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 radioactive 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 épidermoïde 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.
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; Höning, 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; Höning, 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 scarce, 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.

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 denatured 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-indolophosphate (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.

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.
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 antigens, 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-
CONCLUSION

Our results confirm 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.

LITERATURE


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