syrjänen SM, Lamberg MA, Pyrhönen S. Human papillomavirus (HPV) involvement in squamous cell lesions of the oral cavity. Proc Finn Dent Soc 1983;79:1-8.

[18] Khovidhunkit SO, Buajeeb W, Sanguansin S, et al. Detection of human papillomavirus in oral squamous cell carcinoma, leukoplakia and lichen planus in thai patients. Asian Pac J Cancer Prev 2008;9:771-5.

[19] Acay R, Rezende N, Fontes A, et al. Human papillomavirus as a risk factor in oral carcinogenesis: a study using *in situ* hybridization with signal amplification. Oral Microbio Immunol 2008;23:271-4.

[20] Termine N, Panzarella V, Falaschini S, et al. HPV in oral cell carcinoma vs head and neck squamous cell carcinoma biopsies: a meta-analysis (1988–2007). Ann Oncol 2008;19:1681-90.

[21] Liama-Martinz S, Esparza-Gomez G, Campo-Trapero J, et al. Genotypic determination by PCR-RELP of human papillomavirus in normal oral mucosa, oral leukoplakia and oral squamous cell carcinoma sample in Madrid (Spain). Anticancer Res 2008;28:3733-41.

[22] Ting Y, Manos M. Detection and typing of genital human papillomaviruses. Polymerase chain reaction protocols: a guide to methods and applications. San Diego: Academic Press; 1990. p 356–67.

[23] Snijders PJF, van den Brule AJ, Schrijnemakers HF, Snow G, Meijer CJ, Walboomers JM. The use of general primers in the PCR permits the detection of a broud spectrum of human papillomavirus genotypes. J Gen Virol 1990;71:173-81.

[24] Sotlar K, Diemer D, Dethleffs A, et al. Detection and typing of human papillomavirus by E6 nested multiplex PCR. J Clin Microbiol 2004;42:3176-84.

[25] Lubbe J, Kormann A, Adams V, et al. HPV-11 and HPV-16-associated oral verrucous carcinoma. Dermatology 1996;192: 217-21.

[26] Murao K, Kubo Y, Fukumoto D, et al. Verrucous carcinoma of the scalp associated with human papillomavirus Type 33. Dermatol Surg 2005;31:1363-5.

[27] Kay P, Meehan K, Williamson AL. the use of nested polymerase chain reaction and restriction fragment length polymorphism for the detection and typing of mucosal human papillomavirus in samples containing low copy number of viral DNA. J Virol Methods 2002;105:159-70.

[28] Shillitoe EJ. Papillomavirusesas targets for cancer gene therapy. Cancer Gene Ther 2006;13:445-50.

[29] Miller CS, Johnstone BM. Human papilomavirus as risk factor for oral squamous cell carcinoma: a meta-analysis, 1982–1997. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001;91:622-35.

[30] Syrjanen S. Human papilomavirus (HPV) in head and neck cancer. J Clin Virol 2005;32(Suppl 1):59-66.

[31] Kreimer AR, Clifford GM, Boyle P, Franceschi S. Human papilomavirus types in head and neck squamous cell carcinomas worldwide: a systemic review. Cancer Epidemiol Biomaekers Prev 2005;14:467-75.

[32] Sargolzaei S, Keykhaie MR, Moradi A, et al. The association of different genotypes of human papillomavirus with oral squamous cell carcinoma. J Dent School - Beheshti Univ Dent J 2005;23:438-48.

[33] Ghaemmaghami SA, Khalilazar L, Eslami B. The association of different genotypes of human papillomavirus with malignant and premalignant oral lesions. J Dent School - Beheshti Univ Dent J 2003;20:628-40.

[34] Fujita S, Sebna M, Kumatori A, et al. Human papillomavirus infection in oral verrucous carcinoma: genotyping analysis and inverse correlation with p53 expression. Pathobiology 2008;75: 257-64.

[35] Szarka K, Tar I, Feher E, et al. Progressive increase oh human papillomavirus carriage rates in potentially malignant and malignant oral disorders with increase malignant potential. Oral Microbiol Immunol 2009;24:314-8.

[36] Began JV, Jimenez Y, Murillo J, et al. Lack of association between proliferative verrucous leukoplakia and human papilomavirus infection. J Oral Maxillofac Surg 2007;65:46-9.

[37] McKaig RG, Baric RS, Olshan AF. Human papillomavirus and head and neck cancer: epidemiology and molecular biology. Head Neck 1998:20:250–65.

[38] Hansson BG, Rosenquist K, Antonson A, et al. Strong association between infection with human papillomavirus and oral and oropharyngeal squamous cell carcinoma: a population-based case-control study in southern Sweden. Acta Otolaryngol 2005;125:1337-44.