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Detection of HPV DNA by *in situ* hybridization in benign, premalignant and malignant lesions of the oral mucosa

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SUMMARY

Evidence has accumulated in recent years that relates certain types of human papillomaviruses (HPV) to the development of some tumors. We use *in situ* hybridization to study DNA from HPV 6/11, 16/18 and 31/33/35 in 6 squamous papillomas, 18 hyperkeratotic/acanthosis lesions with and without dysplasia (5 and 13 cases respectively) and 27 squamous cell carcinomas of the oral mucosa. HPV DNA was found in 66% of squamous papillomas, 38.4% of hyperkeratotic/acanthosis lesions without dysplasia, 60% of epithelial dysplasia and 37% of squamous cell carcinomas. HPV DNA 6/11 was the most common type found, and in squamous cell carcinomas HPV DNA appear more frequently in well differentiated tumors.

KEY WORDS:

HPV-DNA - *In situ* Hybridization - Oral mucosa

RÉSUMÉ

Au cours de ces dernières années, la relation de certains types de papillomavirus humains (HPV) avec le développement de certaines tumeurs, est devenue évidente. Nous avons utilisé l'hybridation *in situ* pour étudier l'ADN des HPV 6/11, 16/18 et 31/33/35 dans 6 papillomes, 18 lésions hyperkeratosiques/acanthosiques avec et sans dysplasie (5 et 13 cas respectivement) et 27 carcinomes oraux. Nous avons trouvé l'ADN de HPV chez le 66% des papillomes, 38.4% des lésions hyperkeratosiques/acanthosiques sans dysplasie, 60% des dysplasies épithéliales et 37% des carcinomes oraux. L'ADN de HPV 6/11 a été le type le plus fréquemment identifié. En ce qui concerne les carcinomes oraux, l'ADN de HPV a été identifié plus fréquemment dans les tumeurs bien différenciées.

MOTS CLÉS:

HPV-ADN - Hybridation *in situ* - Muqueuse orale

INTRODUCTION

Squamous cell carcinoma of the oral cavity is the most frequent type of malignant tumor arises from the oral mucosa. Its importance stems from its high mortality rate, and because many cases are preceded by a premalignant lesion.

In recent years, several viruses have been implicated in the development of some tumors. Human papillomavirus (HPV) have been related in etiological terms with the development of squamous cell carcinoma, especially in the lower genital tract and larynx (Zur Hausen, 1977; Gissman, 1984; Syrjanen et al., 1988). A relationship between several types of HPV and malignant lesions of the oral mucosa has also been described (Abdelsayed, 1991; Tsuchiya, 1991; Shroyer and Greer, 1991; Watts et al., 1991; Zeuss et al., 1991; Hönig, 1992).

We have use *in situ* hybridization (ISH) to investigate the presence of DNA from HPV 6/11, HPV 16/18 and HPV 31/33/35 in squamous papillomas (SP), hyperkeratotic/acanthosis with and without epithelial dysplasia (ED and H/A respectively) and squamous cell carcinomas (SCC) of the oral mucosa and its relationships with some prognostic histologic aspects.

MATERIALS AND METHODS

51 formalin-fixed, paraffin-embedded oral biopsies from the Department of Pathology of the University Hospital of Granada have been studied. These samples were diagnosed as SP (6 cases), H/A (13 cases), ED (5 cases) and SCC (27 cases), on the basis of light microscopic observations (hematoxylin-eosin stain). The malignant lesion were classified by degree of differentiation (keratin-production, nuclear atypicity, number of mitotic figures, pattern of stromal invasion) as well (17 cases), moderately well (3 cases) or poorly differentiated (7 cases).

Oral SP were found in 2 males and 4 females ranging in age from 32 to 58 yrs (mean 42 yrs). H/A were diagnosed in 7 males and 6 females ranging in age from 34 to 74 yrs (mean 49 yrs). ED was found in 1 male and 4 females ranging in age from 29 to 64 yrs (mean 44 yrs) and lesions diagnosed as SCC were from 24 males and 3 females ranging in age from 38 to 77 yrs (mean 59 yrs) (Table I).

In situ hybridization of HPV DNA was performed on 5 μm sections from formalin-fixed, paraffin-embedded tissue blocks with the HPV Viral Type *in situ* system (Life Technologies, Gaithersburg, Md.), according to the manufacturer's instructions. This system contains three panels of biotin-labeled DNA probes, which recognize viral type 6/11, 16/18, and 31/33/35. Positive HPV hybridization signal were demonstrated as purplish-blue reaction products. Positive and negative DNA control, provided by the manufacturer, were used on each hybridization, as well as cervical biopsies known to be positive for each type of HPV.

TABLE I
Partial and total frequency of positive signals for HPV DNA in the lesions studied.
TABLEAU I
Fréquences partielles et totales d'apparition de l'ADN de HPV dans les lésions étudiées.

Histologic diagnosis	Sex ratio (M/F)	Mean age (yrs) (range)	Total HPV ISH		HPV 6/11 ISH		HPV 16/18 ISH		HPV 31/33/35 ISH	
			cases +	%	cases +	%	cases +	%	cases +	%
SP	2/4	42 (32/58)	4	66	4	100	1	25	2	50
H/A	7/6	49 (34/74)	5	38.4	5	100	0	0	3	60
ED	1/4	44 (29/64)	3	60	3	100	1	33	0	0
SCC	24/3	59 (38/77)	10	37.03	5	50	1	10	5	50
TOTAL	34/17	48 (29/77)	22	43.1	17	33.3	3	5.8	7	13.7

SP: Squamous Papilloma; H/A: Hyperqueratosis/Acanthosis; ED: Epithelial Dysplasia; SCC: Squamous Cell Carcinoma.

RESULTS

The results obtained in our study are shown in Table I. In oral squamous papillomas appeared positive signal in 66% (4/6) of the cases. The type most frequently founded was HPV 6/11 (100%; 4/4), followed by type 31/33/35 (50%; 2/4) and type 16/18 (25%; 1/4). With chi-square analysis, HPV 6/11 appeared more frequent ($p<0.05$) than HPV 16/18 in squamous papillomas (Fig. 1).

HPV DNA was found in 44% of all hyperkeratotic/acanthosis (with and without epithelial dysplasia, globally considerated) (Fig. 2). In this group, HPV DNA 6/11 occurred significantly more frequent (100%; 8/8) ($p<0.01$; chi-square analysis) than HPV DNA 16/18 (12.5%; 1/8) and HPV DNA 31/33/35 (37.5%, 3/8). H/A without dysplasia were positive for HPV DNA in 38.4% (5/13). All positive H/A without dysplasia showed DNA from HPV

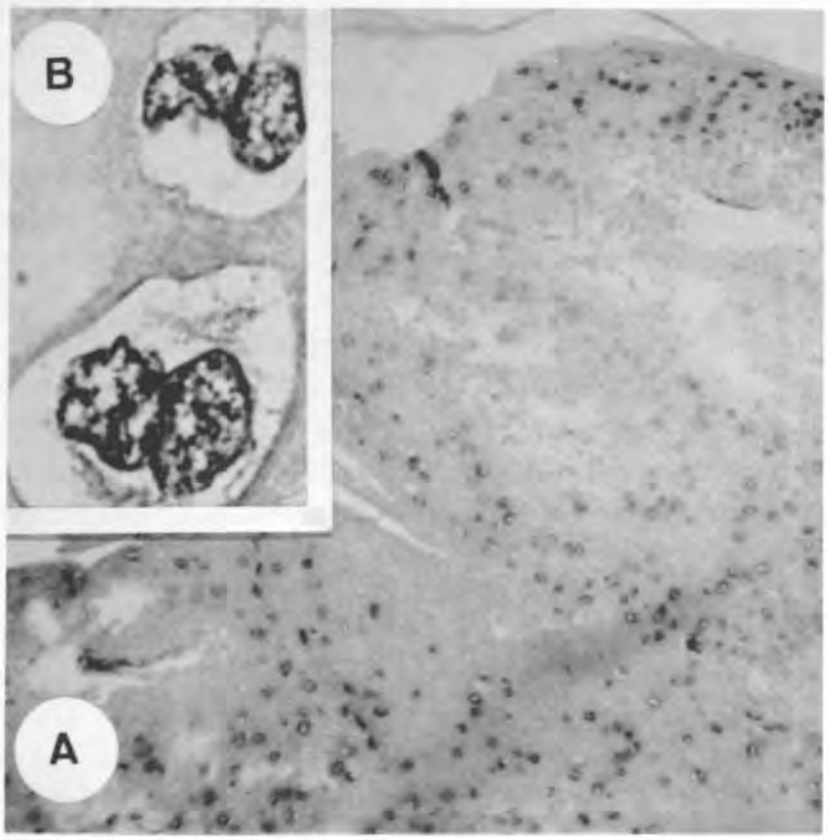

Fig. 1: A. – Positives signals for DNA HPV 6/11 in the nuclei of a squamous papilloma (×60).
B. – Typical cellular lesion of the human papillomavirus infection. Large perinuclear clear halo, cells with simple or multiple nuclei (koylocytic lesion, ×1500) (ISH technique).

Fig. 1: A. – Signaux positifs pour l'ADN de HPV 6/11 dans le noyaux de papillomes oraux (×60).
B. – Lésion cytologique caractéristique de l'infection par le papillomavirus humain. Cellules de grande taille avec un halo cytoplasmatique périnucléaire, noyaux simples ou multiples (koilocytes, ×1500) (technique d'hybridation in situ)

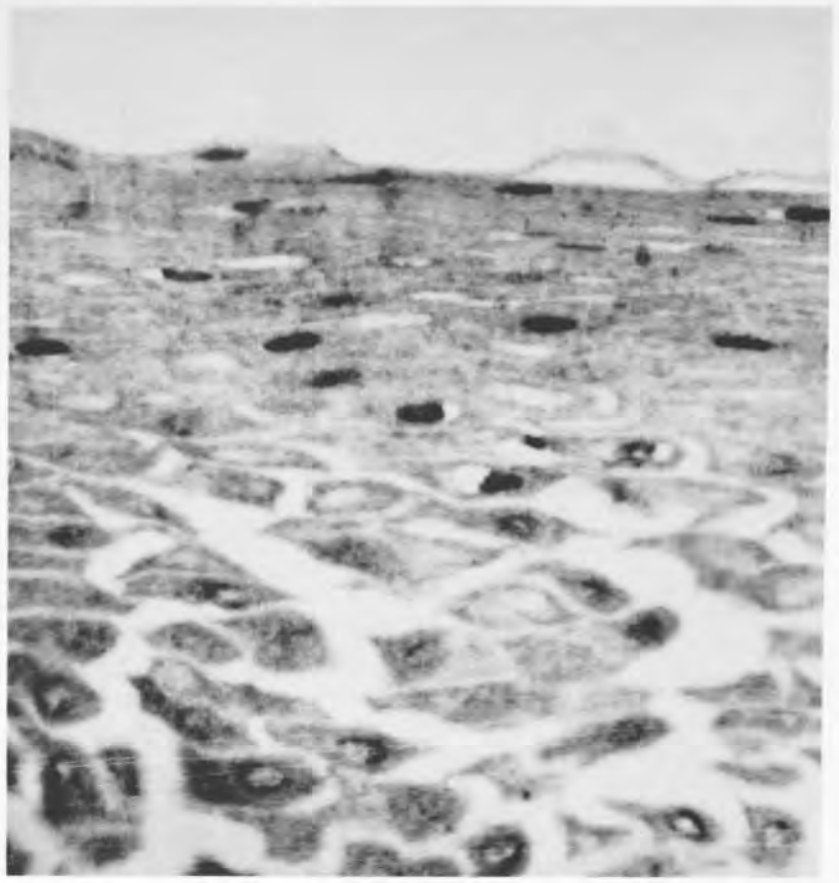

Fig. 2: Positives signals for DNA HPV 6/11 in the nuclei of the cells located in the superficial layer of a hyperkeratotic/acanthosis lesion (×600) (ISH technique).

Fig. 2: Signaux positifs pour l'ADN de HPV 6/11 dans les noyaux de la couche superficielle d'une lésion hyperkeratosique/acanthosique (×600) (technique d'hybridation in situ).

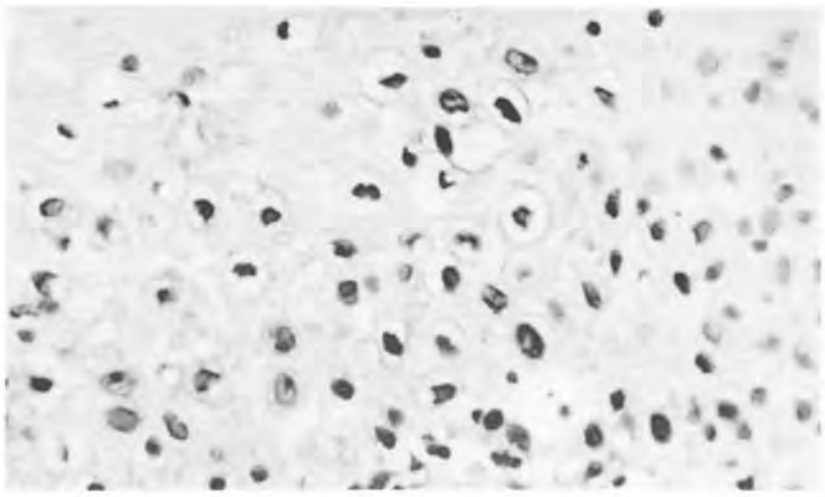

Fig. 3: Nucleous of squamous cells of an oral carcinoma with positives signals for DNA HPV 31/33/35 (×300) (ISH technique).

Fig. 3: Noyaux d'un carcinome oral avec signaux positifs pour l'ADN de HPV 31/33/35 (×300) (technique d'hybridation in situ).

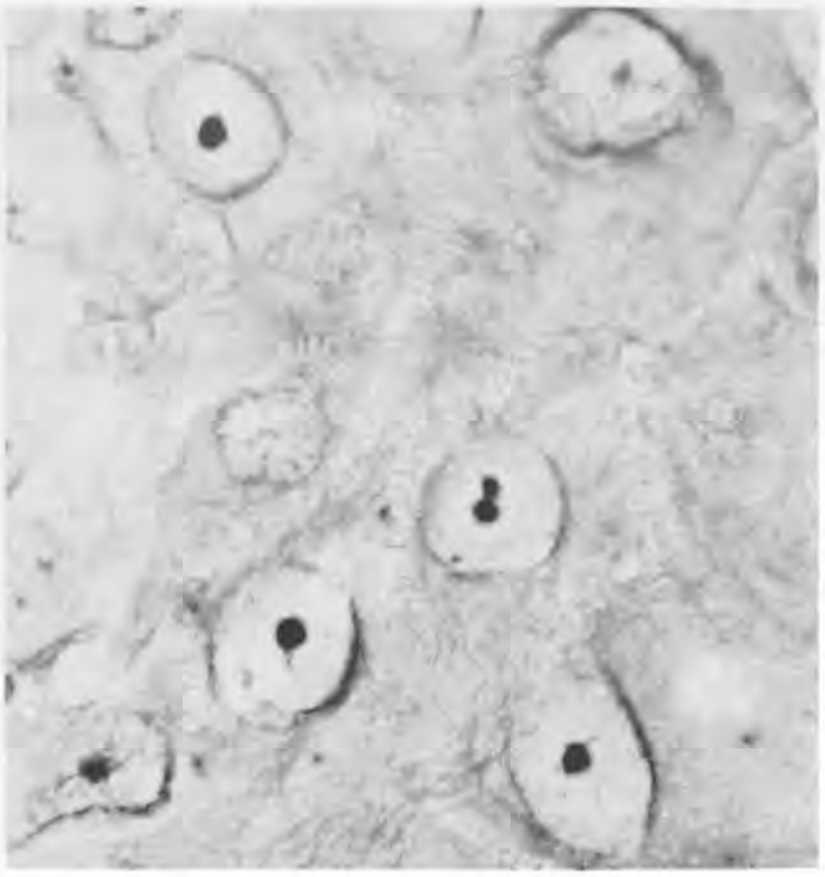

Fig. 4: Positives signals for DNA HPV 16/18 in an oral carcinoma (×1500) (ISH technique).
Fig. 4: Signaux positifs pour l'ADN de HPV 16/18 d'un carcinome oral (×1500) (technique d'hybridation in situ).

6/11 (100%; 5/5) been these types the most frequently found ($p<0.01$; chi-square analysis), whereas HPV DNA 16/18 and 31/33/35 were founded in 0% (0/5) and 60% (3/5) respectively. In lesions with epithelial dysplasia, HPV DNA were detected in 60% (3/5). Epithelial dysplasia showed HPV DNA 6/11 in all positives cases (100%; 3/3), HPV DNA 16/18 in 33% (1/3) and in no cases positive signal were founded for HPV DNA 31/33/35.

Of the 27 SCC studied, 10 (37.03%) were positive for HPV DNA. DNA from HPV 6/11, and HPV 31/33/35 were detected in 50% of the positive tumors (5/10) (Fig. 3), and HPV DNA 16/18 was founded in 10% (1/10) (Fig. 4). In terms of degree of tumoral differentiation, HPV DNA was founded more often in well differentiated tumors (90%; 9/10) than in moderately well differentiated (10%; 1/10) and poorly differentiated SCC (0%; 0/10) ($p<0.05$; chi-square analysis).

DISCUSSION

The detection of HPV DNA in oral squamous papillomas at a rate comparable with that found by other investigators (Sirjänen et al., 1986; Zeuss et al., 1991; Eversole et al., 1897; Welch et al., 1986; Eversole and Lapidis, 1988; Young and Min, 1991; Hönig, 1992) confirms that HPV DNA is common in this lesion. However, the question of the etiological significance of this finding remains unanswered. The prevalence of HPV in normal oral mucosa ranges from 10 to 60%, with HPV 6 and 11 being the less frequently found, whereas HPV 16 and 18 are the most frequent types (Maitland et al., 1987; Kashima et al., 1990; Lawton et al., 1992; Jalal et al., 1992), however in the present study HPV DNA 6/11 is the most frequently found in SP ($p<0.05$). This fact could suggest that these HPV genotypes are harbored in the mucosa for a short period of time before benign proliferation result and probably they play any etiological role.

Oral hyperkeratotic/acanthosis and epithelial dysplasia in our study were positive in 44% (jointly studied); however earlier publications report rates ranging from 0% (Shroyer and Greer, 1991) to 20% (Greer and Eversole, 1990). Because the prognosis of leukoplasias is closely linked to the presence or absence of epithelial dysplasia, we examined the global and partial frequencies of HPV DNA in oral H/A with and without dysplasia. 38.4% of non dysplastic H/A were positive for HPV DNA; this figure increased to 60% in lesions with dysplasia, although without statistical significance ($p<0.1$); however Greer and Eversole (1990) report a lower frequency of HPV in oral dysplasias. HPV DNA 6/11 were the types more frequently founded in positive H/A without dysplasia ($p<0.01$), just the types usually related with better prognosis in other site locations (cervix, larynx), whereas HPV 16/18 were found in 0% of H/A without dysplasia and in 33% of ED, which agree with the idea that those types have more malignant potential.

Although an epidemiological profile of the possible causal agents in the development of squamous cell carcinoma is available (tobacco and alcohol use) (Hoffman, 1985; Bross and Coombs, 1976; Graham, 1977), the role of HPV as carcinogens has been considered only recently. Studies based on animal experimentation (Syverton, 1952; Campo, 1984; Campo et al., 1980) have shown benign tumoral processes of viral origin to undergo malignant degeneration in a high percentage of subjects.

Evidence of relation between HPV and different tumoral lesions has also been sought in humans (Chang et al., 1993; Euvard et al., 1993; Frazar et al., 1993). In this connection the anogenital region is probably the most thoroughly studied. HPV DNA have frequently been associated with dysplasia and genital cancer (Seifert and Burkhardt, 1977; Furihata et al., 1993; Hording et al., 1993; Ibaraki et al., 1993); similarly, HPV has been demonstrated with a variety of methods in malignant and premalignant lesions of the oral cavity (Syrjanen et al., 1983; Jontell et al., 1990).

We analyzed 27 squamous cell carcinomas of the oral cavity, using ISH to detect DNA from HPV 6/11, HPV 16/18 and HPV 31/33/35. The percentage of positive cases in this study (37.07%) is including in the wide range of recently studies (Table II).

TABLE II:
Summary of the frequency of positives signals for HPV DNA in oral carcinomas recently published with different methods.
TABLEAU II:
Résumé des fréquences d'apparition de l'ADN de HPV des carcinomes oraux publiées récemment avec différentes méthodes d'identification.

Author (yrs)	N° HPV +/ N° tested (%)	Detection method
Syrjänen et al., (1986)	1/2 (50)	ISH
De Villiers et al., (1985)	3/7 (43)	SB
Milde & Loning, (1986)	4/7 (57)	ISH
Loning et al., (1987)	5/13 (38)	DB
Deckmecian et al., (1987)	4/4 (100)	ISH
Maitland et al., (1987)	7/15 (47)	SB
Ostrow et al., (1987)	1/3 (33)	SB
Lee et al., (1988)	1/2 (50)	SB
Gassenmaier et al., (1988)	16/58 (23)	ISH
Syrjänen et al., (1988)	6/51 (12)	ISH
Greer et al., (1990)	2/2 (100)	DB
Zeuss et al., (1991)	0/15 (0)	ISH
Young & Min., (1991)	0/17 (0)	ISH
Watts et al., (1991)	14/14 (100)	PCR
Palefsky et al., (1991)	8/25 (32)	PCR
Shroyer & Greer, (1991)	1/10 (10)	PCR
Present study	10/27 (37)	ISH

ISH: *in situ* hybridization; SB: Southern blot hybridization; DB: dot blot hybridization; PCR: Polymerase chain reaction.

Although more than 60 different types of HPV have been isolated, currently available hybridization methods are capable of detecting only a limited number of HPV types. So many of them unstudied or undescribed yet could be present in premalignant or malignant lesions of the oral cavity, as pointed out by Larsen et al. (1991). By the other hand, different results could be related with the sensibility of the technique employed to demonstrate HPV DNA.

One point of interest between our results was that most positive tumors for HPV DNA were well differentiated squamous cell carcinomas ($p<0.05$). In this way, three reasons could be emited to explain this fact: (1) HPV could modifies the genome of the epithelial cells in such a way as to induce well differentiated neoplastic cells able to form keratin; (2) HPV could select keratinizing cells as a preferred site of DNA replication; and (3) poorly differentiated tumors may be positive at the beginning of its development and become negative thereafter.

In some locations, certain types of HPV are related with different prognoses. For example, HPV 16 and 18 are associated with invasive cervical carcinoma (Ostrow et al., 1987; Schneider-Maunouroy et al., 1987). Similar observations have been reported for the oral cavity: Chang (1990) found types 2, 6, 7, 11, 13, 16, 18, 30 and 32 to be significantly more frequent in carcinomas of the upper respiratory tract, and the types 16 and 18 were specially common in invasive carcinomas. Syrjanen (1983) reported differences in prognosis on the basis of the type of HPV present in the tumor, and considered carcinomas with HPV DNA 6 and 11 to behave less aggressively than 16 or 18 positive cases. As well all positives carcinomas for HPV DNA 6/11 in our study were well differentiated tumors.

CONCLUSION

This study confirms that HPV 6/11, HPV 16/18 and HPV 31/33/35, frequently found in genital tissue, infects the oral mucosa, and suggests that HPV 6/11 is the most common type associated with oral squamous papillomas ($p<0.05$) and hyperkeratosis/acanthosis lesions ($p<0.01$).

HPV DNA appear more frequently in well differentiated squamous cell carcinomas ($p<0.05$) than in poorly differentiated ones.

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