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Detection of human papillomavirus DNA sequences in leucocytes: a new approach to identify hematological markers of HPV infection in patients with oral SCC

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SUMMARY

Squamous cell carcinoma of oral mucosa was shown to be associated with human papillomavirus (HPV) infection. The aim of this study was to find a hematological marker for HPV infection in patients with extensive HPV positive squamous cell carcinoma of the anterior oral cavity. In forty patients, referred to our clinic, suffering from histopathologically confirmed squamous cell carcinoma of the oral cavity with a tumor index T3/N2/M0 preoperative blood probes were taken. Human leucocytes were gained by FACS-lysis centrifugation. Paraffin-embedded sections of tumor tissue were deparaffinized and proteolytically digested to expose fixed target DNA.
For detection of human papillomavirus, DNA sequences 6, 11, 16, 18, 31 and 33 a non radiocative *in situ* hybridization technique was used (Biohid®). For the first time we demonstrated HPV DNA sequences in leucocytes of patients with HPV positive oral SCC. Furthermore we found a correlation in type between HPV DNA sequences in tumor tissue and in leucocytes. This might lead to HPV as a hematological marker for follow up of patients with oral SCC and have consequences on therapy.

KEY WORDS:

Human papillomavirus, oral carcinoma, human leucocytes, carcinogenesis, hybridization.

RÉSUMÉ

Il a déjà été démontré que le cancer épidermoïde de la muqueuse orale pouvait être associé à une infection par le virus du papillome humain. Le but de cette étude a été de trouver un marqueur hématologique pour une infection à HPV chez des patients porteurs d'un cancer envahissant de la portion antérieure de la cavité orale HPV positif. Des prélèvements sanguins ont été effectués chez 4 patients adressés à notre clinique, présentant un cancer épidemoïde de la cavité orale histologiquement confirmé, avec un index tumoral T3/N2/M0. Les leucocytes humains ont été isolés par centrifugation FACSlyse. Des coupes incluses à la paraffine de tissus tumoraux ont été déparaffinés et soumises à une digestion par protéolyse afin d'être exposées à la fixation de la cible d'ADN.

Une technique d'hybridation *in situ* non radio-acitve (Biohit ®) a été utilisée afin de détecter les séquences 6, 11, 16, 18, 31 et 33 des virus du papillome humain. Nous sommes les premiers à avoir démontré la présence de séquences d'ADN de HPV dans les leucocytes de patients porteurs de cancers oraux HPV positifs. De plus, nous avons trouvé une corrélation entre les types des séquences d'ADN de HPV du tissu tumoral et des leucocytes. Il nous est possible ainsi de disposer d'un marqueur hématologique pour suivre l'évolution des patients porteurs de cancers de la bouche et d'envisager de nouvelles perspectives thérapeutiques.

MOTS CLÉS:

Virus du papillome humain, cancer de la bouche, leucocytes humains, cancérogenèse, hybridation.

INTRODUCTION

Human papillomaviruses (HPV) are a group of heterogeneous viruses, some of which infect anogenital epithelia and are implicated in the etiology of cervical cancer (Pfister, 1984) as well as with oral cancer (Scully, 1983; Scully *et al.*, 1983; Hönig, 1992; Eberlein-Gonska *et al.*, 1994).

HPV DNA is present in the majority of premalignant lesions (Eberlein-Gonska *et al.*, 1994) and malignant cancers of the oral cavity, with HPV type 16 (HPV 16) and 18 (HPV 18) being the most commonly to be identified in such lesions (Scully *et al.*, 1983; Hönig, 1992; Eberlein-Gonska *et al.*, 1994). Integrated copies of viral DNA are found in invasive carcinomas, while non-integrated (episomal) copies are found in premalignant lesions (Dürst *et al.*, 1985; Gissmann *et al.*, 1983). Genetic analysis has shown that the open reading frames (ORFs) of some papillomaviruses are necessary and sufficient for efficient cellular transformation. The E6 and E7 ORFs of HPV 16 has been shown to possess transforming activity for mouse fibroblasts (Yasumoto *et al.*, 1986) and primary human keratinocytes (Pirisi *et al.*, 1987). The E7 gene product can also cooperate with an activated ras oncogene to fully transform and immortalize primary rodent cells (Matleshewski *et al.*, 1987; Phelps *et al.*, 1988; Storey *et al.*, 1988), and its continued expression has been shown to be required for maintenance of this transformed phenotype (Crook *et al.*, 1988). The E6 and E7 phenotype can also bind to cellular p53 protein (Werness *et al.*, 1990) and the retinoblastoma gene product (pRb-105) (Dyson *et al.*, 1989), respectively.

Both of these proteins belong to a class of tumor suppressor proteins which appear to play an important role in suppressing the transformed phenotype (Finlay *et al.*, 1989; Huang *et al.*, 1988). Finally, HPV-16 and 18 E7 is the most abundant viral protein in CaSki and 'SiHa cells (Seedorf *et al.*, 1987) and in HPV 16-containing cervical carcinoma biopsy tissue (Smotkin and Wettstein, 1987).

It is clear from human and animal studies that papillomaviruses contribute significantly to the development of many carcinomas, but we still do not have a clear understanding of the importance of other interacting viral, chemical or cellular factors. The immunological approach to the prevention of HPV diseases requires a thorough analysis of the viral proteins against which humoral and cellular immune responses are mounted during and after infection. At present, although there is little direct evidence for the efficacy of either humoral or cell-mediated immunity on the control of HPV infection, an important role for T-cell monitoring has been postulated because of the link between HPV disease and cellular immunodeficiency (Frazer *et al.*, 1986; Halpert *et al.*, 1986; Kirchner, 1986; Malejczyk *et al.*, 1989, Morison, 1975; Schneider *et al.*, 1983; Spencer *et al.*, 1970). However, the nature of this response has yet to be defined.

Epidemiologic studies would be important and useful to define the role of HPV 16 in cancer development (Kanda *et al.*, 1992).

Serological tests for the detection of antibodies to HPV proteins in human sera were developed in order to obtain useful diagnostic markers for papillomavirus-associated diseases but serological studies have been scarse, as compared with studies by nucleic acid hybridization techniques, because of the lack of the cell culture producing appropriate viral antigens. The immune response to papillomavirus infections so far is only poorly characterized by the lack of experimental systems for the production of viral proteins that can be used as antigens in serological assays (Gissmann *et al.*, 1992).

For sufficient treatment and prevention of oral cancer in the light of virustatics and chemotherapeutics, we tried to detect HPV-DNA in human leucocytes. The aim of this study was to develop a new and simple technique for serological studies by direct detection of HPV-DNA sequences in human blood cells and to find a relation between HPV infection of human leucocytes and primary tumor tissue in patients with extensive HPV positive squamous cell carcinoma of the anterior oral cavity. Therefore we developed a new procedure for detection of HPV in human leucocytes to evaluate clinical course and leertaste may be to find consequences on treatment and prophylaxis of oral SCC.

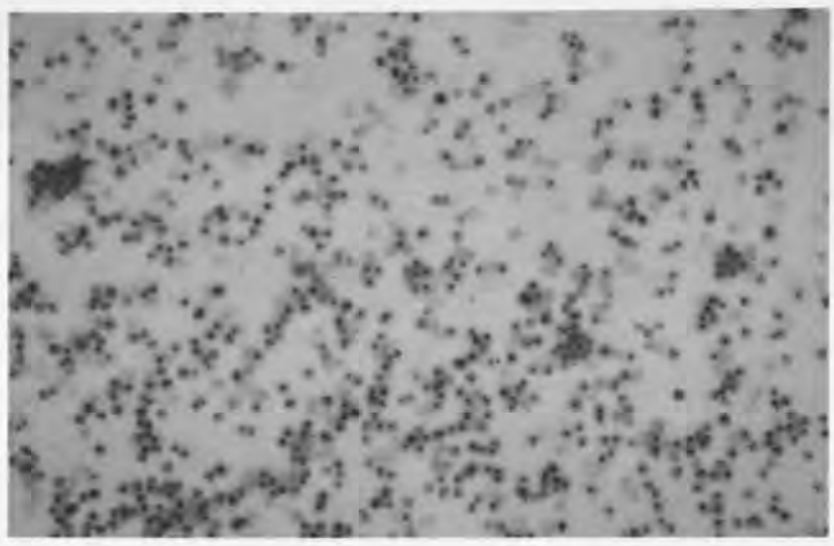

Fig. 1: In situ typing test; positive control. By this test specific HPV-DNA can be identified (×240).
Fig. 1: Test de typage in situ: contrôle positif. L'ADN-HPV peut être identifié par ce test (×240).

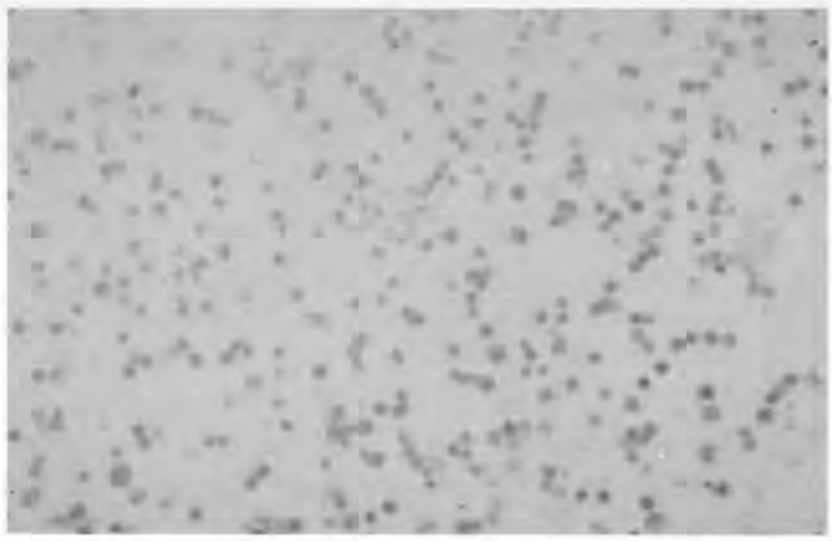

Fig. 2: In situ typing test; negative control (×240).
Fig. 1: Test de typage in situ: contrôle négatif (×240).

MATERIAL AND METHODS

In forty patients, referred to our clinic, suffering from histopathologically confirmed squamous cell carcinoma of the oral cavity with a tumor index T3/N2/M0 preoperative blood probes were taken.

Human leucocytes were gained by simultaneous lysis of red blood cells and partial fixation of white cells was performed by a proprietary buffered solution containing <15% formaldehyde and <50% diethylene glycol (FACS-lysis).

After lysis with FACS-solution and two wash steps with PBS (Phosphate buffered saline without calcium, magnesium or phenol red, containing 0.1% sodium azide) leucocyte suspension was gained containing 7000-10000 cells/μl.

20μl of suspension were pipetted on biotin-covered slides, fixed with ethanol and frozen by −20°C or stained directly. Paraffin-embedded sections of tumor tissue were deparaffinized and proteolytically digested to expose fixed target DNA.

For detection of HPV-DNA sequences 6, 11, 16, 18, 31 and 33 a non radioactive *in situ* hybridization technique was used (Biohit®) (Hönig, 1991).

Nucleic acid is visualized by hybridization of the labeled HPV-DNA probes with target DNA. For visualizing distinct HPV types HPV DNA probes of HPV-6, -11, -16, -18, -31 and -33 were used. A negative control probe containing pBR 322 ensured that the tissue sections contained no false positives (Fig. 2). To assure that the staining procedures were run correctly positive controle slides were provided (Fig. 1).

Biotinylated probes were applied to the section. Double stranded probe and target DNA were simultaneously denaturated to single strands by heating (6-8 min, 95±2°C). The homologous sequences of the probe and target DNA specifically annealed during the subsequent hybridization step.

Hybridization between DNA in specimen and the HPV related probe was determined by the use of enzyme-substrate reaction.

First, a complex of alkaline phosphatase and streptavidin is bound to the biotin of the hybridized HPV DNA probes (Syrjänen *et al.*, 1987 & 1988). In the second step the entire complex is visualized after con-

version of the substrate 5-bromo-4-chlor-3-indolphosphate (BCIP) in the presence of chromogen, nitroblue tetrazolium (NBT), into a HPV DNA localizing purple precipitate.
Examination of target the DNA (Fig. 3-5).

The data were compared to a control group of twenty blood probes taken from healthy patients.

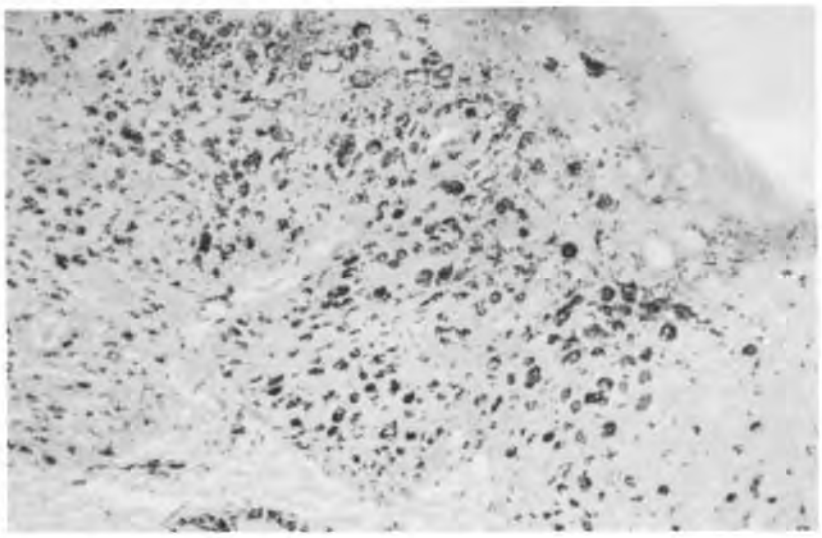

Fig. 3: SCC of the anterior oral floor stained with HPV-18-probe. Localisation of HPV-18 structural proteins in the nuclei (×240).
Fig. 3: Cancer épidermoïde du plancher de la bouche coloré avec le test pour HPV-18. Localisation des protéines de structure de HPV-18 dans les noyaux (×240).

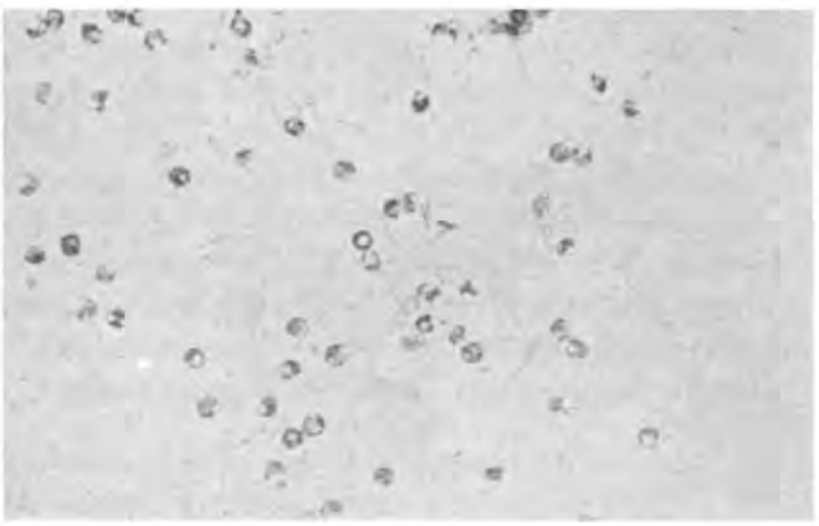

Fig. 4: Human leucocytes of patients with HPV-18 positive oral SCC (Fig. 3); remark the episomal viral DNA revealed by the purple precipitate (×240).
Fig. 4: Leucocytes humains de patients porteurs d'un cancer épidermoïde HPV-18 positif (Fig. 3); remarquer l'épisome viral ADN révélé par la réaction (±240).

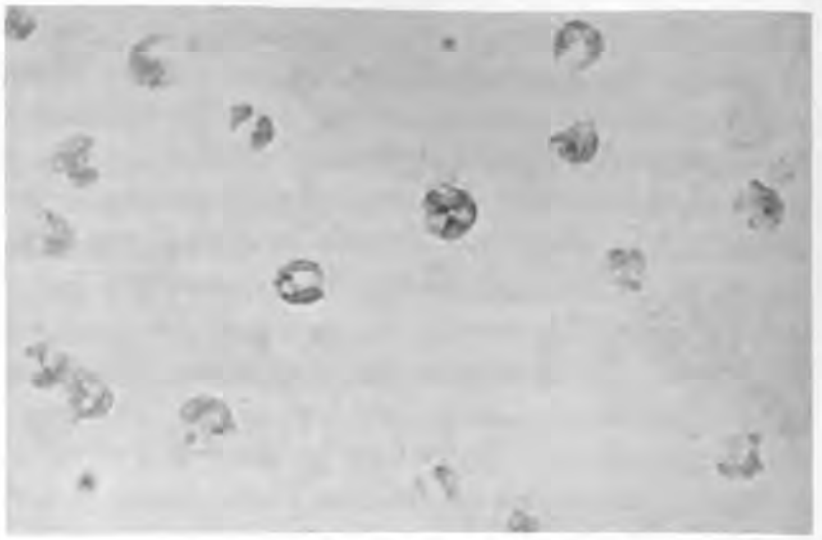

Fig. 5: Human leucocytes stained positive for HPV 16 (purple precipitate) in a patient with HPV positive oral SCC (×300).
Fig. 5: Leucocytes humains positifs pour HPV 16 chez un patient porteur d'un cancer épidermoïde HPV positif (×300).

STATISTICAL ANALYSIS

The data were analysed by t-test. Furthermore the correlation was determined.

RESULTS

In the paraffin-embedded tumor tissue and infiltrated lymphatic nodes in 70% a positive reaction for episomal viral DNA 6, 11, 16, 18 and 31, 33 could be seen (Fig. 4). HPV type 6 was detected in 25%, HPV 11 in 15%, HPV 16 in 60%, HPV 18 in 70%, HPV 31 in 10 and HPV 33 in 30%.

In 92.86% of these cases papillomavirus DNA of the same type could be detected in leucocytes in preoperative blood samples (Fig. 3 and 5).

In 65% of all cases HPV-DNA could be detected in human leucocytes showing an intranuclear purple precipitate (Fig. 6). Especially «high risk» HPV types 16 in 55% and 18 in 65% were the most common types to be found.
Furthermore in 25% HPV 6, in 15% HPV 11 and in 25% HPV 33 were found in leucocytes, whereas HPV 31 was detected in none of all cases.

The correlation (r) between positive detection of HPV in primary tumor tissue and in human leucocytes was 0.987.
HPV-16 and 18 sequences were not detected in leucocytes of healthy patients. In 20% HPV 6 DNA sequences were found.

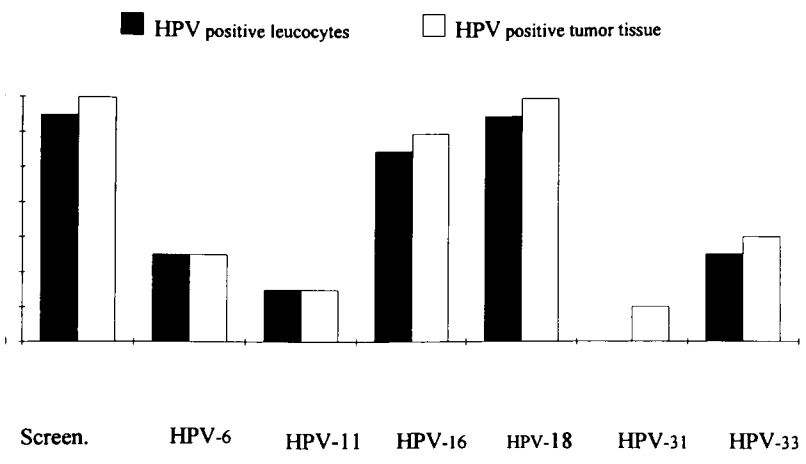

Fig. 6: Prevalence of HPV in human leucocytes and tumor tissue of patients with oral SCC. In nearly all cases of HPV positive tumor tissue HPV of the same type could be detected in leucocytes as well.
Fig. 6: Présence de HPV dans des leucocytes humains et dans la tumeur de patients avec un cancer épidermoïde. Dans presque tous les cas de tumeurs HPV postives, l'HPV de même type peut être identifié dans les leucocytes également.

DISCUSSION

The prevalence of HPV-DNA detected in human leucocytes shows a significant relation ($p < 0.005$) to HPV found in primary tumor tissue. If HPV-16 and 18 could be detected in primary tumor tissue, they could be detected in leucocytes as well. For the first time we demonstrated HPV-DNA sequences in leucocytes from patients with oral SCC and lymph node metastases. Thereby we found a correlation between HPV prevalence in primary tumor tissue, regionaly lymph node metastases and leucocytes ($r = 0.987$), as other ways of infection could be excluded by clinical investigation.

It has been established in several studies that antibodies to HPV-16 and 18 correlate with cancer of the uterine cervix and antibodies against viral proteins are discussed as serological marker (Bleul *et al.*, 1991; Gissmann *et al.*, 1992; Köchel *et al.*, 1991). The inability to propagate HPVs in culture and the paucity of viral particles in clinical lesions have limited the availability of viral proteins to serve as antigen targets in immunological assays. To circumvent this problem, a number of investigators used HPV proteins or peptides expressed by bacteria to detect HPV antibodies (reviewed in Galloway, 1992). However these serological markers were disappointing for following reasons:

- even among cases with HPV-proven cancers, only about 50% were positive for antibodies to HPV (Galloway, 1994),
- there are subtypes of HPV 16 and HPV 18 which differ in their E7 epitope from the prototype whose sequences were used for the respective synthetic protein (Gissmann *et al.*, 1992) and
- in certain number of cases antibody response is impaired, since papillomaviruses interfere with MHC class II expressing cells within infected epithelium (Gissmann *et al.*, 1992),
- there was no increased risk for antibodies to E7 among patients with cervical carcinoma *in situ*, the most proximal precursor to invasive cancer (Gissmann *et al.*, 1992).

The strongest association of E7 seropositivity was the late-stage cervical cancer. Although an effective antibody response against infection with HPV (perhaps directed towards the viral capsid protein) may be important in the early stages of infection, it is likely that a functional T-cell response against other viral antigens is indispensable for the ultimate control of disease. The recognition of T-cell determinants by T cells (particularly T helper cells) contributes significantly to the cell mediated immune response against infectious organisms as T cells cooperate with B cells in the induction and transformed by HPV16E7 and ras is significant, as these epitopes may represented particularly immunodominant regions common to different species (Commerford *et al.*, 1991). Taken together, these results indicate that antibodies to viral antigenes, esp.E7 are not likely to be a prognostic marker to identify individuals at risk of progression or even a reliable mean to identify cancer patients (Galloway, 1994).

In contrast the detection of episomal HPV DNA in human leucocytes seems to be a sensitive and simple method for virus detection in blood of carcinoma patients. The advantages are beside high sensivity, a simple test and a diminishing of false positive results by *in situ* hybridization (Syrjänen, 1990). Especially oral cancer tissue reveals bacterial superinfection as shown in own investigation, leading to false positive signals in a polychain reaction (Syrjänen, 1988).

Nevertheless it remains to be elucidated, wether testing human leucocytes for HPV can be used as screening test for oral SCC, esp. in case of tumor recurrence. We are developing not only a qualitative but a quantitative method by counting positive leucocytes in relation to negatives for further more sophisticated investigation of clinical course in case of HPV-positive oral SCC under standard condi-

tions. Furthermore we are planing a longterm follow-up for investigating HPV under therapeutical means.

Our results may have consequences on pretherapeutic for clinical course or even therapeutic means in case of detecting «high risk HPV» for example in biopsies or leucocytes. HPV or viral proteins might act as maker in human leucocytes or in human serum. Therapeutically the addition of virustatics to conventional chemotherapy might be discussed.

CONCLUSION

Our results confirms the role of human papilloma virus in oral carcinogenesis and might lead to HPV detection in blood as a screening in follow-up examination. Therapeutically the addition of virustatics to conventional chemotherapy might be suggested. Thereby it might be possible to reduce metastases and tumor growth.

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