Increase of proliferating cell nuclear antigen (PCNA) expression in HPV-18 positive oral squamous cell carcinomas

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Abstract

Several studies have established a probable relationship between HPV infections and oral neoplasia. The present study analyze the importance of the integration of DNA of HPV type 18 on the proliferative capability of oral squamous cell carcinoma (OSCC) by the study of PCNA expression. Thirty seven patients with OSCC were studied. A complete clinical history and histopathological study was performed. DNA of HPV-18 was found in 7 of the 37 oral OSCCs (19.1 %). PCNA expression appeared in 75.7 % of OSCCs (28 cases). 9 OSCCs (24.3 %) were completely negative, whereas 13 (35.1 %), 8 (21.6 %) and 7 (18.9 %) show minimal, moderate and intense PCNA expression. 4 HPV-18 positive OSCCs showed a intense PCNA expression, 1 OSCC showed moderate PCNA expression, whereas 2 OCSSs showed minimal PCNA expression. Statistical correlations between PCNA expression and DNA HPV-18 amplification showed a more intense PCNA expression in HPV-18 positive OSCCs (p=0.023). Further studies are needed to establish whether the increase of cellular proliferation induced by HPV-18 has prognostic consequences.

KEY WORDS

HPV, Oral squamous celle carcinoma, Proliferating celle nuclear antigen.

INTRODUCTION

Human papillomavirus (HPV) has a well known association with dysplasia and carcinoma of the uterine cervix and vulva [40]. In recent years, several studies have established a probable relationship between HPV infections and oral neoplasia [25]. HPVs include at least 60 genotypes, of which HPV types 6, 11, 16 and 18 are most commonly isolated from oral lesions. In the oral cavity, HPV types 6 and 11 are frequently associated with squamous cell papillomas, whereas types 16 and 18 are associated more frequently with dysplasia and carcinomas [42].

HPV types 16 and 18 can immortalize primary human keratinocytes in cell culture [30, 18, 21, 45, 23, 16]. Products from E6 and E7 ORFs of HPV types 16 and 18 have been shown to form complexes with the normal cellular proteins p53 and the retinoblastoma gene product Rb [12, 44]. In addition, HPV-infected cells show increased levels of some cyclins when compared with non-infected cells [31]. It has been suggested that the effects that these complexes have on cell cycle control could contribute to malignant progression in cells harbouring HPV sequences [12, 44, 7, 43]. Proliferating cell nuclear antigen (PCNA) – also called cyclin – is an acid nuclear protein of 36 KDa with an isoelectric point of 4.8 KDa which is expressed by neoplastic and non-neoplastic cells undergoing division [2]. PCNA acts as cofactor for delta DNA polymerase and its distribution in the cell cycle, increasing through G1, peaking at the G1/S-phase interface, decreasing through G2, and reaching low levels which are virtually undetectable by immunohistochemical methods in M-phase and quiescent cell [6]. The recent availability of monoclonal anti-PCNA antibodies that function in routinely fixed and processed tissues has permitted the assessment of the proliferative capability of a variety of lesions [24] which are difficult to obtain as fresh or unfixed tissues.

The purpose of this study was to analyze the importance of the integration of DNA of HPV type 18 – a high risk neoplastic transformation type-on the proliferative capability of oral squamous cell carcinoma (OSCC) by the study of PCNA expression.

MATERIAL AND METHODS

Thirty seven patients with OSCC were studied in the Oral Medicine Department of the University of Granada (Spain) between 1989 and 1991, and were revised in June 1994. 70.3 % (26 cases) were males and 29.7 % (11 cases) were females, all within an age range of 22 to 87 years (mean : 60 years, standard deviation : 14). A complete clinical history was performed and the patients were questioned about some habits such as tobacco use, consumption of alcohol, oral hygiene and traumatic factors affecting the oral mucosa. Also the condition of cervical nodes at the moment of diagnosis, tumoral location, tumoral size, clinical appearance of the lesion, existence of precancerous lesions, and TNM stage were noted.

Samples of all OSCCs were formalin-fixed and paraffin-embedded. Sections were H & E stained. The histopathological study by an experienced pathologist included type of tumoral growth (solid mass, cords, little groups or dissociated malignant cells), keratin production (absent, minimum, moderate, maximum), number of cells in mitosis, and cytological atypia. The methods of quantifying parameters noted above are shown in table 1.

Descriptive statistics for the variables analyzed were performed by using BMDP [10].

TABLE 1
Histopathological description of the studied tumors (n= 37).

Variable	Distribution	n (%)
Tumoral growth[a]	Solid	25 (67.6%)
	Cords	3 (8.1%)
	Small groups of tumoral cells	7 (18.9%)
	Dissociated cells	2 (5.4%)
Keratin production[a]	Absent	2 (5.4%)
	Minimum	1 (2.7%)
	Moderated	18 (48.6%)
	Maximum	16 (43.2%)
N° of mitoses	0-1/8 fields/40x	23 (62.2%)
	1-2/8 fields/40x	6 (16.2%)
	2-5/8 fields/40x	6 (16.2%)
	>5/8 fields/40x	2 (5.4%)
Cytological abnormality[a]	Grade I	8 (21.6%)
	Grade II	25 (67.6%)
	Grade III	4 (10.8%)

[a] The quantifying of theses parameters was based on the personal experience of the oral pathologist.

CELL CULTURE

The HeLa cell line was obtained from the American type culture collection (Rockville, MD) and cells were cultured in RPMI-1640 medium supplemented with 10 % fetal calf serum. HeLa cells contain HPV 18 sequences [19, 36].

AMPLIFICATION OF HPV SEQUENCES

Multiple (3 to 5) 10 µm sections of the paraffin-embedded tissues obtained from each patient were packed into 0.5 mL eppendorf microcentrifuge tubes, dewaxed with xylene, washed with ethanol and digested for 24 hr at 37° C in an extraction buffer containing 50 mM KC1,

10 mM Tris-HC1 (pH= 8.3), 2.5 mM KC1, 0.1 mg/mL gelatin, 0.45 % nonidet-P40, 0.45 % Tween 20 and 60 µg/mL proteinase K. The samples were heated to 95° C for 8 min to inactive the proteinase K, centrifuged (10,000 g/10 min) to pellet the debris, and 10 µL of the supernatants analyzed by PCR. PCR amplifications were performed using a DNA thermal cycler (PERKIN-ELMER-CETUS, Norwalke, CT). The total reaction volume was 100 µL, containing 10 mM Tris-HC1 (pH 8.3), 50 mM KC1, 1.5 mM $MgC1_2$, 200 µM each of dATP, d CTP, dGTP, and dTTP, as well as 1 µM of each primer. Primers specific for a segment of gene E_6 (HPV 18) were synthesized by Eurogentec (Liège, Belgium) (see Table 2).

Finally, 2.5 units of thermostable Taq polymerase (AMPLITAQ, PERKIN-ELMER-CETUS) were added and the mixture was submitted to 32 cycles of amplifications with the following profile; denaturation for 2 min at 94° C; primer annealing for 2 min at 55° C, and primer extension for 1 min at 72° C.

PCR assay was performed using both positive and negative controls. Positive controls consisted of DNA extracted from HPV-containing cell line HeLa.

TABLE 2
Oligonucleotide primer and probe sequences used to detect HPV 18 DNA by PCR.

HPV type	Primer location	Sequence	Product length (bp)
18-upstream	E6 (102-125)	5'-ACT ATG GCG CGC TTT GAG GAT CCA-3'	
18-downstream	E6 (417-436)	5'-GGT TTC TGG CAC CGC AGG CA-3'	334
18-probe	E6 (361-399)	5'-ATG GAG ACA CAT TGG AAA AAC TAA CTA ACA CTG GGT TAT-3'	

The reaction products were analyzed by a dot-blot hybridization assay using 32p-labelled oligomer probes specific for HPV 18 (Table 2) [28]. The filter was prehybridized for 1 hr at 42° C in 3xSSPE, 5x Denhardt's, 0.5 % SDS before adding 32p-end-labelled oligomers [33] and then hybridized for 1 h. at 42° C in the same solution. The filter was washed in 1x SSPE, 0.1 % SDS 3 times for 5 min at room temperature, once at 55° C for 10 min and finally once at room temperature for 5 min. Autoradiography was performed at –70° C on Kodak X-AR film and exposed for 1 h. Samples showing amplification below or equal to the negative controls cells were considered negative.

IMMUNOCYTOCHEMISTRY

Immunocytochemistry was performed with PC10 (Biomeda), a monoclonal antibody that detects PCNA in dividing cells [17]. After dewaxing and pretreatment with 3 % hydrogen peroxide, reactions were incubated overnight at 4° C with PC10 at 1:20 dilution. The secondary antibody was rabbit anti-rat (Supersensitive Multilink-HRP/DAB, Biogenex). Reactivity was detected using peroxidase-streptavidin conjugate (label supersensitive, Biogenex) at 1:1000 dilution, followed by reaction with an aminoethylcarbamazole staining Kit (Liquid Oab Chromogen) for 6 min at room temperature. The sections were then counterstained with haematoxylin.

RESULTS

Table 3 shows clinical descriptions of OSCCs collected. The histopathological results are shown in Table 1.

TABLE 3
Clinical variables of the studied tumors (n= 37).

Variable	Distribution	n (%)
Tumoral location[a]	Tongue base	3 (8.1%)
	Lateral margin of the tongue	6 (16.2%)
	Floor of the mouth	5 (13.5%)
	Gingiva	9 (24.3%)
	Buccal mucosa	14 (37.8%)
Clinical appearance[a]	Exophitic	5 (14.3%)
	Endophitic	3 (8.6%)
	Ulcerated	24 (68.6%)
	Verrucous	3 (8.6%)
Tumor arise from precancerous lesion	No	31 (83.8%)
	Yes	6 (16.2%)
Tumoral size	< 2 cm	4 (10.8%)
	2-4 cm	9 (24.3%)
	> 4 cm	24 (64.9%)
Affect on cervical nodes at diagnosis	N0	22 (59.5%)
	N1	11 (29.7%)
	N2a	1 (2.7%)
	N2b	2 (5.4%)
	N2c	1 (2.7%)
Stage (TNM)	I	4 (10.4%)
	II	5 (13.8%)
	III	11 (29.7%)
	IV	17 (45.9%)

[a] 2 unknown.

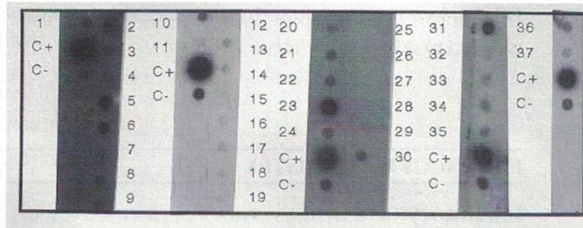

Fig. 1
Autoradiograph of the PCR amplification product.

DNA of HPV-18 was found in 7 of the 37 oral OSCCs (19.1%). The autoradiograph of the PCR amplification product is presented in figure 1.

PCNA expression appeared in 75.7 % of OSCCs (28 cases). 9 OSCCs (24.3%) were completely negative, whereas 13 (35.1%), 8 (21.6%) and 7 (18.9%) (figure 2) show minimal, moderate and intense PCNA expression.

4 HPV-18 positive OSCCs showed a intense PCNA expression, 1 OSCC showed moderate PCNA expression, whereas 2 OSCCs showed minimal PCNA expression.

Statistical correlations between PCNA expression and DNA HPV-18 amplification showed a more intense PCNA expression in HPV-18 positive OSCCs (p=0.023). No statistical relationship was found when other parameters were compared.

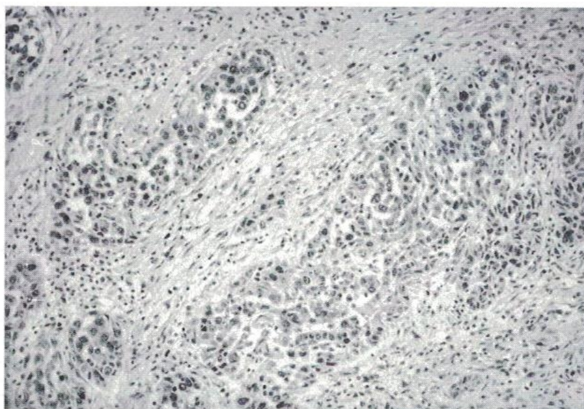

Fig. 2
Intense PCNA expression in OSCCs.

DISCUSSION

Human papillomavirus is a group of DNA viruses that show predilection for epithelial tissues of different organs of the body. In oral tissues, sixteen HPV-DNA genotypes have been isolated from oral lesions [29, 3]. HPV types 6, 11, 13 and 32, so called low-risk HPVs, are related to benign papillomatous lesions of the oral cavity. In contrast, high-risk HPV genotypes [16, 18, 31, 33, 35] have increased malignant potential and are frequently associated with oral squamous cell carcinoma (OSCC) [37, 46, 25, 4]. Infection with HPV may result in stimulation of oncogenes and proto-oncogenes, overexpression of growth factor receptors and inactivation of certain tumor suppressor genes [47, 34, 22].

Interaction between these viruses and the cell division cycle of oral epithelial cells are complex and it is not the same in different HPV types [9]. This different behaviour, is probably related to the molecular interactions between HPV-DNA and cellular proteins that regulate the proliferation and differentiation processes.

The role of HPV in the pathogenesis of oral squamous cell carcinoma (OSCC) remains unclear [15]. However, different reports have analyzed OSCCs for HPV DNA finding a mean prevalence of 26.2 %. High-risk HPVs-(16 and 18) were detected significantly more often (80.0%) in HPV-positive OSCCs than in low-risk HPVs-(6/11) (16.3%) [27]. The frequent occurrence of high-risk HPV types 16 and 18 is suggestive of a causative role for HPV in a considerable number of OSCCs [32].

In the context of the multi-step theory of malignant progression, the cellular integration of the HPV DNA alone probably cannot lead to the oncogenic transformation state. HPV 16 and 18 sequences affect cell cycle control by the interaction of E6 and E7 HPV oncoproteins with cellular p53 and Rb protein [11, 30]. These proteins are suspected of playing a role either in the entry of cells into GO, or preventing them from making the transition from G1 to the S phase of the cell cycle [7, 20, 8, 5]. E6 and E7 oncoproteins form a potentially inactivating complex with p53 and Rb cellular proteins [12, 44]. Maintenance of proliferative activity could contribute to the multistep carcinogenic process by allowing epithelial cells to go through an abnormally high number of mitoses, increasing the chances of a secondary tumorigenic event might occur.

This increase of cell proliferation can be tested by different methods, including detection of PCNA with a simple immunohistochemical assay. PCNA is an auxiliary protein of delta DNA polymerase, and its expression is associated with the S phase of the cell cycle [14, 1, 2]. In the present study a significant correlation (p=0.023)

between PCNA expression and HPV-18 positive OSCCs was found and so the authors are in agreement with the theory postulating that high-risk HPV could be related to the oncogenic process by interactions of the oncoproteins E6 and E7 with cellular protein regulators of the cell cycle.

Increased PCNA expression has been related to histologic grade, mitotic index, and lymph node metastasis [38, 39, 25]. Elevated PCNA levels also are correlated with decreased disease-free state and survival [39, 35]. These results, according to data reported in other studies [26], suggest that this increase in PCNA expression could be a consequence of integration of HPV 18 DNA in the genome of epithelial cells. TSUJI *et al.* [41] found significant differences in PCNA score between malignant and non-malignant lesions, suggesting a difference in growth activity. The mean PCNA score decreased significantly from 20 % to 8 % after cancer chemotherapy.

FLAITZ *et al.* [13] found PCNA expression in 88 % of tumour cells of the cases included in their study. This high degree of expression of PCNA is indicative of an aggressive neoplasm associated with a decrease in disease-free periods, the tendency to metastasize, and the decrease in overall survival.

In the present study a significant correlation (p=0.023) between PCNA expression and HPV-18 positive OSCCs was found and so the authors are in agreement with the theory postulating that high-risk HPV could be related to the oncogenic process by interaction of the oncoproteins E6 and E7 with cellular protein.

These results suggest an increased proliferative activity of HPV-18 positive OSCCs conditioned through PCNA expression. Further studies are needed to establish whether the increase of cellular proliferation induced by HPV-18 has prognostic consequences.

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