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Association of human papillomavirus with preinvasive and invasive cervical carcinoma

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ABSTRACT Several human papillomavirus (HPV) types have been implicated in the development of cervical carcinoma worldwide. The use of molecular techniques have facilitated the detection and typing of HPV in cervical lesions.

Seventy four formalin-fixed tissues of various cervical lesions were analysed by *in situ* hybridization using digoxigenin-labelled probes for HPV 16 and 18 DNA sequences. HPV DNA was found in 76% of the cervical tissues. The prevalence of HPV DNA sequences increased with severity of the CIN lesions ranging from 25% in CIN 1 and 67% in CIN 2 to 85% in CIN 3/CIS. HPV DNA was detected in 80% of both adenocarcinoma and squamous cell carcinoma cases. Analysis of the data by viral type revealed that HPV 16 prevailed in 25% of CIN 1, 67% of CIN 2, 55% of CIN 3/CIS, 20% of adenocarcinomas and 67% of squamous cell carcinomas. HPV 18 was detected in 25% of CIN 1, 53% of CIN 2, 85% of CIN 3/CIS, 80% of adenocarcinomas and 67% of squamous cell carcinomas. The detection rate of HPV DNA increased from 25% in low grade lesions (CIN 1) to 77% in high grade lesions (CIN 2 and CIN 3/CIS). In squamous cell carcinoma HPV 16 DNA was prevalent as frequently as HPV 18 DNA but in adenocarcinoma HPV 18 DNA was detected more often than HPV 16 DNA. Mixed infections with both HPV types occurred more often than single infections with either type.

The increasing association of HPV 16 and 18 with enhanced severity of the precursor lesions suggests that these high risk oncogenic viruses may have a role in the development and progression of cervical cancer.

ABSTRAK Beberapa jenis virus papiloma manusia (HPV) telah dikaitkan dengan karsinoma serviks di seluruh dunia. Penggunaan pelbagai teknik molekul telah membantu dalam mengesan dan mengenalpasti jenis-jenis HPV yang hadir di dalam lesi servikal.

Sejumlah 74 tisu parafin yang terdiri dari beberapa gred lesi servikal telah dikaji dengan menggunakan teknik hibdridisasi *in situ*. Kajian ini telah menggunakan prob berlabel digoxigenin yang spesifik untuk HPV 16 dan HPV 18. DNA HPV telah berjaya dikesan dalam 76% dari kesemua lesi servikal yang dikaji. Kehadiran DNA HPV didapati meningkat dengan keterukan lesi, iaitu dari 25% untuk CIN 1, 67% untuk CIN 2 kepada 85% untuk CIN 3/CIS. DNA HPV juga telah dikesan dalam 80% daripada kes-kes adenokarsinoma dan karsinoma sel skuamus. HPV 16 telah dikenalpasti di dalam sebanyak 25% daripada lesi CIN 1, 67% dari lesi CIN 2, 55% dari lesi CIN 3/CIS, 80% dari adenokarsinoma dan 67% dari karsinoma sel skuamus. HPV 18 pula telah dikesan di dalam 67% dari lesi CIN 1, 53% dari lesi CIN 2, 85% dari lesi CIN 3/CIS, 80% dari adenokarsinoma dan 67% dari karsinoma sel skuamus. Pengesanan DNA HPV meningkat dari 25% dalam lesi gred rendah (CIN 1) kepada 77% dalam lesi gred tinggi (CIN 2 dan CIN 3/CIS). Bagi karsinoma sel skuamus, DNA HPV 16 telah dikesan dengan kadar yang sama dengan DNA HPV 18. Walaubagaimanapun, DNA HPV 18 telah lebih kerap dikesan dalam adenokarsinoma berbanding dengan DNA HPV 16. Infeksi bercampur dengan kehadiran kedua-dua jenis HPV adalah lebih kerap berlaku berbanding dengan infeksi tunggal dengan mana-mana satu jenis HPV. Peningkatan kadar pengesanan HPV 16 dan 18 dengan keterukan lesi prekursor mungkin menunjukkan penglibatan virus onkogen risiko tinggi ini dengan pembentukan dan perkembangan kanser servikal.

(*in situ* hybridization, digoxigenin, human papillomavirus, cervical carcinoma, cervical intraepithelial neoplasia, HPV)

INTRODUCTION

Human papillomaviruses (HPV) are associated with a wide range of clinical, subclinical and latent disease, spanning from benign warts to premalignant and malignant lesions. The HPVs are site-specific DNA viruses which induce epithelial or fibroepithelial proliferation (hyperplasia) of the skin and/or mucosa [1].

With the advent of molecular cloning, it has become apparent that specific HPV types are associated with anatomically distinct diseases. Thus, the heterogeneity of the HPV group probably reflects an adaptation of these viruses to specifically differentiated tissue [2].

Over 70 HPV types have been identified and of these about 30 have been isolated from the genital mucosa in males and females [3, 4, 5, 6]. However, accumulated evidence shows the frequent involvement of HPV 6, 11, 16, 18 and 31 in a large majority of genital tract infections [5, 6, 7, 8]. HPV 6 and 11 are mainly associated with the exophytic condyloma acuminata and rarely associate with high grade dysplasia and invasive cancers [9,10]. Moreover, recent studies demonstrate that these two viruses may persist

in tissues visibly unaffected by cytopathogenic changes [11, 12, 13]. In contrast HPV 16, 18, 31, 33 and 35 are commonly associated with high grade cervical intraepithelial neoplasia (CIN) lesions and invasive carcinomas [5, 6, 14, 15, 16]. This frequent association of the viruses with the disease has led to the suggestion of the involvement of high risk HPVs in cervical carcinogenesis [17, 18]. Nevertheless, some CIN and cervical carcinoma tissues do not harbour any detectable HPV DNA sequences. These findings suggest that such carcinomas may have evolved independently of any HPV infection [19]. Thus, further investigations need to be conducted to elucidate the role of HPVs 16, 18, 31, 33 and 35 in the development of cervical cancer.

In Malaysia, little is known of the geographic distribution of the oncogenic HPV types 16 and 18 in cervical pathology. This investigation was undertaken to clarify the relationship of HPV infection in cervical disease ranging from low grade to high grade CIN to cervical carcinomas. Here we report the application of *in situ* hybridization technique using digoxigenin-labelled HPV 16 and 18 DNA probes to analyse the prevalence of these HPVs in cases of cervical dysplastic, precancerous and cancerous lesions.

MATERIALS AND METHODS

Clinical specimens

Seventy four formalin-fixed and paraffin-embedded biopsies of various lesions of the cervix were obtained from Melaka General Hospital and Sultanah Aminah Hospital, Johor Bharu. Thirty of the 74 biopsies had the histologic features of a squamous cell carcinoma (SCC), 5 were adenocarcinomas (ADC) and 39 cervical intraepithelial neoplasia (CIN)/carcinoma *in situ* (CIS) lesions comprising of 20 cases of CIN 3/CIS, 17 cases of CIN 2 and 4 cases of CIN 1. Of the 74 randomly selected cases, 16 were Malays, 15 were Chinese and 4 were Indians.

Paraffin Sections

The paraffin-embedded tissues were cut into 5 µm sections, mounted on prewashed and pretreated slides [20]. Adjacent sections were stained with hematoxylin and eosin for histologic assessment and confirmation. The slides with sections were stored in a box at room temperature in the dark until used for *in situ* hybridization studies.

The positive controls utilized in this study were the cervical cancer-derived CaSki (containing the HPV 16 genome) and HeLa (containing the HPV 18 genome) cell lines. These cells were cultured in RPMI 1640 and harvested when in log phase growth. They were washed in PBS and deposited on prewashed and gluteraldehyde-treated slides. The cells were fixed in cold ethanol and stored at -20°C for later use.

Non-radioactive probe preparation and labelling

Probes for HPV 16 and 18 were prepared by polymerase chain rection (PCR) from plasmids (with HPV DNA inserts). During PCR the amplicons were labelled with digoxigenin (DIG) using the Non-Radioactive DNA Labelling and Detection Kit (Boehringer Mannheim, Germany). The HPV L1 consensus primer pair, MY09 and MY11 (Perkin Elmer Cetus, USA) which were utilized in PCR were degenerated. They had the following sequence:

Primer 1 (MY09) - 5′-
CGTCCMARRGGAWACTGATC-3′
Primer 2 (MY11) - 5′-
GCMCAGGGWCATAAYAATGG-3′ where
M=A or C, R=A or G, W=A or T and Y=C or T.

The two resulting probes were the 450 bp digoxigenin-labelled L1 DNA region of HPVs 16 and 18.

In situ hybridization

The technique of *in situ* hybridization for the detection of HPV 16 and 18 sequences in formalin-fixed, paraffin-embedded cervical tissue and cell lines were performed according to the protocol described by Boehringer Mannheim [20] with some modifications. Sections were deparafinized at 56°C and dewaxed in xylene. From this point onwards, slides with CaSki and HeLa were included routinely as positive controls. Negative controls were tissues untreated with the probe only. After rehydration in decreasing concentrations of ethanol, the slides were washed in distilled water for 5 minutes. Treatment with 0.2 N HCL were carried out for 20 minutes, followed by washes in distilled water and PBS, at 5 minutes each. The tissues were then subjected to digestion with 100 µg/ml of Proteinase K (Sigma Co, St Louis, USA) at 37°C for 20 minutes. After washes with 0.2% glycine and PBS for 5-10 minutes each, the tissues were fixed with 4% formaldehyde and the wash was repeated with PBS and 1 x SSC. Following dehydration in increasing concentra-

tions of ethanol, the tissues were treated with prehybridization solution for 1 hour at room temperature. The probe and target DNA were denatured at 95°C for 6 minutes, and then hybridization was conducted with hybridization solution (1 x Denhart's solution, 5% dextran sulfate, 50% formamide, 4 x SSC, 0.2 mg/ml sonicated salmon sperm DNA and 500 ng digoxigenin-labelled probe) at 42°C over night. Following hybridization, high stringency washes were performed with 2 x SCC and 1 x SCC for 1 hour each at room temperature and two 0.5 hour washes with 0.1 x SCC, one at 68°C and one at room temperature.

For colour development [21], the tissues were pre-equilibrated with buffer A (100 mM Tris-HCl, 150 mM NaCl pH 7.5) for 1 minute and followed with buffer B (0.5% w/v blocking reagent in buffer A) for 30 minutes. Thereafter, the slides were rinsed with buffer A and incubated with the diluted antibody-conjugated solution (anti-digoxigenin-AP) for 30 minutes, after which the slides were rinsed twice for 5 minutes each with buffer A and equilibrated for 2 minutes with buffer C (100 mM Tris-HCl, 10 mM NaCl, 50 mM $MgCl_2$, pH 9.5). The slides were incubated overnight in freshly prepared substrate (0.45% nitroblue tetrazolium salt, 0.35% 5-Bromo-4-chloro-3-indoyl-phosphate in buffer C) and the colour development was stopped with buffer D (10 mM tris-HCl, 1 mM EDTA, pH 8.0). The slides were counter-stained in Mayers hematoxylin, mounted in glycergel and examined under the microscope (Olympus, Japan).

RESULTS

CIN 1, CIN 2 and CIN 3/CIS

Seventy four formalin-fixed and paraffin-embedded biopsies from the various lesions of the cervix were analyzed by *in situ* hybridization. Digoxigenin-labelled HPV 16 and HPV 18 probes were used separately to detect the presence of HPV DNA.

Positive hybridization signals were localized in the nuclei of infected cells for both HPV 16 and HPV 18. However these signals were widely variable in both intensity and topographical distribution within the lesions. In some CIN 1 and 2 lesions, the distribution of HPV DNA involved the basal and parabasal layers and in others extended to the overlying, more differentiated intermediate layers. Staining was strongest in the koilocytes (Figs. 1 and 2). In cases of CIN 3/CIS, intensely stained nuclei were abundant, producing a crowded appearance above the basal and parabasal layers to the surface of the epithelium. Positive staining was either very weak or not present at all within the nuclei of cells deep in the basal and parabasal layers (Fig. 3).

In HPV-positive invasive lesions (Fig. 4), stained nuclei were abundant and distributed uniformly throughout the lesions. In majority of the cases, staining intensities varied from cell to cell within the same lesion. In many cases, the HPV DNA-positive cells were present only within the lesions but not all affected cells stained for HPV DNA. Thus, the HPV DNA-positive malignant cells did not differ morphologically and could not be distinguished from their adjacent HPV-negative counterparts. The absence of staining in the nuclei of stromal cells confirmed the specificity of the probes used in this study.

Two cell lines, namely, CaSki and HeLa, which were the positive controls, hybridized intensely with HPV 16 and 18 DNA probes, respectively. Specificity of the probes were tested by cross-hybridization with the probes in HeLa and CaSki cell lines. Thus, HeLa did not react with HPV 16 DNA probe and CaSki did not react with HPV 18 DNA probe. Similarly, no hybridization was detected on the negative control slides which comprised of tissues untreated with the probe.

Of the 74 cases, 56 (76%) were positive for HPV DNA sequences (Table 1). The HPV 16 and 18 DNA were de-tected in 58% and 68% of the cases, respectively (Table 2). In 18 samples HPV DNA was absent. These negative tissues were subjected to repeat *in situ* hybridization runs and the results were confirmed as negative. Of a total of 18 tissues negative for the HPVs, 6 were squamous cell carcinomas, 1 was an adenocarcinoma, 3 were CIN 3/CIS, 5 were CIN 2 and 3 were CIN 1.

Table 1. Detection of HPV DNA in cervical intraepithelial neoplasia and invasive carcinoma of the uterine cervix.

Histological subtypes	No of Cases	HPV DNA positive Number (%)
Squamous cell carcinoma	30	24 (80)
Adenocarcinoma	5	4 (80)
CIN 3/CIS	20	17 (85)
CIN 2	15	10 (67)
CIN 1	4	1 (25)
Total	74	56 (76)