

HUMAN PAPILOMAVIRUS DETECTION IN CERVICAL DYSPLASIAS OR NEOPLASIAS AND IN CONDYLOMATA ACUMINATA BY *IN SITU* HYBRIDIZATION WITH BIOTINYLATED DNA PROBES

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SUMMARY

Specimens from cervical dysplasias or carcinomas and genital condylomata acuminata were retrospectively analysed by *in situ* hybridization (ISH) with biotinylated DNA probes for human papillomavirus (HPV) types 6, 11, 16 and 18. In the control group no case was positive for HPV DNA. In mild/moderate dysplasias, 4 cases (14%) were positive for HPV 6 or 11 and 2 cases (7%), for HPV 16. In the severe dysplasia/*in situ* carcinoma group, 9 cases (31%) showed presence of DNA of HPV types 16 or 18. Six invasive carcinomas (20%) were positive for HPV type 16 or 18. Among condylomata acuminata, 22 cases (73%) were positive for HPV types 6 or 11.

In all ISH-positive cases only one viral type was detected. No correlation between HPV DNA positivity and histological findings of HPV infection was observed.

Although less sensitive than some other molecular biology techniques, *in situ* hybridization with biotinylated DNA probes proved to be simple and useful for detecting and typing HPV in samples routinely received for histopathological analysis.

KEY WORDS: Human papillomavirus; *In situ* hybridization; Cervical dysplasia; Cervical cancer; Condylomata acuminata.

INTRODUCTION

More than one decade ago, two research groups (MEISELS et al., 1977 and PUROLA & SAVIA, 1977)^{15, 23} showed that cervical koilocytotic lesions diagnosed as mild or moderate dysplasia are in fact associated with a sexually transmitted virus and could be an early step in the natural history of cancer of the cervix. Thereafter, many studies confirmed that assumption and now a growing body of data implicates human papillomavirus (HPV) in the etiology of cervical neoplasia²⁴. One of the strongest evidence of HPV oncogenic role is the close association of particular viral types with proliferative lesions of different malignant behavior^{9, 10, 13}. Among more than 60 HPV types presently known, 22 have already been isolated

from genital lesions, of which types 6 and 11 are commonly linked to benign or low grade lesions, while types 16, 18, 31, 33 and 35 are the most prevalent types in high grade or malignant lesions^{12, 13}.

HPV infection can be detected in clinical samples by a variety of methods, being cervico-vaginal cytology the most used one. Cervical biopsy, usually guided by colposcopy, can also be used. The outstanding microscopy feature of HPV infection is koilocytosis, which is characterized by superficial or intermediate squamous cells presenting nuclear atypia and perinuclear halos, commonly associated with bi or multinucleation. Electron microscopy of viral particles and immunohistochemistry using

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polyclonal or monoclonal antibodies directed to specific viral proteins are alternative ways to recognize the virus. However, all these methods have shortcomings and, in general, their sensitivity or specificity is not high. Furthermore, these procedures cannot precisely identify the virus type, which, as seen above, is of paramount importance in the study of HPV infection.

By detecting viral DNA directly in a sample, molecular biology techniques have been shown to have high sensitivity and specificity in diagnosing HPV infection and are the only methods capable of typing the virus^{7,8}. *In situ* hybridization (ISH), in particular, is technically simple, has high efficiency and permits not only the detection and typing of HPV but also the precise determination of the topography of the virus within cells and tissues. Using DNA technology, it has been demonstrated that HPV is present in a considerable number of lesions of different anatomical sites, especially in uterine cervix^{9,10,13}.

The majority of studies designed to detect HPV DNA have used radioactive probes. Although very sensitive, these probes present considerable drawbacks, namely high cost of reagents, short half-life, health risks and the logistic problems of handling radioactive materials in a clinical laboratory setting. To avoid that, many non-isotopic labelling systems have been developed^{11,16}. Among them, biotinylated probes are gaining worldwide acceptance and have made molecular hybridization available to routine laboratories^{1,5,17}.

There are only few studies dealing with the association between HPV and cervical or anogenital lesions in Brazilian people^{12,14,29,30}. In all of them the data were obtained by Southern blot using radioactive DNA probes. The present work consisted of a retrospective analysis by ISH of the prevalence of HPV infection in genital condylomata acuminata and cervical biopsies comprising the whole spectrum of proliferative lesions, from mild dysplasia to squamous cell carcinoma, in order to know the frequency of HPV in these conditions in Brazil.

MATERIAL AND METHODS

Clinical samples were obtained from patients referred to Hospital das Clínicas UFMG, Brazil, and were collected from 1984 to 1990. For inclusion in the study, all biopsies or surgi-

cal specimens from uterine cervix or condylomata acuminata from the perineal region, vulva or vagina examined in the Histopathology Laboratory were reviewed in 5 µm sections stained by hematoxylin and eosin. The samples had been fixed in 10% formalin, processed and embedded in paraffin. All consecutive specimens having enough amount of tissues and showing good preservation were selected for ISH analysis, which comprised 118 cervical samples and 30 condylomata acuminata. Cervical specimens were classified in four groups: control, in which no histologic signs of HPV infection or proliferative lesions were present, mild/moderate dysplasia, severe dysplasia/*in situ* carcinoma and invasive carcinoma. Histopathological diagnosis was based on the criteria proposed by MEISELS et al. (1987)¹⁵ and the World Health Organization²¹.

From each selected case four adjacent sections 5 µm thick were mounted on microscope slides coated with a solution (0.5mg/ml) of poly-L-lysine (Sigma). After 60 minutes baking at 60°C, the sections were immersed in xylene for 30 minutes and in absolute ethanol for 10 minutes, air dried, and incubated sequentially in proteinase K (200 µg/ml in 50 mM Tris-HCl, pH 7.5 and 5 mM EDTA) and 0.1 N HCl, for 15 minutes, at 37°, to permeabilize the cells, allow probe entry and expose the target sequence. To prevent non-specific binding of the probes, sections were incubated with a blocking solution composed of 0.1M Tris-HCl pH 7.5, 0.5M NaCl, 1% casein, 3% gelatin from calf skin and 0.05% Tween 20 for 20 minutes, at 37°C. Then, they were washed in 0.1M Tris-HCl pH 7.5 and 0.15M NaCl, dehydrated in ethanol and air dried.

DNA probes for HPV types 6, 11, 16 and 18 cloned in plasmid pBR322 were kindly provided by Drs. E.M. de Villiers, L. Gissmann and H. zur Hausen, from the Deutsches Krebsforschungszentrum, Heidelberg, Germany. The recombinants were amplified and purified according standard procedures²⁷ and labelled with biotin by nick translation, using the Bio-Nick™ Labeling System (GIBCO-BRL). Each section received 20-30 µl of hybridization solution containing 50% formamide, 20% dextran sulphate, 1X SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 250 ng/µl sonicated DNA salmon sperm and 10-20ng of

each biotinylated DNA probe. They were then covered with a glass coverslip. Sections and probes were simultaneously denatured at 100°C for 10 minutes and then hybridized at 37°C for two hours in a humid chamber. The slides were washed sequentially in 1X SSC at 42° three times, 15 minutes each, and in Tris-HCl 0.1M, pH 7.5, containing 0.3% bovine serum albumine. The sections were incubated with 40-50 µl of streptavidin-alkaline phosphatase conjugated (BluGENE™, BRL) for 20 minutes at 37°C. After washing in 0.1M Tris-HCl, pH 9.5, 0.1M NaCl and 50mM MgCl₂ for 15 minutes, the sections were incubated for 60 minutes at 37°C in the dark with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium. Finally, the sections were rinsed in water, stained briefly with eosin, dehydrated in ethanol, clarified in xylene and mounted.

RESULTS

Typically, the ISH signal was seen as a dark blue stain in the nucleus. In condyloma acuminata and in dysplasias the signals were stronger and seen more frequently in intermediate or superficial layers, notably in koilocytotic cells (Fig. 1). In less differentiated cells of carcinomas, the signal was weaker and had no preferential distribution (Fig. 2). The number and distribution of positive cells varied from case to case. In some condylomata a large number of stained cells could be seen in most part of the lesion; in others and in dysplasias, groups of hybridized cells were present; in some other cases only scattered individual positive cells

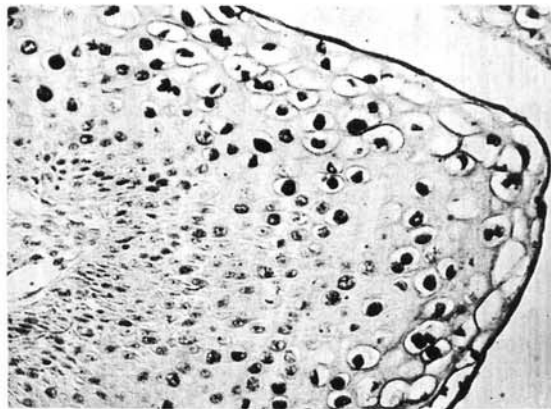


Fig. 1 – *In situ* hybridization. Condylomata acuminata. HPV 11 probe. Intense nuclear staining in intermediate and superficial layers, especially in koilocytotic cells.

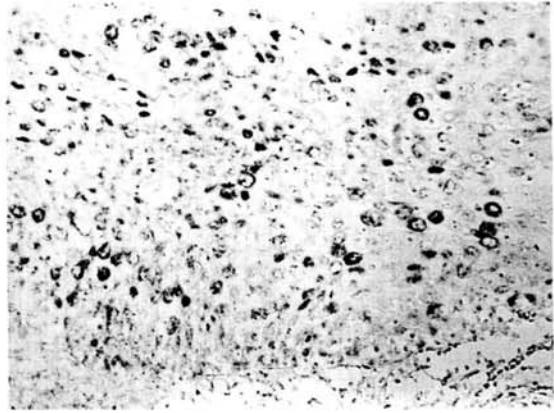


Fig. 2 – *In situ* hybridization. Cervical squamous cell carcinoma. HPV 16 probe. The signals are weaker and have no preferential location inside the lesion.

were visible.

All cervical biopsies of the 30 patients of the control group (without dysplasia or neoplasia) were negative for HPV DNA. None of these cases had histological signs of HPV infection. In the mild/moderate dysplasia group, 6 cases (21%) showed HPV DNA, being 4 (14%) for types 6 or 11 and 2 (7%) for types 16 or 18. Nine cases (30%) of severe dysplasia/*in situ* carcinoma group showed HPV DNA, all of them of the 16 or 18 types. Among invasive carcinomas, 6 individuals (20%) had HPV types 16 or 18. Twenty-two condylomata acuminata (73%) were positive for HPV types 6 or 11. In all positive cases only one viral type was found. These data are summarized in table 1.

Except for the exclusive finding of HPV 16 or 18 in high grade lesions and invasive carcinomas, no particular histological aspect could be associated to a specific viral type. Even the presence of HPV DNA was not correlated with histological findings of HPV infection. Overall, in cervical dysplasias or carcinomas, koilocytosis was present in 12 and absent in 9 of 21 positive cases for HPV DNA. On the contrary, among 67 ISH-negative cases, 23 showed koilocytosis and 44 lacked this element (Table 2). In condylomata acuminata histological signs of HPV infection were encountered in all cases, of which 73% were positive by ISH.

DISCUSSION

The present results are qualitatively quite similar to other studies performed in other geographic regions of the world. The hybridi-

TABLE 1
Frequency of HPV in cervical specimens and in Condylomata acuminata

Groups	Nº of positive cases	HPV type			
		6	11	16	18
Control group	0/30* (0)	0	0	0	0
Mild/moderate dysplasia	6/29 (21%)	3	1	2	0
Severe dysplasia/ <i>in situ</i> carcinoma	9/29 (31%)	0	0	6	3
Invasive carcinoma	6/30 (20%)	0	0	4	2
Condylomata acuminata	22/30 (73%)	10	12	0	0
TOTAL	43/148 (29%)	13	13	12	5

* Number of cases tested

TABLE 2
Frequency of Koilocytosis and positivity of HPV DNA by ISH in cervical dysplasias or carcinomas

	<i>In situ</i> hybridization		Total
	+	-	
Koilocytosis +	12*(34%)	23(66%)	35
-	9(17%)	44(83%)	53
TOTAL	21(24%)	67(76%)	88

* Number of cases

zation pattern was the same described by other authors, in that the signals were more common and evident in intermediate or superficial differentiated cells, where many viral copies are usually present^{7,22}. This study also lends support to the notion that there is no morphological aspect that may be attributed to a particular HPV type. Even koilocytosis, the supposedly pathognomonic feature of HPV infection¹⁵, was not correlated with the presence of HPV DNA. These data reinforce the concept that histopathological analysis alone is not enough to recognize all cases harboring HPV. On the other hand, the fact that some cases with koilocytosis but negative by ISH might be explained by: 1) infection by a viral type not probed. With the stringency conditions employed, only viral types with considerable homology with the

probes could be detected; 2) low amount of the virus, which might not have been recognized by the biotinylated probes; 3) loss of HPV DNA due to inadequate fixation. Since the tissues tested were originally fixed for routine pathological analysis under no completely standardized conditions and for varied periods of time, some DNA loss may have occurred. However, it should be mentioned that only 80% of koilocytotic cells in samples conveniently fixed have HPV DNA detected by ISH¹⁹; 4) the koilocytosis is not real. Although clearly defined, in many occasions accurate koilocytosis recognition is not easy and there may be considerable disagreement among observers about its existence.

This study also confirms the strong association between some HPV types with lesions of different malignant behavior. HPV types 6 and 11 were found only in condylomata acuminata or in low grade cervical dysplasias, whereas types 16 and 18 were present predominantly in high grade or malignant lesions^{12,18}. In high grade dysplasia or in carcinoma the predominant HPV type was 16, the same finding reported by other studies^{6,10,13,28}.

The frequency of HPV in cervical lesions found in this study is lower than the majority of similar investigations performed in different parts of the world^{7,20,26}. By Southern blot, HPV is also detected in higher frequency both in low and high grade lesions^{12,25}. However, one group, using the same methodology, found HPV

types 16 or 18 in 90% of samples from England¹³ and in only 40% from Brazilian women¹⁴. Taken together, these data might suggest that the frequency of HPV in cervical lesions is lower in Brazil compared to other geographical regions. However, another study done in Brazilian samples does not support this view¹². So, the low frequency now reported should be attributed largely to the low sensitivity of the method, to infection with HPV types not recognized by our probes or to a poor DNA preservation due to an inappropriated fixation. Since the reported sensitivity of ISH with biotinylated probes varies in different studies, including those that have used commercial kits²⁶, it might be possible to increase the HPV detection rate by introducing changes in the labeling protocol. One alternative would be the use of random primers⁴. For all of this, prospective studies, using fresh samples properly fixed and also including other approaches, are necessary to know accurately the true frequency of HPV in our region.

There is no previous report on HPV prevalence and typing in condylomata acuminata from Brazilian people. Our finding of 73% positivity of HPV DNA in anogenital condylomata is comparable to many studies made elsewhere^{3,3,18,25}. However, in the current report only HPV types 6 or 11 were detected, in contrast to some series in which other types or multiple infection were found^{3,7}.

The present study also supports the usefulness of ISH with biotinylated DNA probes to detect and type HPV in biopsies routinely sent for pathological examination and kept in paraffin blocks for many years. Due to its simplicity, ISH is becoming increasingly a practical and valuable tool for pathologists. By permitting retrospective analysis, as done in this report, ISH has a positive impact on HPV research and contributes significantly for better understanding of this important infection.

RESUMO

Detecção de papilomavirus humano em displasias ou neoplasias cervicais e em condilomas acuminados por hibridização *in situ* com sondas de DNA biotiniladas.

Amostras de displasias ou carcinomas do colo uterino e de condilomas acuminados da região genital foram analisados retrospectiva-

mente por hibridização *in situ* (HIS) com sondas de DNA biotiniladas de papilomavírus humano (HPV) tipos 6, 11, 16 e 18. Nenhum caso do grupo controle foi positivo para DNA de HPV. Em displasias leve/moderada, 4 casos (14%) foram positivos para HPV 6 ou 11 e 2 casos (7%), para HPV 16. No grupo de displasia acentuada/carcinoma *in situ*, 9 casos (31%) tinham DNA de HPV tipos 16 ou 18. Seis carcinomas invasores (20%) foram positivos para HPV tipos 16 ou 18. Entre os condilomas acuminados, 22 casos (73%) foram positivos para HPV tipos 6 ou 11.

Em todos os casos positivos pela HIS somente um tipo viral foi encontrado. Não foi observada correlação entre a positividade para DNA de HPV e achados histológicos de infecção por HPV.

Apesar de menos sensível que algumas outras técnicas de biologia molecular, a hibridização *in situ* com sondas de DNA biotiniladas mostrou-se simples e adequada para detecção e tipagem de HPV em amostras enviadas rotineiramente para estudo histopatológico.

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