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Non radioactive *in situ* hybridization for detection of human papilloma virus DNA in squamous cell carcinoma of tongue

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SUMMARY

Previous studies indicate that HPV type 16 and 18 (HPV) are associated with squamous cell carcinoma of the head and neck.

In this investigation we evaluate in our hospital 253 patients with oral squamous cell carcinoma of the tongue not related to tobacco or alcohol with a tumor index of T2 NO MO between 1981 and 1991. From 12 patients we were able to obtain tissue. For detection of human papilloma virus, DNA sequences 6, 11, 16, 18, 31 and 33 in paraffin-embedded human tissue biopsies a non-radioactive in situ hybridization procedure was utilized.

Approximately 60% of the carcinoma of the tongue are positive for episomal viral DNA 6, 11, 16 and 18. These results confirms that HPV infection may play a possible role in the multifactural etiology of carcinogenesis of squamous celle carcinoma of the tongue and probably acting synergisticly with other carcinogenesis.

KEY WORDS:

Tongue carcinoma, HPV-DNA, paraffin sections, non radioactive *in situ* hybridization.

RÉSUMÉ

Des études précédentes ont démontré que les types 16 et 18 du virus du papillome humain (HPV) sont associés au carcinome épidermoïde de la tête et du cou.

Au cours de cette investigation nous avons évalué dans notre hôpital de 1981 à 1991, 253 patients présentant un carcinome épidermoïde de la langue, non relié au tabac et à l'alcool, avec un index T2 NO MO. Nous avons pu obtenir des fragments de tissus chez 12 patients. Nous avons appliqué une technique d'hybridation *in situ* non radioactive pour la détection des séquences ADN 6, 11,16, 18, 31 et 33 du virus du papillome humain sur des biopsies incluses à la paraffine.

Environ 60% des cancers de la langue sont positifs pour l'episome de l'ADN viral 6, 11, 16 et 18. Ces résultats confirment que l'infection par le virus du papillome est susceptible de jouer un rôle dans l'étiologie multifactorielle de la cancérogénèse du carcinome épidermoïde de la langue, et qu'elle peut agir en synergie avec d'autre carcinogènes.

MOTS CLÉS:

Carcinome de la langue, HPV-ADN, sections à la paraffine, hybridation *in situ* non radioactive.

INTRODUCTION

Malignant tumors of the oral mucosa constitute up to 50% of malignancies in most countries of the Western hemisphere. It is generally accepted that there is a multifactoral etiology of cancer with different environmental and/or genetic risk factors. This is particularly true of all squamous cell carcinomas, a fact which has particularly been related to tobacco and alcohol consumption (Scully et al., 1991, 1990; Boyle et al., 1990, a, b; Zaridze et al., 1985; Bross and Combs, 1976). Although these factors play a vital role, it has been suspected that oral carcinoma and premalignant lesions may, in some cases, have an infectious etiology (Scully et al., 1985, 1988).

Since papilloma virus infections of the oral mucosa were first demonstrated in animals more than 60 years ago (Demoubreun and Goodpasture, 1932), increasing attention is been focused on the pathogenic role of human papilloma virus in carcinoma (Zur Hausen, 1977; Gissman, 1984; Dürst et al., 1983, 1985; Loening et al., 1985; Eisenberg et al., 1985; De Villiers et al., 1985, Syrjänen et al., 1990, 1992). A connection can no longer be ignored in the light of increasing evidence for a viral association in the various other human malignancies, including some that may effect the head and neck (Scully, 1983 and 1988). There is also evidence of a viral association in malignant neoplasia of other sqamous epithelial like cervix carcinoma (Zur Hausen, 1980).

The possible role of HPV infection in the etiology of oral cancer was not appreciated until 1983 when Syrjänen et al. described cytopathic changes in HPV (koilocytosis) in oral cancers identical to those previously found in precancerous lesions and carcinomas of the uterine cervix. These morphological findings were further confirmed by immune histochemical demonstrations of HPV (capsid) antigens in that lesions.

Human papilloma viruses are small DNA viruses which are dependent on terminally differentiating epithelial cells (keratinoid cysts) for the completion of their life cycle. Papilloma virus infection of the oral cavity has become recognized and various clinical forms have been characterized, including verruca vulgaris, focal epithelial hyperplasia (Heck's disease) (Syrjänen, 1984) multiple papillomas, condylomata accuminata (Praetourius-Clausen, 1972; Jenson et al., 1982).

Papilloma viruses normally infect the basal layers of the skin or mucosa, most propably via microlesions. In the cells viral DNA can be detected in very low copy numbers. During the subsequent cellular differentiation stages, replicating viral DNA is normally found in large copy numbers. In the superficial keratinized cells, abundant capsid proteins are found which contain assembled viral particles.

The oncogenic potential of the papilloma virus has been clearly demonstrated both in vivo (Jarett et al., 1978) and in vitro (Moar et al., 1981; Campo and Spandidos, 1983) and was first detected in squamous cell carcinoma in Germany in 1985 (Loening et al., 1985; De Villiers et al., 1985; Milde and Loening, 1986).

Due to the absence of differentiating cells in malignant lesions, no late viral antigens are present and therefore vital particles are almost always not assembled. Furthermore, the absence of intact viruses does not mean that viral nucleic acid might not be present. It does appear, however, that high expression of the viral gene is not obligatory in carcinoma cells (Lehn et al., 1985, Yee et al., 1985). This might imply an initiator rather than a maintainance role of HPV-associated carcinogens.
It has been suggested that the integration of HPV-DNA into the host cell chromosome and the type of HPV play an important role in the development of malignancy (Zur Hausen 1977, Boshart et al., 1984; Pfister, 1984; Munoz et al., 1988).

Following the major progress made in the field of HPV research during the last 10 years mucosal lesions of the oral cavity have become a subject of renewed interest (Scully et al., 1985; Syrjänen et al., 1983, a, b; Loening et al., 1985).

The aim of this study is to investigate the presence of HPV genotypes (6, 11, 16, 18, 31 and 33) in a group of 12 patients with carcinoma of the tongue who had not used tobacco or alcohol.

MATERIAL AND METHODS

Between 1981 and 1991 we evaluated in our hospital 253 patients with oral squamous cell carcinoma not related to tobacco or alcohol with a tumor index of T2 NO MO. From 12 patients we were able to obtain tissue. After paraffin embedding, the tissues were examined for evidence of HPV. The data were compared to non-neoplastic mucosal biopsy

specimens taken from healthy patients who gave their consent after undergoing an osteotomy of their wisdom-teeth.

For detection of human papilloma virus, DNA sequences 6, 11, 16, 18, 31 and 33 in paraffin-embedded human tissue biopsies a non-radioactive in situ hybridization procedure was utilized with Biohit[R] in situ typing test for initial testing of samples of tongue carcinoma for the presence of HPV DNA. The test used is based on a non-radioactive in situ hybridization technique to detect HPV-DNA sequences in biopsies. Nucleic acid is visualized by hybridization of the labeled HPV-DNA probes with the target DNA. Paraffin-embedded biopsies were cut and placed in the walls of the pretreated slices. One section was used for each type-specific probe and an additional section was used as negative control. The paraffin-embedded sections were deparaffinized and proteolytically digested to expose the fixed target DNA. Deproteinization could be omitted, depending on the fixative and fixation time. Biotinilated probes were placed on the section. Double-stranded probes and target DNA were simultaneously denatured to single strands by heating. The homologous sequences of the probe and the target were then specifically annealed during the subsequent hybridization step. Specific hybridization between HPV-DNA probes and DNA in specimens are determined by enzyme-substrate reaction.
Finally, the sections were counter-stained allowing morphological examination along with visualization of the target DNA (Fig. 1).

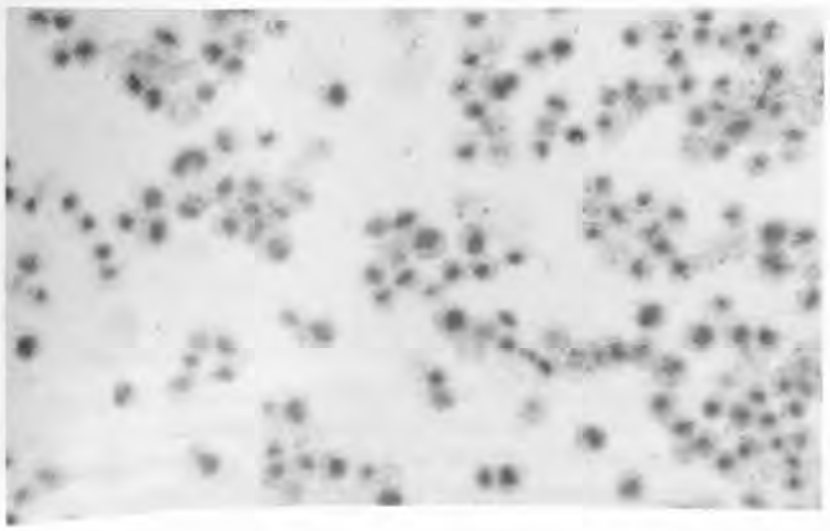

Fig. 1: In situ typing test; positive control. With this test the specific HPV-DNA types can be identify.

Fig. 1: Test de typage in situ: controle positif. A l'aide de ce test, la spécificité des types de l'ADN-VPH peut être identifiée.

The Biohit[R] HPV in situ screening test was used to visualize several HPV types. A negative control ensured that the tissue sections contained no false-positives. To assure that the staining procedures were performed correctly, a positive control slides were provided (Fig. 2).

Fig. 2: In situ typing test; negativ control.

Fig. 2: Test de typage in situ: contrôle négatif.

RESULTS

12 equal squamous cell carcinoma from 12 patients with carcinoma of the tongue and 12 biopsy specimens from 12 healthy patients without oral lesions were analyzed using Biohit[R] in situ typing and the Biohit[R] in situ screening test for HPV-DNA 6, 11, 16, 18, 31 and 33. The results of this investigation are summarized in Tab. 1 and representive cases are shown in Fig. 3 to 8.

The 12 cases in the group of patients with tongue carcinoma who hadn't used alcohol or tobacco stained positive for HPV-DNA 16, 18 and 11. Specific staining was detected in squamous epithelial nuclei in 7 of 12 cases of squamous celle carcinoma. HPV-DNA 16, 18 and 11 proved to be the most common virus types. No viral HPV 16, 18 or 11 was found in biopsies taken from the tumor-free groups. None of the detected HPV types were exclusively confined to a specific morphological appearance of the lesions.

TABLEAU I:

Human papilloma viruses (HPV) 6, 11, 16, 18, 31 and 33 DNA in paraffin-embedded squamous cell carcinoma tissue of the tongue, detected by in situ hybridisation.
ADN des Virus du Papillome Humain (VPH) 6, 11, 16, 18, 31 et 33 dans les tissus de carcinome épidermoïde de la langue inclus à la paraffine, détectés par hybridation in situ.

	Screening test	HPV 6	HPV 11	HPV 16	HPV18	HPV 31	HPV 33
1) U6317	–	–	–	–	–	–	–
27 U8227	–	–	–	–	–	–	–
3) U3496	–	–	–	–	–	–	–
4) U7747	+	–	+	+	+	–	–
5) U1614	+	–	+	+	–	–	–
6) U2907/91	–	–	–	–	–	–	–
7) U4667/91	–	–	–	–	–	–	–
8) U7733	+	–	+	+	–	–	–
9) U9994	+	–	–	–	+	–	–
10) U1213	+	–	–	+	+	–	–
11) U8671	+	–	+	+	–	–	–
12) U4311	+	–	–	–	+	–	–

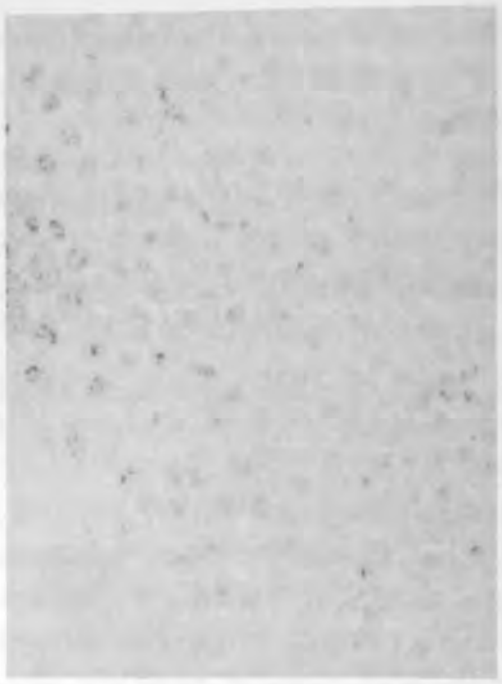

Fig. 4: Squamous cell carcinoma of the tongue. In situ hybridisation with HPV-16 DNA. Nuclear labelling of many cells within the tumor.
Fig. 4: Carcinome épidermoïde de la langue. Hybridation in situ avec l'ADN-VPH-16. Marquage nucléaire de plusieurs cellules tumorales.

Fig. 3-8: Detection of HPV-DNA in paraffin embedded section following in situ hybridisation.
Fig. 3-8: Détection de l'ADN-VPH sur des coupes à la paraffine après hybridation in situ.

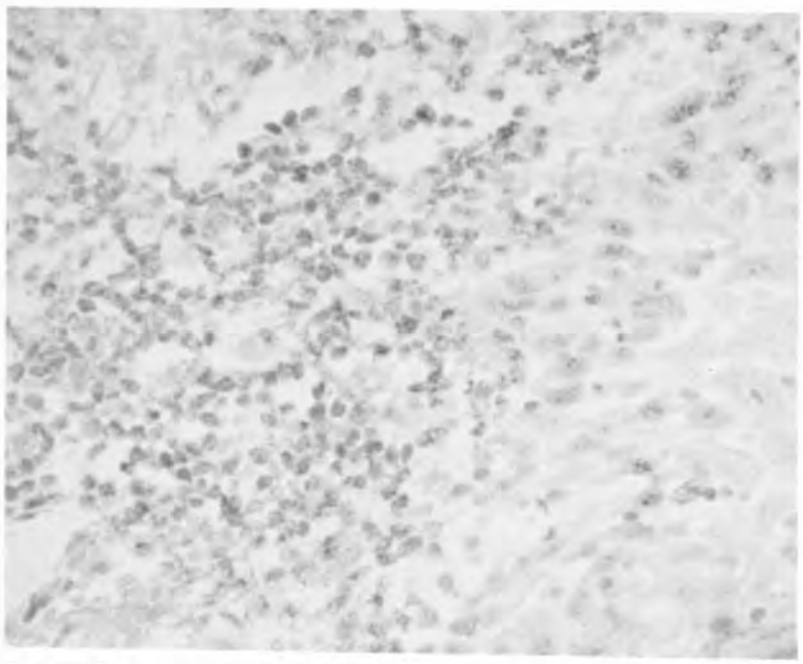

Fig. 3: Screening test. This screening test showing several HPV-DNA positive nuclei.
Fig. 3: Test de criblage. Le test de criblage montre plusieurs noyaux ADN-VPH positifs.

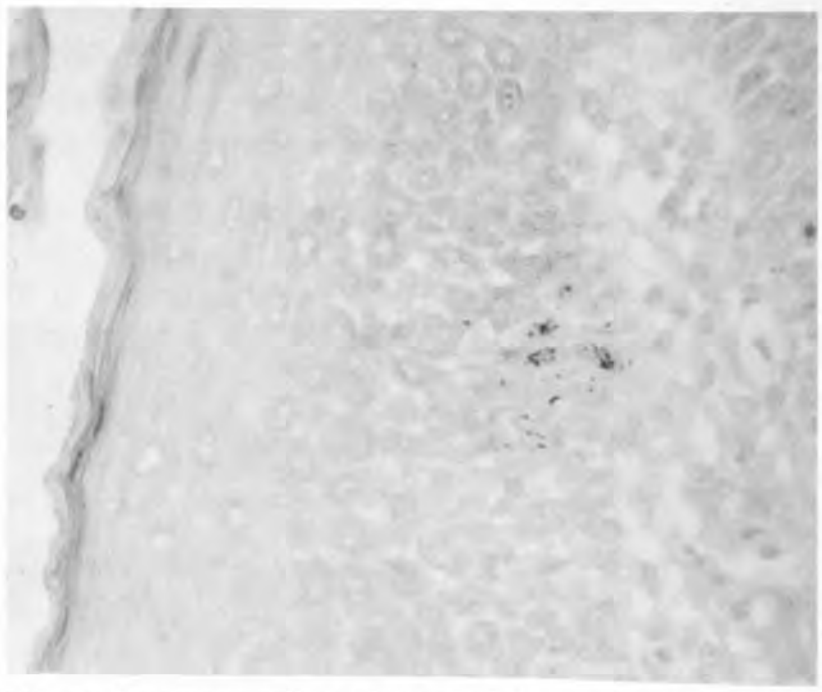

Fig. 5: Squamous cell carcinoma of the tongue. The tissue contains HPV-18 DNA infected cells even parabasal.
Fig. 5: Carcinome épidermoïde de la langue. Le tissu contient des cellules même parabasales infestées par de l'ADN-VPH-16.

Fig. 6: Squamous cell carcinoma of the tongue with HPV-11-probe. Original brown precipitates appaer black. Many more granules (mostly related to the nuclei) could be seen in the different planes of optical focus.
Fig. 6: Carcinome épidermoïde de la langue avec la sonde VPH11. Les précipités qui à l'origine sont bruns apparaissent noirs. Un grand nombre de granules (la plupart en relation avec le noyau) peuvent être observés dans différents plans de mise au point.

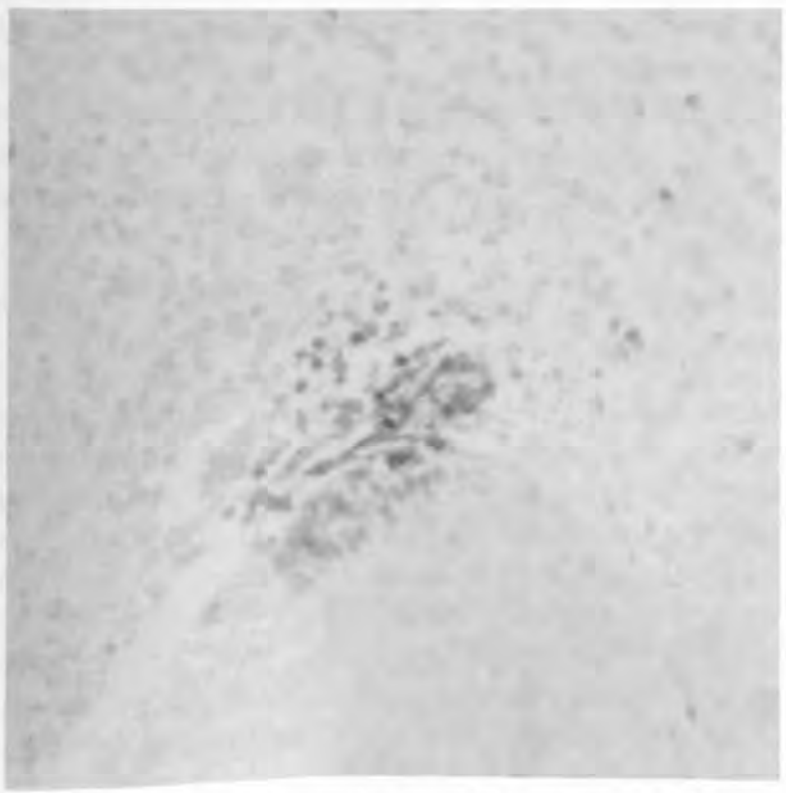

Fig. 7: Squamous cell carcinoma of the tongue. HPV-16 is localized in the deeper layer of the tissue.
Fig. 7: Carcinome epidermoïde de la langue. Le VPH-16 est localisé dans les couches profondes du tissu.

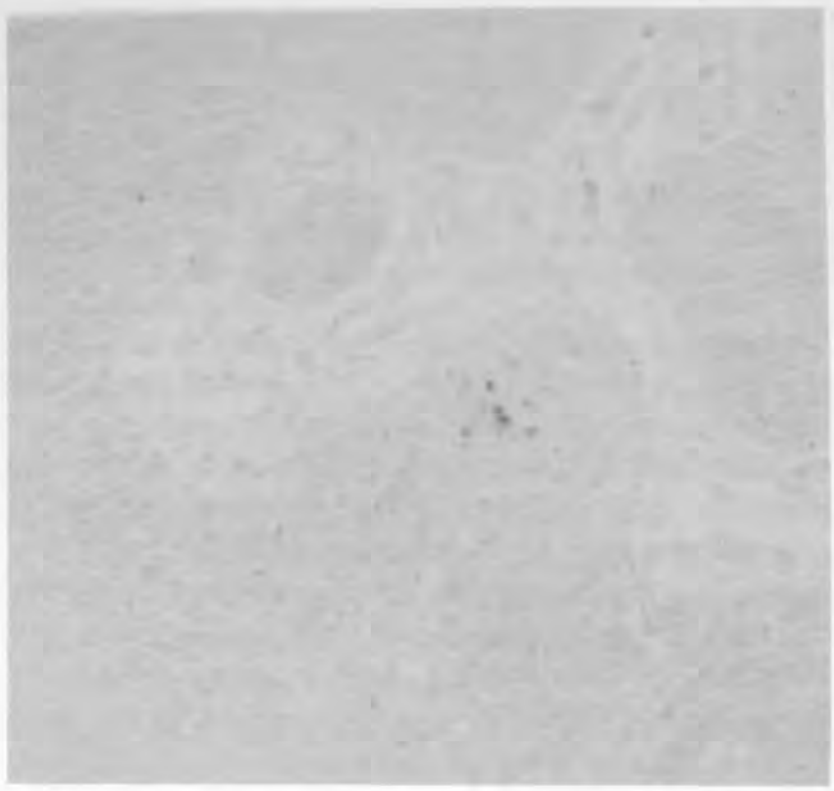

Fig. 8: Squamous celle carcinoma of the tongue. Lokalisation of HPV-18 structural proteins in the nuclei (arrow).
Fig. 8: Carcinome épidermoïde de la langue. Localisation des protéines de structure du VPH-18 dans les noyaux.

DISCUSSION

Current methods for detecting papilloma virus are limited. No diagnostic serological tests are available as yet and HPV cannot be isolated from clinical specimens using current cell or tissue culture methods (Pfister and Fuchs, 1987; Spalholz and Howley, 1989; Syrjänen, 1987, Beutner, 1989). Histochemistry can detect HPV capsid antigens, but only in productive infections, this seriously limiting its diagnostic usefulness (Syrjänen, 1984).

The diagnosis of clinically manifest HPV infections has been based on histological examination of tissue biopsies. In unequivocal cases, the morphological characteristics and cytopathic changes in HPV should be revealed (Meisels et al., 1976, Purola and Savia, 1977; Syrjänen, 1984; Syrjänen et al., 1987). These techniques are insufficient, however, to disclose most of the subclinical and all of the latent infections as well as being unable to provide any information about the type of HPV involved (Syrjänen 1989, Wickenden et al., 1987; Toon et al., 1986).

Hybridization analysis of nucleic acid is the best procedure currently available for the diagnosis of HPV infections (Syrjänen, 1987; Zur Hausen, 1988;

Roman and Fife, 1989; Syrjänen, 1990). In situ hybridization allows for simultaneous identification and localization of infectous HPV cells and evaluation of the morphology lesions (Syrjänen, 1986). A sensitive and specific non-radioactive in situ test which leads to reliable detection and minimizes false-positive results can be used.

The viral genome of human papilloma virus particles is a double-stranded circular DNA surrounded by capsid made of structural proteins. In the vast majority of currently known HPV isolates, the size of the genome is about 7.9 Kb. Squamous epithelial cells are the only known target of HPV. In primary infections, the basal cells are suspected of being the sites of viral entry into the epithelium (Spalholz and Howley, 1989; Pfister and Fuchs, 1987). In productively infected squamous epithelium, viral DNA is found through out the entire epithelium, but mature HPV virions are found in the outer layers only, parallel with the expression of capsid proteins.

Although viral DNA is always present in every type of HPV infection, major viral particles are only produced in permissive infections. All other types of HPV infections are called non-permissive.

Accordingly, viral DNA can be found either in HPV virions, in episoms, or as host-cell genome-integrated sequences, depending upon the type of infection and in close correlation with the grade of lesion (Pfister and Fuchs, 1987; Spalholz and Howley, 1989, Dürst et al., 1987). Insofar, the DNA of a few HPV types has been found to be integrated in the host cell DNA, as we were also able to demonstrate.

HPV 6 and 11 are associated with benign lesions, whereas HPV 18 and 16 are associated with cervical intraepithelial neoplasia (CIN) and invasive cervical and oral cancers. The viral DNA in premalignant CIN lesions is episomal, freely replicating and produces infectious viruses. In malignant cells like those in the cases reported, the DNA is randomly integrated into the viral chromosomes. It appears, therefore, that there are two groups of HPV, one with low and one with oncogenic potential and that other factors, such as tobacco or alcohol, may be necessary for progression to malignancy.

In accordance to other investigations where HPVs predominantly of types 2, 11, 16 und 18 in head and neck squamous cell carcinoma have been identified, especially in oral carcinomas of the buccal mucosa and the floor of mouth by hybridization studies (Loening et al., 1985; Syrjänen et al., 1986, Maitland et al., 1987; De Villiers et al., 1985; Adler-Storthz et al., 1986, Syrjänen et al., 1989; Brandsma and Abramson, 1989), this study documents the findings of significantly high frequency of HPV-DNA types, particularly 6, 11, 16 and 18 in the carcinoma of tongue in non-smoking and drinking persons. In the present study, we demonstrated HPV viral genomes carcinoma of the tongue, We interpret these findings as a strong evidence against HPV being an inadvertent or incidental infection.

Our data point to HPV as an important etiological factor in the histogenesis of squamous cell carcinoma of the tongue.

The mechanism of HPV oncogenesis remain hypothetical but are interlocked with the virus distinctive infectious cycle and synergism between the infection and other initiators (Zur Hausen, 1982). HPV seem to require cells that are still capable of dividing, like oral mucosa, for successful infections. Uptake of the cells results in its episomal persistence in turn stimulating and enhanced proliferation. Independent viral replication seems to be locked by host-cell factors that are released or become less stringent as the epitelial cells differentiate.

Based on the above data, it is difficult to prove a direct involvement of the potentially carcinogenic virus in oral cancer development. A number of other DNA viruses, for which the evidence of carcinogenic potentional in human cancer is considerably than that for HPV, are also present in high proportion of the population and few develop cancer like Epstein Barr virus, which is related to nasopharyngeal carcinoma.

Therefore other factors must play a role if virus is related to oncogenesis.

According to Zur Hausen 1986, the development of carcinoma is a result of failing or a failed intracelular control of persisting viral genomes in proliferating cells. It has been hypothesed that a defect in intercellular surveillance mechanism may be important in HPV associated oncogenesis (Zur Hausen, 1986; Schwarz et al., 1987) and that a specific cellular defence mechanism acting against cancer development, known as antioncogene, might possibly be mutated by viruses.

Alignment of HPV-DNA sequences, which were divided into early (E) and late (L) regions reveals a similar genetic organization of regions that for viral

proteins designated open reading frames (ORFS) (Broker, 1987). The region are designated E1 to E7 appear to be express soon after infection and code for proteins involved in the induction and regulation of DNA synthesis (Mc Cane, 1988).

It has been shown that for instance HPV 16 E6 and E7 geneproducts may act against the antioncogenes (Mc Cane, 1988). They are also able to bind to various human geneproducts, particularly the P53-genes, which appear to regulate cell proliferation and differentiation (Dyson et al., 1989; Levine et al., 1991). Removal of this gene by complexing with a viral coded product could disrupt normal control mechanism, which could result in a reduced capacity to differentiate and greater potentional to replicate (Scully, 1992).

On the other site, HPV may act synergistically with tobacco and alcohol. This hypothesis is consistent with the conclusion by Gissman 1984 that HPV alone is insufficient or incapable of induction or initiation of malignant growth but seems to require the synergistic action of chemical and/or physical cocarcinogens.

Further investigations are necessary to identify and characterize the virus in order to elucidate other cofactors (Hayes et al., 1987; Gayant and Yeole, 1987; Zaridze et al., 1985) and identify additional viruses in the mechanism of malignant transformation in oral cancers, the goal being sufficient treatment and prevention of oral cancer. Our patients in whom HPV 16+18 were detected receive targeted follow-up care and management.

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