Application of the PCR method for identification of the HPV in squamous cell carcinoma of the oral cavity

Aplicação do método PCR para identificação do HPV em carcinoma de células escamosas da cavidade bucal

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ABSTRACT

Introduction: The development of squamous cell carcinoma of oral cavity (SCC) is a multifactorial process, where the cellular mutagenesis is determined by co-carcinogenetic agents like the human papillomavirus (HPV). Objective: the objective of this study is to identify the HPV in the SCC by means of polymerase chain reaction (PCR). Methods: twenty cases of SCC have been analyzed where the material used was extracted from specimens included in paraffin blocks of the Pathology Laboratory file of the São Vicente de Paulo Hospital of Passo Fundo/RS in the period of 2003 to 2007. They were all male cases and the 6th decade of life was the most prevalent. The lower lip was the region that comprises most of the defects and the histological grade “well-differentiated” from the World Health Organization was predominant. It has been detected the presence of HPV's DNA in four of the 20 cases of the SCC (20%). Conclusions: it can be assured that, by the fact of the HPV has been identified in a considerable percentage of cases of SCC, it can have influence on the genesis of the referred malignant neoplasia. So, more studies are necessary to define what is the possible role of HPV in the oral carcinogenesis.

Keywords: Oral cancer. HPV. PCR.

RESUMO

Introdução: O desenvolvimento do carcinoma de células escamosas da cavidade bucal (CCECV) constitui-se num processo multifatorial, no qual a mutagênese celular é determinada por agentes co-carcinogênicos como o papilomavirus humano (HPV). Objetivo: o objetivo deste estudo é identificar o HPV no CCECV por meio da reação de cadeia em polimerase (PCR). Métodos: vinte casos de CCECV foram analisados, tendo sido o material utilizado extraído de espécimes incluídos em blocos de parafina do arquivo do Laboratório de Patologia do Hospital São Vicente de Paulo de Passo Fundo/RS no período de 2003 a 2007. Todos os casos levantados pertenciam a pacientes do sexo masculino e a sexta década de vida foi a mais prevalente. O lábio inferior foi a região que apresentou o maior número de lesões e o grau histológico “bem diferenciado” da Organização Mundial da Saúde foi predominante. Detectou-se a presença de DNA do HPV em quatro dos vinte casos de CCECV estudados (20%). Conclusões: pode-se concluir que, pelo fato do HPV ter sido identificado numa porcentagem significativa de casos de CCECV, ele pode ter influência na origem da referida neoplasia maligna. No entanto, mais estudos são necessários para definir qual é o possível papel do HPV na carcinogênese bucal.

Palavras-chave: Câncer bucal. HPV. PCR.
INTRODUCTION

The oral epithelium is formed by a basal or germinative layer that composes the most profound area, limited to the chorion, and by a prickly layer composed by various stratum of several strata of poliedric cells. Basal cells are more undifferentiated and the richest in metabolic activity as well10.

Cells multiplication is carefully regulated and it responds to body specific needs. Therefore, when the special controls which regulate cell multiplication collapses, the cell begins to grow and divide in such irregular manner, then forming a tumoral mass. Multiple mutations are necessary so that a normal cell becomes malignant4.

Human cell regulation with the virus may be caused by the cellular fusion, by believing that, by means of this process, the virus contributes to carcigenesis29. It is estimated that 15% of all human cancers in the world can be attributed to the virus12. The DNA as well as the RNA virus have shown that they are able to cause cancer in human beings. The Epstein-Barr virus, the human papillomavirus (HPV), hepatitis B virus and human-8 herpes virus are the four DNA virus that are involved in carcinogenesis. But the RNA virus linked to this process is the human virus T-lymphotropic type 1 and the one of the hepatitis C3.

The association of the HPV oncogenic with the cervix carcinoma is already well-established27, however, the relation of this pathogen with the SCC is still uncertain, and it has been documented by several researchers9,11,13,23,26,28. About 100 types of HPV have been identified, where in the oral cavity the HPVs 6, 11, 16 and 18 were the most prevalent18. Fifteen types of HPV have been classified as high risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) for cervix cancer24.

The advance in the field of genetic and molecular biology allows that, by means of this technique, the use of scarce sources such as tissue fixed in formol and included in paraffin5,8,15.

Before such presuppositions, the objective of this present study was to identify in 20 samples of SCC recorded in the Pathology Laboratory file of the São Vicente de Paulo Hospital of Passo Fundo/RS (PLHSVP) (period of 2003 to 2007), the presence of HPV through application of the PCR, in order to correlate this virus with the infirmity in study.

MATERIAL AND METHODS

Ethical considerations

This work was submitted and aproved for the Research Ethical Comitee of University of Passo Fundo, RS, Brazil (protocol number 333/2006).

Clinical samples

Twenty fragments of SCC fixed in formol 10% and embedded in paraffin, randomly collected in the files of the PLHSVP, in the period of 2003 and 2007, with data related to age, gender, anatomical location and histological grade of neoplasia were used, according to the WHO. Paraffin blocks comprising tissue samples of biopsy concerning each case have been cut in microtome in 10 μm thickness until it has obtained 30 to 40 mg of material, which were stored inside micro-tubes with 2.000 μL, capacity, type Eppendorf.

Cuts preparation

The material stored in the micro-tubes was submitted, twice, to a bath of 1.000 μL xilol for 30 min at 65°C, centrifuged at 14.000 rpm per 5 min, discharging the supernatants in both stages. After it has been paraffin embedded, the tissue was hydrated in ethyl alcohol descendent chain (absolute ethanol 95%, ethanol 70%) and deionized water. In each one of the stages, the material was centrifuged at 14.000 rpm for 5 min, and the supernatant discharged.
DNA extraction

The protocol used for extraction of the DNA of the SCC cases was based on the information provided by the manufacturer of the extraction kit of the DNA Wizard Genomic Purification™ (Promega Corporation, Madison, WI, USA), with some modifications to the technique optimization. From the material hydrated, proteins digestion and the extraction itself of the DNA have begun. For this, 600 μL cellular lysis and 300 μL nuclear lysis solutions were added, incubating at 65°C for 60 min. The digestion of the proteins was accomplished by adding a solution of proteinase K (10 μg/μL), in a proportion of 3.5 μL of the solution to each 1 mg of paraffin material, incubating overnight at 50°C-60°C. After that, it has been added 3 μL of RNase solution and incubated for 15 to 30 min at 37°C. In the next stage, 200 μL of protein precipitation solution was added, homogenized with vortex, 15 min in ice was incubated, centrifuged at 14,000 rpm for 4 min and the supernatant transferred to a new tube of 2,000 μL containing 600 μL of isopropanol, inverting it softly from 5 to 6 times. After, it was centrifuged at 14,000 rpm for 5 min. Then, the supernatant was removed, adding 600 μL ethanol 70% iced. Subsequently, it was centrifuged at 14,000 rpm for more than 5 min; the tubes were inverted on the towel-paper and left open for 15 min to dry. Next, 100 μL rehydration solution of DNA was added, incubating for 1h at 65°C. These samples were stored at 4°C.

DNA amplification

In this work, the consensus primers MY09: 5′ CGTCCMAARGGAWACTGATC3′ and MY11: 5′ GCMCAGGGWCATAAYAATGG 3′ were used, which specifically enlarge a part of the gene L119. The reaction of amplification was accomplished in final volume of 50 μL, being comprised of 5 μL tampon 10x, 2.5 μL of MgCl2, 200 μM of each deoxynucleotidyl-transferase (TdT), 12.5 pmoles of each primer and 3 U of Taq DNA polymerase. For each amplification reaction, 5 μL of the solution were used, containing the DNA previously extracted. In each amplification battery by the PCR, a negative control has always been used (water MiliQ) and positive (sample of HPV positive ceded by the Simbios Biotechnology Laboratory), comprised of the same concentrations and same reagents of the amplification reaction, except the DNA. The amplification system of the DNA was achieved in tubes of 200 μL, which were transferred to the thermocycler (MJ Research PTC 150). The PCR program was consisted of the following stages: 95°C for 5 min, 40 amplification cycles (95°C for 1 min, 55°C for 1 min, 72°C for 1 min) and 72°C for 5 min, according to what has been described by BAUER et al.3 (1991) and MANOS et al.14 (1989). The product visualization of the PCR has been achieved in electrophoresis of agar gel (1% in TA) stained with ethidium bromide under UV light.

The results obtained have been evaluated by means of descriptive statistics of frequency.

RESULTS

From the total of 20 cases evaluated, all were male, the 6th decade of life was the most prevalent, the lower lip was the anatomical region that has been most affected and the histological grade of the WHO “well-differentiated” was the prevalent. The presence of DNA viral (HPV) occurred in 4 of the 20 cases of SCC, totalizing 20% (Table 1 and Fig. 1).
Indicators: male *, female **; 1-floor, 2-lower lip, 3-oropharynx, 4-tongue border, 5-soft palate, 6-retromolar; 1 - WHO I, 2 – WHO II, 3 – WHO III; + = HPV positive, - = HPV negative; DND – non-avialble

DISCUSSION

The carcinogenesis is a process in which several genetic events that change the normal functions of the proto-oncogenes and tumor suppressor genes, occurs. This may result in an increase of the growth factors production or the number of the cellular surface receptors, in the reinforcement of the messenger intracellular signaling and in the increase of transcription factors production.

The relationship of the HPV with the SCC has been supported by the high risk capacity of the HPVs (16 and 18) of increasing the cellular proliferation in vitro. In the present study, the presence of the DNA viral (HPV) in 4 of the 20 cases of SCC, diagnosed by the PCR was identified, totaling 20%. This way, it can be assured that, due to the HPV has been identified in a considerable percentage of SCC cases, it may have influence on the genesis of the referred malignant neoplasia. Yet, it is asserted that there was no correlation of the presence of the HPV as for gender, age, anatomical location and histological grade in the samples studied.

KREIMER et al. (2005) have reviewed 60 studies of PCR in oral carcinoma of oropharynx and larynx, where 35 works related the HPV with the SCC, corresponding to 2.642 of the cases studied. So, the average found of HPV in SCC was 23.5%. The HPV 16 was the most prevalent, corresponding to 68.2% of the HPVs presented in the SCC.

PINTOS et al. (2007) have evaluated 72 cases of oral cancer and oropharynx by means of the PCR, detecting the HPV in 14 cases (19.4%), where the HPV 16 was the most prevalent with 8 cases (11.1%).

SMITH et al. (2004) have investigated the relationship of the HPV in head and neck cancer by means of the PCR, verifying the presence of HPV in 22.9% of the 201 patients evaluated. The HPV 16 was the most prevalent with 85% of the cases.

GONZALEZ (2007) have verified the presence of HPV by means of the PCR in 7 of the 16 SCC samples analyzed (43.7%). The HPV 16 was the most prevalent in lesions.

RIVERO and NUNES (2006) have correlated 40 samples of SCC with the presence of HPV by means of the PCR, not identifying the presence of HPV in any of the samples.

All studies previously described were carried out with the PCR and presented results related to the presence of HPV in SCC similar to those obtained in the present work (20% of the cases).

The PCR was invented by the scientist Kary Mullis in 1983, being considered as the best method developed and widely used for amplification of nucleic acids, providing great sensitivity and keeping high specificity.

Nevertheless, other techniques are used to detect the HPV in oral cancer. XAVIER et al. (2005) have evaluated 20 cases of SCC, verifying the HPV in 15 (75%), using the histopathological examination to the presence of koilocytosis.

TINOCO et al. (2004) using the immunohistochemical, have analyzed 38 cases of SCC, detecting the HPV in 16 (42.5%).

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TABLE 1 – Description of the data related to age, gender, anatomical location, histological grade and presence of HPV in twenty cases of SCC analyzed

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>Anatomical Location</th>
<th>Histological Grade (WHO)</th>
<th>Presence of HPV</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>58</td>
<td>*</td>
<td>1/4</td>
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<td>3</td>
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<td>8</td>
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<td>9</td>
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<td>49</td>
<td>*</td>
<td>4</td>
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</table>
VIDAL et al.27 (2004) when verifying the presence of the HPV in SCC by means of hybrid capture have observed that from 40 individuals, 11 showed the HPV in SCC (27.5%).

In this study, it could be observed the variety of positiveness concerning the presence of HPV in SCC, what can occur due to different sizes of samples or also, by due to the use of methods with variable sensitivity and specificity.

As for the type of tissue used in the study (tissue fixed in formol and included in paraffin), it is known that it can provide a DNA fragmented and/or the possibility that the formol mediate cross reaction of nucleic acids themselves and with proteins. However, such conditions have been considered in the present study, verifying that the primordial factor that will be taken into account, so that it can be tried to avoid such situation, is the incubation time and the temperature in the stage of the DNA extraction. This way, in this study, these variables were compatible to what has been proposed by previous studies6.

CONCLUSIONS

It is concluded that the present work have verified the presence of the viral DNA (HPV) in 4 of the 20 cases of SCC analyzed, totaling 20%. Also, it has been verified that the use of tissue fixed in formol and included in paraffin, when the accomplishment of retrospective laboratory researches with the PCR, is effective.

REFERENCES

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