Pathology – Research and Practice 213 (2017) 759–765

Contents lists available at ScienceDirect

Pathology – Research and Practice

journal homepage: www.elsevier.com/locate/prp

Original article

Plasma HPV DNA is detectable in oral leukoplakia patients

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ARTICLE INFO

Article history:
Received 21 October 2016
Received in revised form 5 March 2017
Accepted 12 April 2017

Keywords:
Leukoplakia
Plasma
Tissues
DNA
Papillomaviridae
Polymerase chain reaction

ABSTRACT

Background: Oral leukoplakia is considered a potentially malignant lesion for the development of squamous cell carcinoma, and various risk factors may be related to its development and malignant transformation, including the human papillomavirus (HPV). The aim of this case-control study was to detect the presence of HPV in fresh tissue, plasma and saliva samples obtained from patients with and without oral leukoplakia, and verify the correlation of the presence of DNA of HPV between different sources of materials.
Methods: In this study, 32 patients with oral leukoplakia and 24 patients selected in a case-control manner were included. DNA extraction from the samples was performed, and afterwards it was amplified by nested polymerase chain reaction (nPCR) for the detection of HPV (nPCR: MY09-MY11/GP05+-GP06+).
Results: The DNA of HPV was found in 68.75% of the fresh tissue samples; in 50% of plasma, and in 62.5% of saliva samples in the group of patients with leukoplakia; in comparison with 45.8%, 54%, and 45.8%, in the fresh tissue, plasma and saliva samples, respectively, in the control group.
Conclusion: Based on the present study, there was no difference in the rate of HPV detection in patients with or without oral leukoplakia. However all sources tested in this study were considered suitable for HPV detection, especially plasma samples, which showed be an important non-invasive source of HPV detection in leukoplakia patients.

1. Introduction

Leukoplakia is the most common potentially malignant lesion in the oral cavity, and may lead to the development of squamous cell carcinoma (SCC) [1]. The World Health Organization (WHO) has described it as a plaque or white stain that cannot be clinically or pathologically characterized as any other disease [2]. Scheifele and Reichart [3] showed that patients with oral leukoplakia presented 36 times more probability of developing SCC than those in the general population.

The etiology of leukoplakia is multifactorial, but it is mainly associated with oral habits, such as smoking and the habit of chewing tobacco, similarly to SCC [1]. HPV is now recognized as an important risk factor for the development of oropharyngeal cancers, and results of previous studies have demonstrated epidemiological and molecular evidence of the presence of the HPV genome in pre-malignant oral lesions in SCC tissues, especially of the subtypes of HPV-16 and -18 [1,4,5]. There are over 150 known genotypes of HPV and it is believed that at least 15 have oncogenic potential. The genotypes of HPV are classified as low risk (for example HPV 06-11-13-32), medium risk (HPV 31-33-35-51), and high risk (HPV 16-18) according to the degree of correlation with certain malignant neoplasias, with those of medium and high risk frequently being found in potentially malignant and malignant lesions of the anogenital tract, skin and upper aerodigestive tract [2,6].

Tissue would be the best source for analyzing the rate of HPV infection, since it is an epitheliotropic virus, and presents its proliferation in the cells of the basal and parabasal layer of the epidermis or mucosa, establishing its infection in the more superficial layers of the epithelium [7]. For a long time it was believed that HPV could not be transmitted to various locations of the body through the blood, however, various studies have demonstrated that the DNA of HPV may be found in the blood circulation, including the

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http://dx.doi.org/10.1016/j.prp.2017.04.005
0344-0338/

peripheral blood mononuclear cells (PBMC), serum, plasma, umbilical cord and arterial blood [8]. Blood plasma has been shown to be an attractive medium for the detection of infection by HPV, since it is a non-invasive method, and blood is the only fluid that is in direct contact with all the organs [9], however, there are no studies that have made the association of the rate of detection of HPV in blood plasma with that of tissue obtained from patients with oral leukoplakia.

Saliva is a body fluid that has been shown to be adequate for the detection of HPV [10,11], and because it is a non-invasive method for collecting material, it can easily be performed in any population. In addition to being easy to obtain saliva, it presents cells of different regions of the oral mucosa, thus facilitating the analysis when seeking HPV, since it is known that HPV presents predilection for certain areas of the oral cavity, such as the oropharyngeal region and tonsillar pillar [11]. When analyzing saliva samples from patients with oral SCC, SahebJamee et al. [12] found the presence of HPV in 40.9% of the patients and in 25% of the controls. Sayyah-Melli et al. [13] evaluated the saliva of women with genital lesions associated with HPV and detected HPV in 64.4% of the saliva samples.

In the literature there is a notable discrepancy in the results of studies conducted in oral lesions investigating infection by HPV, with a variance from 0% to 100% [4]. This is due to the difficulty of comparing results from one study to another, as a result of the different techniques used; type of source of material used and quality of collecting the material; availability of fresh, carefully collected cytologic samples; and in many cases, patients differing in age, pathology and specific risk factors [4,6].

Further studies are necessary to elucidate the relationship of the role of HPV in potential malignant lesions, especially oral leukoplakias. Therefore, the aim of this study was to detect the presence of HPV in fresh tissue, blood and saliva samples obtained from patient with and without oral leukoplakia, by means of nested polymerase chain reaction (nPCR) and verify whether there is correlation of the positivity of HPV with the different sources of materials.

2. Materials and methods

This study was approved by the Research Ethics Committee of the Araçatuba School of Dentistry, (FOA)-UNESP, (Process No. FOA-01034/2011) in June 06, 2011. Informed consent was obtained from all individual participants included in the study.

The patients that formed the study group and control were selected between the years 2011 and 2013. In order to form the Study Group, 32 patients were selected, with oral leukoplakia diagnosis confirmed after undergoing biopsy that was subjected to histological exam.

For the control group, 24 individuals without leukoplakia were selected. They were paired by sex and age with a difference of up to 5 years, and required oral surgery procedures (distal wedge or pre-prosthetic surgery).

Excluded from this study were persons who presented any type of auto-immune diseases (such as, for example: Addison's disease, celiac disease, dermatomyositis, lichen planus, multiple sclerosis, myasthenia gravis, pernicious anemia, rheumatoid arthritis, Sjögren's syndrome, systemic lupus erythematosus, scleroderma, vasculitis, psoriasis, Behçet's disease), who were in a gestational period, and those who had been treated for any type of malignant neoplasia. These are factors that change the patient's immunity and could interfere in the HPV infection.

2.1. Data and material collection

Individuals who smoked regularly during some time of their life were considered smokers, even those who had currently quit smoking. Non-smokers were considered those who had not made regular use of tobacco at any time of their life. For the classification of alcoholics and abstainers, the same criteria were used.

A term of free and informed consent was obtained from all the patients included in this study. The patients, both those in the study and control group were equally submitted to the collection of materials for the research. Before saliva collection, the patients were instructed not to consume beverages or water up to 30 min before the collection was made. To obtain saliva samples, the patients were asked to spit saliva into a 15 ml (milliliter) falcon tube for 10 min, in order to obtain a minimum of 5 ml of saliva. In addition 10 ml of blood were collected from each patient, which were deposited in tubes containing 10% tripotassium EDTA. The samples were immediately centrifuged at 1500 rpm for 20 min at 4 °C, to obtain the plasma.

Biopsies were performed for the purpose of diagnosis and treatment, and the samples obtained were divided into two parts: one part was conserved in formol in order to perform the routine histopathological exam and the other part was conserved in liquid nitrogen for later biomolecular exams for the detection of DNA of HPV. After obtaining the histopathological exam results, the patients with leukoplakia were divided according to the degree of dysplasia of the epithelium into: absence of dysplasia, mild, moderate and severe dysplasia. The surgical procedures (distal wedge or pre-prosthetic surgery) in control patients were performed irrespective of the research, as they were considered treatment.

All the samples were identified and stored in a freezer at −80 °C immediately after collection, for later submission to laboratory procedures.

2.2. DNA extraction

DNA was extracted from the samples in accordance with the instructions of the DNA QIAamp® mini extraction kit manufacturer (QIAGEN Ltd., Crawley, UK). The DNA obtained from all the samples was submitted to spectrophotometry (NanoDrop® ND-1000 UV–Vis) that confirmed the presence of a good quality DNA in all the samples. Subsequently, all samples were standardized and diluted in the same concentration of DNA before the PCR step.

2.3. PCR for control human gene

The presence and integrity of the DNA of all the samples were tested by means of amplification of the human β-globin gene, using the oligonucleotides PCO3-PCO4 which amplifies 110 base pair (bp) product [14]. As positive control for the β-globin gene, a previously tested blood sample was used and as negative control, DNA was replaced by ultrapure water. The presence of human DNA was analyzed by means of electrophoresis on 2% agarose gel in a 1× TBE buffer (Horizontal electrophoresis – Amersham Pharmacia Biotech model EP3501, Sweden) (Fig. 1). For all the samples the presence and integrity of the genomic DNA was confirmed, and afterwards they were submitted to research of the HPV gene by means of PCR in two stages (nPCR) with the primers for the DNA of HPV. All the samples were tested in duplicate.

2.4. nPCR for amplification of HPV

For HPV detection, the pairs of primers MY09/11 were used in combination with the pair GP5/6+ of Invitrogen Life Technologies®, Brazil, resulting in a fragment of 140 bp [15,16], and they allowed the detection of a series of types of HPV, among them HPV-6, -11, -16, -18, -31 e -33 [16]. As positive control for infection by HPV, a sample of HeLa, a lineage uterine cervical carcinoma cells with up to 4 copies of HPV-18 per cell were used. The negative control consisted of a mixture of amplification and ultrapure water. The