Association of human papillomavirus with preinvasive and invasive cervical carcinoma

Z. A. Nurnayati\(^1\), M. Yadav\(^2\), J. M. Daniels\(^3\) and A.W. Norhanom\(^4\)

\(^1\)Institute of Advanced Studies, University of Malaya, 59100 Kuala Lumpur, Malaysia
\(^2\)Department of Genetics & Cellular Biology, University of Malaya, 59100 Kuala Lumpur, Malaysia
\(^3\)Department of Pathology, Melaka General Hospital, Melaka, Malaysia
\(^4\)Centre for Foundation Studies in Sciences, University of Malaya, 59100 Kuala Lumpur, Malaysia

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 molekuler telah membantu dalam mengesan dan mengenalpasti jenis-jenis HPV yang hadir di dalam lesi servikal.

Sejumlah 74 tisu parafin yang terdiri dari beberapa grend lesi servikal telah dikaui dengan menggunakan teknik hibridisasi 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 dikaui. Kehadiran DNA HPV didapat 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 skuamas. HPV 16 telah dikenalpasti di dalam sebanyak 25% daripada lesi CIN 1, 67% daripada lesi CIN 2, 55% daripada lesi CIN 3/CIS, 80% dari adenokarsinoma dan 67% daripada karsinoma sel skuamas. HPV 18 pula telah dikesan di dalam 67% daripada CIN 1, 53% daripada CIN 2, 85% daripada CIN 3/CIS, 80% daripada adenokarsinoma dan 67% dari karsinoma sel skuamas. Pengesahan DNA HPV meningkat dari 25% dalam lesi grend rendah (CIN 1) kepada 77% dalam lesi grend tinggi (CIN 2 dan CIN 3/CIS). Bagi karsinoma sel skuamas, 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 berlaku dengan kehadiran kedua-dua jenis HPV adalah lebih kerap berlaku berbanding dengan infeksi tunggal dengan mana mana satu jenis HPV. Peningkatan kadar pengesahan HPV 16 dan 18 dengan keterukan lesi prekursor mungkin menunjukkan penglibatan virus onkogen risiko tinggi ini dengan pembentukan dan perkembangan karsen 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 cytopathogenetic 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 cancersous 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 glutaraldehyde-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 reaction (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' GCMACGGGWCATAAYATGG-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 deparaffinized 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 tem-
perature. 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% 
edextran 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 hybri-
dization, high stringency washes were performed with
2 x SCC and 1 x SCC for 1 hour each at room tem-
perature 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 buf-
fer C (100 mM Tris-HCl, 10 mM NaCl, 50 mM MgCl2,
pH 9.5). The slides were incubated overnight in fre-
shly prepared substrate (0.45% nitroblue tetrazolium
salt, 0.35% 5-Bromo-4-chloro-3-indoly-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 hematoxy-
lin, mounted in glycergel and examined under the mi-
roscope (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 differentiat-
ed 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, stain-
ing 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 morphologi-
cally and could not be distinguished from their adja-
cent HPV-negative counterparts. The absence of stain-
ing in the nuclei of stromal cells confirmed the specifi-
city 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 pro-
bes 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 com-
prised 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, respec-
tively (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 neo-
plasia and invasive carcinoma of the uterine cervix.

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