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DETECTION OF HUMAN PAPILLOMAVIRUS DNA IN BIOPSIES OF ORAL PAPILLOMAS

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Abstract:

Introduction: There are more than 120 genotypically different forms of human papillomaviruses (HPV), of which more than 40 HPV types can infect genital and oral mucosa in both males and females. While it is proven that HPV infections play a major role in the pathogenesis of head and neck squamous cell carcinomas, there is little information regarding the role of HPV in oral papillomas. The aim of this study was to highlight the importance of HPV testing in oral, oropharyngeal and laryngeal papilloma.

Materials and methods: From 2012 to April 2018, there have been 105 patients diagnosed with oral, oropharyngeal and laryngeal papilloma. After DNA isolation, PCR was performed for ß-globin gene to ensure quality of the tested DNA. The presence of HPV DNA was determined using general primers Gp5+/Gp6+. Amplification of HPV types 6/11, 16 and 18 was carried out using type-specific primers. All PCR products were subjected to electrophoresis and analyzed.

Results: HPV 6/11 was found in 20 (19%) patients. None of the samples was positive for HPV 16 nor HPV 18. Samples of 12 (11%) patients could not be genotyped.

Conclusion: Only HPV 6/11 was identified in tested biopsies. HPV testing should be recommended for all oral lesions.

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INTRODUCTION

Human papillomaviruses are a heterogeneous group of DNA viruses. Although similar in genomic organization, there are nearly 200 different strains of HPV, most of which are harmless, with more than 120 genotypically different forms, which share nearly 90% homology. All known types infect specific epithelia and often cause proliferation at the site of infection. The most important sub-groups are mucosal (genital) types, mostly infecting areas of the genital system and cutaneous types of HPV, which commonly infect the extra-genital sites.[1, 2]

From five major known HPV genera (α-, β-, γ-, mu- and nu-papillomavirus), the alpha type is clinically the most relevant with mucosal (genital) types as main representatives.[3] Mucosal HPV types have been well-studied, and subsequently categorized into low-risk, intermediate-risk and high-risk types, according to their potential for causing malignant proliferation.[4]

There are more than 40 HPV types that can infect genital and oral mucosa in both males and females. 2 The most common low-risk HPV (LR-HPV) genotypes are HPV 6 and HPV 11 and the most common high-risk HPV (HR-HPV) genotypes are HPV-16, 18, 31 and 33.[4]

Nearly 75% of sexually active men and women world-wide have been exposed to genital HPV at some point in their lives. The immune system naturally clears most HPV within 2 years, but the ones that persist can cause serious diseases.[2]

Numerous studies have proven that HPV infections play a major role in the pathogenesis of head and neck squamous cell carcinomas.[5] Meanwhile, the role of HPV in papillomas is still uncertain due to scarce information.[6] It was shown that head and neck cancers and papillomas tend to show a different HPV status.[7]
Also, it was shown that oral and oropharyngeal papillomas are not associated with HR-HPV, but what remains unclear are the roles of different HPV types in their development.[8]
Oral, oropharyngeal and laryngeal papillomas are benign exophytic papillary lesions in the oral cavity, oropharynx and larynx, whose development is associated with HPV infection. They can occur as single or multiple lesions with a smooth or verrucal surface whose color (white, pink or red) depends on the degree of keratinisation.[9] Oral squamous papillomas develop in every part of the oral mucosa but more frequently at the surface of the tongue, the soft palate and the lips.[10]
The aim of this study was to highlight the importance of HPV testing in oral, oropharyngeal and laryngeal papillomas well as to determine the prevalence of low (HPV-6 and -11) and high (HPV-16 and -18) risk HPV genotypes in biopsies of oral papillomas.

MATERIAL AND METHODS

HPV DNA testing on papillomas is routinely conducted at the Institute of Pathology, University of Zagreb School of Medicine, Croatia. In the period from 2012 to April 2018, 105 patients diagnosed with oral, oropharyngeal and laryngeal papillomas (40 male and 65 female) have been tested for HPV.

DNA isolation

DNA was isolated from formalin-fixed paraffin-embedded (FFPE) tissue sections (6x10 µm) according to the QiAmp DNA Mini Kit protocol (QIAGEN, Hilden, Germany). Briefly, paraffin was removed by xylene (1200 µL, centrifugation 14 000 g, 5 min) and washed two times with ethanol. Tissue sections were digested with proteinase K (100 µg/µL) at 56°C overnight. After adding ethanol, samples were transferred to filter columns, washed twice and purified DNA was separated from the membrane with 50 µL of elution buffer into sterile Eppendorf tubes. Extracted DNA was immediately stored at +4°C.

Polymerase chain reaction, PCR

All amplification reactions were carried out in the GeneAmp PCR System 9700 (PE Applied Biosystems), using a specific set of primers with AmpliTaq Gold DNA Polymerase (PE Applied Biosystems, Foster City, CA, USA), GeneAmp 10X PCR Gold Buffer and 25 mM MgCl2 (PE Applied Biosystems) with the addition of 10 mM dNTP mix.
Positive and negative controls were also amplified in every reaction. Positive controls were samples that were previously positive for a given reaction. The negative control in all reactions was distilled water.
Quality control of isolated DNA was performed by amplification of the ß-globin gene using the following primers: F 5´->3´ gaa gag cca agg aca ggt ac and R 5´->3´ caa ctt cat cca cgt tca cc. Amplification of the ß-globin gene was performed under following conditions: 94°C 5', 40 cycles of 95°C 30'', 55°C 30'' and 72°C 45'', followed by 72°C 7' and 4°C∞.
The presence of HPV DNA was determined by amplificating a part of the L1 region using general primers Gp5+/Gp6+: Gp5+ 5´->3' ttt gtt act gtg gta gat act ac and Gp6+ 5´->3´gaa aaa taa act gta aat cat att c. 11Amplification of Gp5+/6+ was carried out under the following conditions: 95°C 4', 40 cycles of 94°C 1', 40°C 2' and 72°C 1'30'', followed by 72°C 4' and 4°C ∞. The obtained amplicon was used to perform auto-nested PCR with Gp5+/6+ under the same conditions.
HPV 6 and HPV 11 were confirmed using a single set of primers HPV 6/11: F 5´->3' tac act gct gga caa cat gc and R 5´->3' aca tcc aca gca aca ggt ca.
The presence of HPV 18 was tested using the following set of primers: F 5´->3' cac gca cac gct tgg cag gt and R 5´->3' aag gat gct gca ccg gct ga.
Amplification of HPV 6/11 and HPV 18 was carried out under the same conditions: 94°C 5', 40 cycles of 95°C 30'', 58°C 30'' and 72°C 45'', followed by 72°C 7' and 4°C ∞.
Primers for amplification of HPV 16 were: F 5´->3' cgt gtt ctt gat gat ctg caa and R 5´->3' tca aaa gcc act gtg tcc tga. The conditions under which amplification of HPV 16 was performed were the same as for the ß-globin gene.

Electrophoresis and analysis

Aliquots (10 µl) of each PCR product were subjected to electrophoresis on 2% agarose gel stained with ethidium bromide and analyzed under UV light (expected fragment length was 265bp). Digital images were taken with Kodak DC290 Zoom Digital Camera, Kodak EDAS System (Kodak).

RESULTS

The gender distribution of a total of 105 patients included in the study revealed a slight female predominance, with a ratio 1.62:1. The mean age was 40.6 years (ranging from 5 to75 years).
Positive ß-globin amplification was confirmed in samples from all patients.
HPV DNA was not detected in 73 (70%) samples. Samples of 12 (11%) patients were positive only for

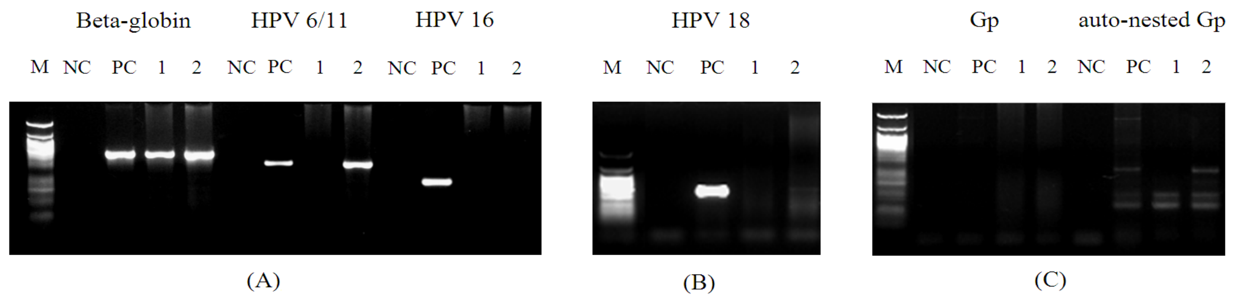

Figure 1. Agarose gel electrophoresis of PCR products after amplification of samples 1 & 2 with (A) primers for Beta-globin, HPV 6/11 and HPV 16; (B) with primers for HPV 18, and (C) with primers for Gp and products of subsequent auto-nested Gp amplification.

Gp5+/6+ thus declared as HPV X (unknown type of unknown risk).

HPV 6/11 was found in 20 (19%) patients, from which 8 (7%) were also Gp5+/6+ positive. Regarding the gender distribution, 11 men (28%) and 9 women (14%) were HPV 6/11 positive. None of the samples was positive for HPV 16 or HPV 18 (Figure 1).

DISCUSSION

There were 40 male and 65 female patients tested for HPV DNA. HPV 6/11 was found in 20 (19%) patients, with a higher incidence in men. Even though this result suggests possible gender-dependent distribution of HPV, further analyses should be done with an increased number of patients. None of the samples was positive for HR-HPV. Samples of 12 (11%) patients could not be genotyped.

Though several studies report that benign lesions were more common in males than in females,[12] this study shows opposite results. However, the higher incidence of HPV positivity in men revealed here is in accordance with observations of Frigerio and co-workers. Unfortunately, they have not done HPV typization to further fortify our results.

In our population, there was a lower percentage of confirmed low risk HPV types than in previous studies which found HPV 6/11 in 34 -56% of biopsies.[6, 8]

Because not all HPV6/11 positive samples were positive for Gp5+/6+, it is possible that the L1 region was lost due to integration of viral DNA.[12] As for the cases which were Gp5+/6+ positive, this could be a part of the same HPV type. The other possibility is that these are cases of multiple infections as described in laryngeal papilloma.[13] However, findings on multiple infections are ambiguous. While some studies state that no multiple infections were found in papillomas,[7] others declare infection with both 6/11 and 31/33/53 subtypes.[6] Also, there are studies that state that HR-HPV and LR-HPV infections seem to be mutually exclusive in papillomas of the oral cavity and oropharynx.[8] The percentage of only Gp5+/6+ positive samples in our study is not imperceptible, which raises questions whether these samples could be some other than tested LR- HPV types, or even some other HR-HPV.

A lot of studies found no high-risk types in papillomas,[7, 8] which we can only confirm for HPV 16 and HPV 18. Nevertheless, in previously mentioned studies,[6 14] some types of HR-HPV were found.

Non-typable samples are a result of limitations of the techniques used in this study, which are that only 4 of nearly 200 HPV types could be identified. This could probably be resolved by using other detection methods, which, although numerous,[15] are mostly designed to detect HPV from fresh, not FFPE samples. Those that are intended for FFPE samples are not economical for small groups of samples such as the one in this study.

The importance of HPV genotypic variants in this model is to differentiate LR and HR HPV patients, not so much to differentiate HPV genotypes per se. This could provide better treatment options and possibly better treatment outcomes.

Obtaining data on treatment outcomes could emphasize the importance of HPV DNA detection and reveal a possible correlation between recurrent papillomas and HPV. Additionally, results in published studies are diverse, which is probably a result of different techniques. As there are not so many studies researching oral HPV infections, and various different methods are used, little is known to date. Standardizing methods for HPV detection would ensure comparability between future oral HPV studies.[16]

In conclusion, no HR HPV types have been identified, while LR HPV was found in 19% of patients, with higher incidence in men. Further investigations are needed to identify the non-typable samples, as well as a higher sample pool to fortify obtained results. As the incidence of HPV-associated cancers of the oral cavity and oropharynx is increasing,[17, 18] HPV testing should be recommended for all oral lesions as well.

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