



Screening of Papillomavirus Gene Sequences in Oral Cancer Patients in the Federal District, Brazil

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ABSTRACT

The role of HPV in other cancer localization besides the genital apparatus is under scrutiny. Biopsies of oral neoplasms of 26 patients attending the dental clinics of the Regional Hospital of North Wing in Brasilia were analyzed by histopathological methods, screened for HPV genomic sequences utilizing the DIGENE hybrid capture 2 HPV DNA kit, and also, tissue fragments were processed for cell culture. The Oral Squamous Cell Carcinoma was diagnosed among 84.6% (22/26) of the patients, localized in the floor of the mouth (5/22); in the inferior lip (2/22); in the tongue (5/22); in the oropharynx (6/22); in the hard palate (3/22), and in the soft palate (1/22). The remaining patients (4/26) had tissue alterations compatible with Herpes Simples Virus infection (1/26), hyperplasia/metaplasia of the epithelial tongue (1/26), reactive lymphoid hyperplasia (1/26) and actinic cheilitis (1/26). The HPV DNA sequences were not detected in any of the patients. Three primary cell lines were obtained from different patients' tumor biopsies, which were viable during 12 months, showing morphology typical of malignization. Poor socioeconomic conditions and low educational background seems to be associated to OSCC, despite the fact that the majority of the patients were in the range of 60 to 69 years old (34.6%), when it is more frequent the cancer development. It is questioned here if the absence of HPV DNA segments would be influenced by the traditional sex behavior observed among the patients or if after neoplasia establishment, the episomal HPV DNA was lost or cleared in the cell.

Keywords: Oral neoplasia, Squamous cell carcinoma, Human papillomavirus, Epidemiology, Brasilia.

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INTRODUCTION

The human papillomaviruses (HPVs) are etiologically linked to diverse benign proliferative disorders of skin and mucosae,¹ but just in 1983 that Zur Hausen, whose group repetitively failed to detect herpes simplex DNA in cervical cancer biopsy specimens, found in experimental data, evidences of the causal role of HPV in genital cancer.² In this way, carrying out molecular analysis based on other HPV types, it was proposed a new type, the HPV-16, designated after the hybridization of DNA fragments from one biopsy sample of invasive cancer of the cervix with human HPV type 11 DNA sequences, only under nonstringent conditions. Anyway, beforehand in 1976, Zur Hausen also, had already suggested the role of HPV in malignant transitions from genital warts.³ The etiological link of papilloma viruses with animal cancer, likewise Shope papillomavirus in rabbits, fibrosarcomas, esophageal and ocular carcinomas in bovine, cutaneous carcinomas in sheep, among other animal neoplasia, conducted research work toward the findings of HPV DNA sequences in human neoplasia tissue.⁴

Besides the role of HPV in genital neoplasia, both in women and men, and mostly significative the cancer of cervix, other cancer localizations, in the neck and head have been exhaustively studied concerning the HPV etiological and pathophysiological aspects. Previously to molecular evidences of HPV causal role in cancer, biopsies of laryngeal papillomatosis had shown to display HPV antigens by immunohistochemical assays.⁵ Sequentially, additional studies substantiated by molecular analysis demonstrated the presence of HPV antigens and DNA in tissues of squamous cell carcinoma of the larynx in man.⁶⁻⁸ Also, in the esophagus, the occurrence of squamous cell carcinoma was suggestive of HPV involvement.⁹ Experimental data pointed out to a genital origin of laryngeal papillomavirus infection.¹⁰

Similarly to the members of the Herpesviridae family, it was hypothesized that HPV could be found in health tissues, in a quiescent state, and be activated during the host immunosuppression, physical and psychological stress, inadequate nutrition or infection by other pathogens.¹¹ Cumulative experimental data in the malignant transformation of nasal, oral and anal squamous cell papillomas reinforces the role of HPV in the human

squamous cell carcinogenesis.¹²⁻²⁰ After all the data gathered, the etiology of two squamous cell carcinoma of the head and neck besides the association mainly with smoking and alcohol use, HPV assumed an important role.²¹

The members of the papillomaviridae family comprise viral agents infecting mammals, birds and nonavian reptiles, showing tropism for epithelial tissues. The papillomavirions are small, measuring about 50 nm, nonenveloped DNA tumor viruses with a circular genome of nearly 8 kB,^{22,23} present in an episomal form in transformed cells.¹ Despite the slow mutation rate, the genome of approximately 240 distinct HPV types were sequenced allowing to trace back this family for more than 100 million years.^{24,25}

The human papillomaviruses are classified in low and high-risk types, according to their oncogenic potential. Usually the low-risk HPV types are benign, rarely involved in malignization while the high-risk are etiologically associated to anogenital and cervical cancer of uterus, and head and neck tumors. Molecular events differentiate the low-risk HPV types from the highrisk, as the former disrupt the regulation of viral gene expression, inducing exaggerated mitosis cycles, inhibiting efficient mechanisms of DNA repair, which causes chromosomal abnormalities of host cells leading to transformation and malignization. The main target of these cell virus interactions are the basal/parabasal epithelial tissues. HPVs infected cells usually express 2 transforming proteins, E6 and E7, which play important role in the immortalization of keratinocytes by the expression of telomerase act. These proteins interfere with the regulation of cell cycle interacting with p56 and Rb tumor suppressor pathways. The E6 proteins modulate the activity of p53 and PDZ-domain proteins, and the E7 proteins exhibit the differential ability to target the several different members of the retinoblastoma protein family.²⁶ Miller et al²⁷ showed that catalytically inactive hTERT, elongation-defective hTERT, and telomere recruitment-defective hTERT also add to E7 in the mechanism of surpassing senescence blockade, consequently establishing cell immortalization.

The oral squamous cell carcinoma (OSCC) has a remarkable incidence worldwide. Many factors have been implicated in the etiology of OSCC. Smoking habits, alcohol abuse and the existence of premalignant lesions are the most common associated factors in the etiology of OSCC in the oral cavity, 3 larynx, oropharynx and hypopharynx, mainly among elderly people, while genetic factors and HPV infection seems more likely to be among young patients, eventhough synergic action of chemical, physical and biological factors could be working to the genesis of OSCC.²⁸⁻³² It has been reported the increased incidence of human papillomavirus, mainly the HPV-16,

in oropharyngeal cancers, in the developing world.³³⁻³⁵ According to St Guily et al³⁶ the HPV prevalence in 12 French centers was 46.5% in oropharyngeal carcinomas, higher in female than in male cases, and all HPV types had prevalence below 5%, except HPV 16, which was the most prevalent. In Japan, Matsushita et al³⁷ reported HPV DNA detection in the oral cavity of 12 (6.1%) female sex workers; additionally the HPV 56 was the most common type found (7/12), while HPV-52, 16 and 56 were the most usually detected in the cervix. De Spíndula-Filho et al,³⁸ in Brazil, did not find any statistical correlation between OSCC and HPV infection. Horewicz et al³⁹ could not detect HPV 16 DNA in 104 gingival samples of subjects with periodontitis, despite the fact that some authors claim that the same virus type would be involved in periodontal breakdown allowing the virus to infect and persist in the oral tissue triggering later several oral lesions, including squamous cell carcinoma, condyloma acuminatum, verruca vulgaris, focal epithelial hyperplasia and periodontal diseases.^{40,41}

In this research work, we tried to ascertain the findings of some authors^{36,37,42} of HPV DNA detection in samples with malignant characteristics. The oral malignized tissue samples were biopsied and histologically examined, and to reinforce the diagnosis of malignant nature of the oral tissues sampled, long-term cell cultures were established. All biopsied samples were investigated for the presence of high-risk and low risk HPV DNA type.

MATERIALS AND METHODS

Patients: The patients were attended in the public hospitals, representing 23.6% (26/110) of new cases of oral cancer in the Federal District, in Brazil, in 2010. Of the 26 patients looking for clinical dental assistance, willing to participate in the study, in the Regional Hospital of North Wing (Hospital Regional da Asa Norte/HRAN), 69.2% (18/26) were male, and the remaining, female (08/26). All the subjects were Brazilian whose, 15 out of 26 (57.7%) had caucasian 4 predominant traits; 38.5% (10/26) were of mixed racial characteristics, and 3.8% (01/26) presented negroid phenotype. The smoking addiction and alcohol consumption was declared by 80.8% (21/26) and 65.4% (17/26) of the patients, respectively. All patients were between 30 and 89 years old, being most of them, in the range of 60 to 69 years old (34.6%). As previously described, according to the age distribution, the patients' occupation rank firstly as retired (38.5%), agricultural labourers, homeworkers and mason, each representing 11.5%, and the others, 3.8%, painter, unskilled worker, plastic artist, merchant, housekeeper, stallholder and artisan. Most of the patients were married (57.7%), and the others, single and widowed, corresponding each to 15.4%, and divorced



11.5%. This research work was approved by the Human Ethical Committee in the Health State Department in the Federal District, Brazil (Secretaria de Saúde Pública do Distrito Federal). The patients enrolled in this study signed the term of informed consent stating that they were aware of the research work, and allowed one of us, a dentist surgeon, to biopsy the lesion in their oral cavity.

Tissue samples: The macroscopic observation of altered tissue were biopsied, and a small piece of neoplasm was removed in order to carry out the histopathological analysis, the HPV DNA screening and cell culture.

Histopathological analysis: The samples were collected and fixed in 4% formaldehyde solution, washed in distilled water and dehydrated in sequentially progressive alcohol concentration from 30 to 100%. Previously to embedding in paraffin, the tissues were cleared in a mixture of ethanol:toluene and toluene:paraffin. The paraffinized blocks were cut in microtome, and the fixed slices, in glass slides, were stained with hematoxylin and eosin and analyzed under light microscope.

Hybrid capture assay: According to the digene hybrid capture 2 HPV DNA (Digene, Gaithersburg, MD, US) kit instructions, cryopreserved tissue samples (liquid nitrogen) were sliced, added to denaturing reagent, vortexed and allowed to reach 65°C in a water bath, in 2 cycles. The denatured samples were transferred to a microplate, and the RNA probe hybridization cocktail for high risk (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and low risk HPV type (HPV-6, 11, 42, 43 and 44) were added independently to each sample. The microplate containing the controls, the calibrators and the samples 5 under analysis were briefly shaken and incubated at 65°C. Sequentially, the microplates were incubated at 65°C. All samples (the controls, the calibrators and the analyzed samples) were transferred to the capture microwells and shaken in a rotatory shaker and, the supernant removed. Attached hybridized RNA/HPV DNA samples to antibody were washed out, dried and immediately added to the substrate solution, allowed to react for 15 minutes and read in a luminometer. The samples were considered positive for high risk HPV types if the relative light unit/cutoff (RLU/CO) were ≥ 474.33 , and positive for low risk HPV types if the RLU/CO were ≥ 300 taking into account the controls and calibrators values.

Cell culture: The fresh biopsied tissues were kept on sterile Dubecco's Modified Eagle Medium (DMEM, Invitrogen, Life Technologies, Grand Island, NY, US) supplemented with 1% of penicillin and streptomycin (Sigma Co., St. Louis, MO, US). Sequentially, the tissues were washed with sterile medium and mechanically sliced with scalpel and scissors. The minced tissues were enzymati-

cally treated with 0.1% prewarmed collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ, US) and incubated for 1 hour at 37°C. After the incubation period, incomplete DMEM chilled medium was added to the digested cell solution and centrifuged for 5 minutes at 1500 rpm. The pelleted cells were treated with 0.25% trypsin in 1 mM EDTA solution (Invitrogen Life Technologies, Grand Island, NY, US) for 15 minutes at 37°C, and chilled DMEM incomplete medium was added and centrifuged as previously described. The recovered cells were washed and centrifuged thrice with incomplete DMEM. The cells were reconstituted in complete DMEM medium with 10% fetal bovine serum, 1% penicillin/streptomycin (Sigma, St. Louis, MO, US) and transferred to 25 cm² plates. After reaching the confluence, a new fresh medium replaced the cell's old medium. Cell growth and morphology were observed everyday under inverted microscope.

RESULTS

Of 26 patients suspected of oral cancer, none had the HPV. high risk and low risk DNA sequences detected in the biopsies of oropharynx tissues by the HPV nucleic acid hybridization utilizing the digene hybrid capture 2 HPV.

DNA test: The histopathological examination of processed samples revealed that 84.6% (22/26) of the patients had the images of Oral Squamous Cell Carcinoma (OSCC), which of them, 5 were found in the floor of the mouth, 2 in the inferior lip, 5 in the tongue, 6 in the oropharynx, 3 in the hard palate and 1 in the soft palate (Figs 1 to 4). The remaining patients had tissue alterations compatible to Herpes Simplex Virus infection (01/26), hyperplasia/metaplasia of the epithelial tongue (01/26), reactive lymphoid hyperplasia (01/26) and actinic cheilitis (01/26). Distinct attached primary cell cultures of long term, more than 12 months growth and passage, were obtained from malignized oral tissue samples of 3 patients, showing abundant cytoplamic material (Fig. 5) and evidenciated nucleus (Fig. 6). After the 7th passage, fast metabolic activity and rapid cell growth was observed. Besides other factors, poor socioeconomic conditions and educational background seems to be associated to OSCC, and also the age, as the majority of the patients were in the range of 60 to 79 years old (53.8%).

DISCUSSION

Jarboe et al⁴³ compared the QIAGEN/Digene HR-HPV Hybrid Capture 2 with the HR-HPV *in situ* hybridization test, and concluded that the QIAGEN kit is a reliable and effective method to detect HPV DNA sequences in tumors of the oropharyngeal squamous cell carcinoma.

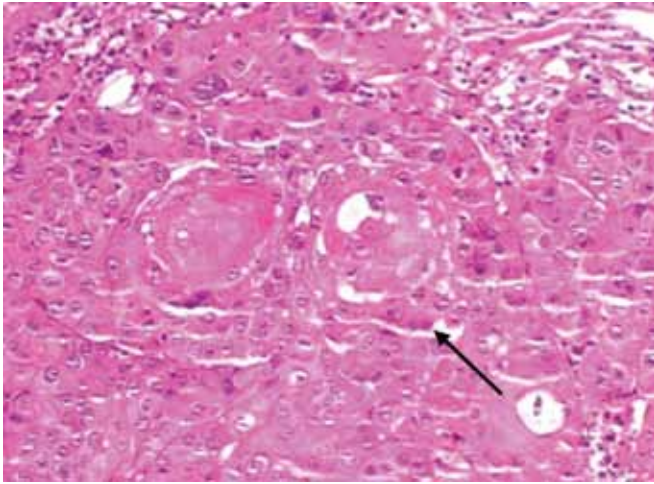


Fig. 1: Tumoral epithelium exhibiting general disorganized tissue architecture

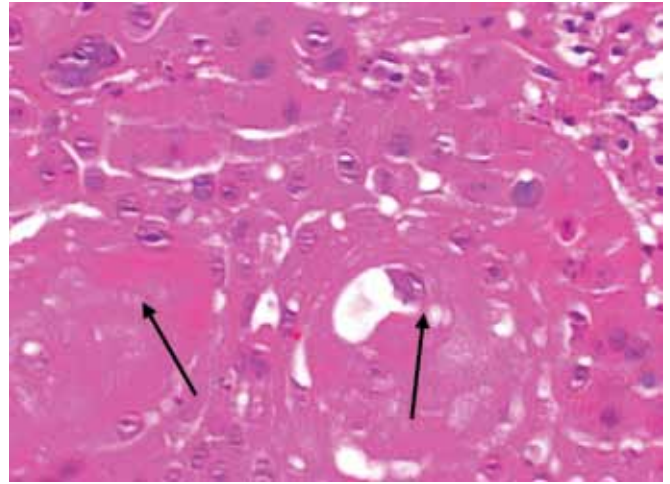


Fig. 2: Corneal pearls formation characterizing well-differentiated epidermoid carcinoma

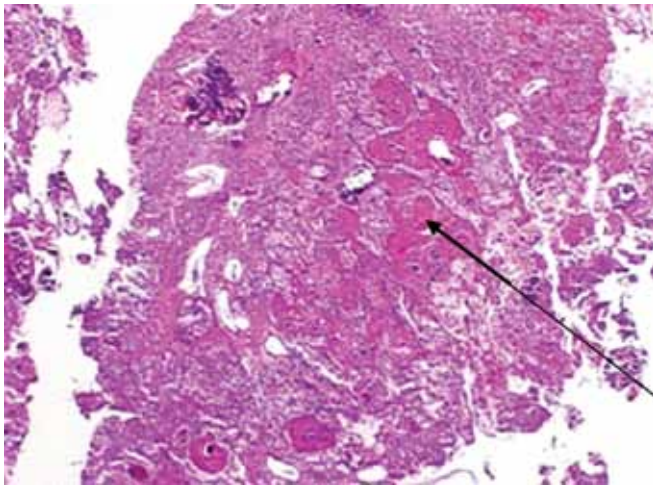


Fig. 3: Well-differentiated epidermoid carcinoma exhibiting corneal pearls

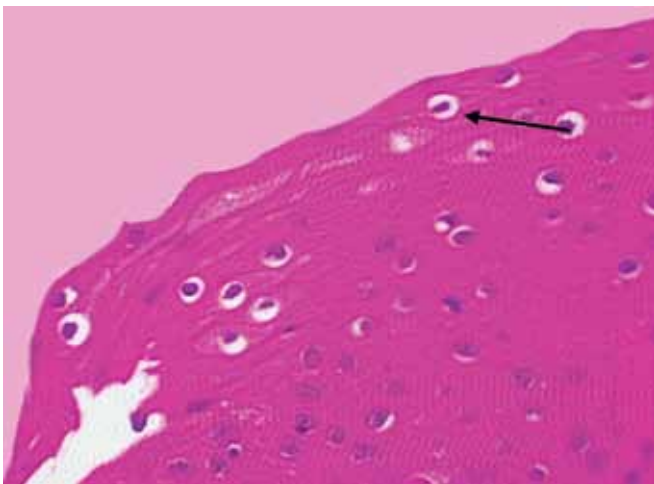
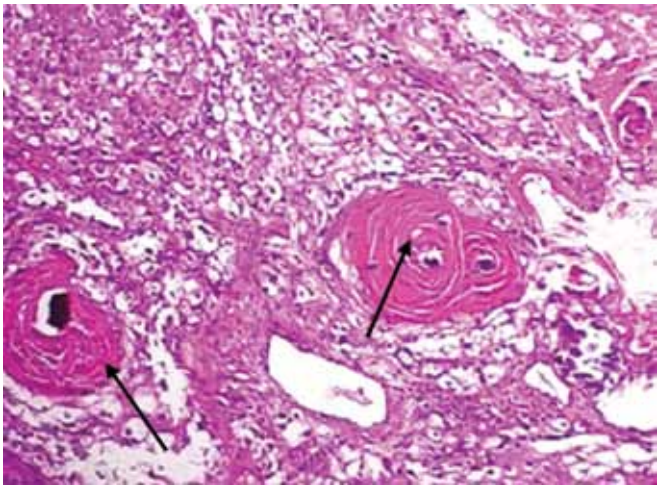


Fig. 4: Typical epithelium tissue exhibiting perinuclear halo resembling koilocytotic atypia

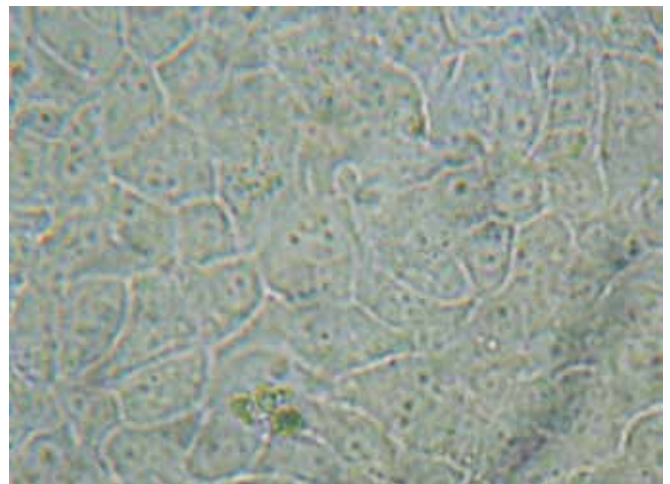


Fig. 5: Cell culture of patient's biopsy at passage #10 (100x)

Also, the QIAGEN/Digene HR-HPV Hybrid Capture 2 is the only HPV diagnostic assay licensed by the United States Food and Drug Administration for use in diagnostic purposes.⁴⁴ Despite so many comparative studies, showing the highest specificity and sensitivity, being the

most used method to detect HPV, Poljak et al⁴⁵ claimed that the Abbott Real Time High Risk HPV test showed excellent analytical specificity, and it did not show any cross-reactivity with low risk HPV genotypes that tested positively with the QIAGEN/Digene HR-HPV Hybrid

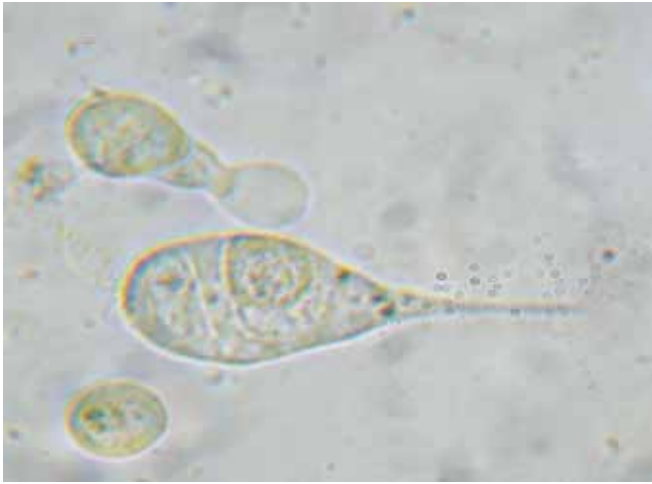


Fig. 6: Individualized cell at passage #10 (400x)

Capture 2 Test. Further analysis of the DNA samples extracted from patients' tissues will be carried out in order to confirm the negative results we obtained utilizing the Qiagen/Digene Hybrid Capture 2 Test.

In spite of the results obtained in our study, related to HPV gene sequences, there are important issues to be addressed. As extensively discussed in the literature, cancer arises driven by multifactorial interactions in multifaceted microenvironment,⁴⁶ as mechanistically demonstrated by Wei et al.³² Not differently, in the limited number of clinical cases here, but significantly representing the annual incidence of oral neoplasia cases in the Federal District in 2010 (23.6%), multiple factors intermingling with each other, likewise smoking addiction, alcoholism and age, seem to be the main determinants in the malignant neoplastic development. Other factors, certainly play an important role in the etiology of cancer, particularly OSCC, like HPV infection and chemicals as pesticides contamination in food and in drinkable water.⁴⁶ The failure to detect HPV gene sequences in the tumor biopsies, does not necessarily mean that the virus did not play any action in the tumorigenesis, or if the methodology employed was not sensitive enough to detect DNA virus segments, or even, the etiology of these OSCC could not be linked to the high and low risk HPV screened in this study as the results obtained by Pereira et al.⁴⁷ According to our data, the patients here, presented a sexual traditional behavior, originated from rural areas and religiously dedicated to ritual celebrations so, the sexual promiscuity seems not to be a common practice among the majority of the patients here studied, therefore, it is suggested that in agreement to the above observations, it could be explained the reason of the failure to detect HPV DNA segments in the biopsies examined as previously stated elsewhere.⁴⁷ As well-known, HPV infection and cervical cancer are sexually influenced. Some data have shown a positive correlation between

HPV DNA sequence detection in specimens from genitalia and oral cavity among subjects actively involved in oral sex practice as corroborated by Beder Ribeiro et al,⁴⁸ and stated by Rosenquist⁴⁹ that, the sexual behavior seems to play a major role in HPV infection in unusual anatomical sites, even though Matsushita et al³⁷ claimed that HPV oral transmission is an independent event of genital HPV infection as supported by other authors.^{50,51} Most striking are the data obtained by Shew et al⁵² finding HPV gene segments in the vagina of young adolescents, not yet involved in sexual intercourse, and also Flake et al⁵³ detected HPV DNA fragments in the saliva of young male adolescents, both cases reported in the US. Therefore, it is not yet clear if HPV constitute a normal virus biota of oral and genital mucosae or, if these viruses besides infection by oral and sexual contact, are also acquired by contact with contaminated surfaces as reported by Strauss et al.⁵⁴ In both situations, HPV pathogenicity seems to be still obscure. Also, it could not be dismissed the possible clearance of virus antigens and DNA after malignization establishment, eventhough it is reported in the literature that among subjects infected by HPV, determined proportion became cleared of infection while others evolve to tissue malignization.⁵⁵

CONCLUSION

In our research work, besides other factors, poor socioeconomic conditions and low educational background are usually associated to OSCC, despite the fact that the majority of the patients were in the range of 60 to 79 years old (53.8%), which certainly contributed to the OSCC incidence.

The long term cell culture of biopsied tissues, for more than 12 months, and the cells' pictures characteristic of the malignant nature of the oral biopsy samples here analyzed, would allow us to further investigate the cellular and molecular mechanisms involved in the etiology of OSCC.

In order to clarify our doubts definitely, we will proceed to amplify HPV genetic sequences utilizing generic primers and, if amplicons were obtained, they will be sequenced and compared to published HPV sequences to identify the virus type. Also, the number of patients will be increased to improve the chance to detect the virus.

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