# IMMUNOLOGY AND MOLECULAR EPIDEMIOLOGY OF HUMAN PAPILLOMAVIRUS IN 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 of Malaysian patients. The analysis of HPV protein expression and immune response to HPV proteins on the other hand, have been made possible by the use of immunohistochemical and serological techniques.

DNA preparations from 40 cervical carcinoma, 30 normal cervical scrapes and 30 non-cervical specimens were analyzed by polymerase chain reaction (PCR) for the presence of HPV DNA. Subsequent Southern blot hybridization typing of PCR amplified HPV DNA using digoxigenin-labelled HPV 16 and 18 probes revealed the presence of HPV 16 in 57.5% and HPV 18 in 57.5% of cervical cancer specimens but none in the non-cervical cancer specimens. These viruses were also detected in 26% of the normal cervical scrapes.

Seventy four paraffin-embedded tissues of various cervical lesions were analyzed 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 types 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 61% 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).

Combined data indicate that the prevalence of HPV 16 DNA (57.9%) did not differ significantly from that of HPV 18 DNA (64%) in all cervical lesions analyzed. In squamous cell carcinomas HPV 16 was prevalent as frequently as HPV 18 DNA but in adenocarcinoma HPV 18 DNA (80%) was detected more often than HPV 16 DNA (20%). Mixed infections with both HPV types were present in all histological grades of cervical lesions. Mixed infections occurred more often than single infections with either type.

Immunohistochemical analysis revealed that the expression of HPV 18 E6 and HPV 16 L1 proteins in cervical lesions. The expression of HPV 18 E6 protein increased with enhanced severity of the cervical lesions, ranging from 53.3% of CIN 2 to 90% of CIN 3/CIS, 100% of adenocarcinoma and 83.3% of squamous cell carcinoma cases. The protein was not detectable in low grade lesions but increased to 75.7% of the high grade lesions and continued to be highly expressed in invasive carcinomas. This reflects the higher E6 transforming activity in the more severe lesions.

Of the 74 cervical lesions examined, 52.7% exhibited the presence of HPV 16 L1 protein. The protein was expressed in 26.6% of CIN 2, 50% of CIN 3/CIS, 80% of adenocarcinoma and 70% of squamous cell carcinoma cases. An increase in the L1 protein positivities was observed with severity of the cervical lesions indicating that the L1 gene may not be totally disrupted with integration of the HPV DNA into the host genome.

Serologic response to HPV 16 and 18 in sera obtained from 80 cervical cancer patients, 32 healthy pregnant women and 30 healthy university students were evaluated with ELISA assays using six synthetic peptides, synthesized based on

sequences deduced from the early and late regions of HPV 16 and 18. Anti-HPV antibodies were found in all three groups of women studied. None of the antibodies to peptides L1:13, L1:30, L2:49 and E7:5 of HPV 16 seemed to be sufficiently disease specific to be used as markers for cervical cancer. Although anti-HPV 16 E7:5 antibodies were also prevalent in healthy subjects, the data revealed that the HPV 16 E7 protein was produced in elevated amounts in cervical cancer patients. This reflects the higher E7 transforming activity in these patients.

Anti-HPV 16 E2:245 IgA and anti-HPV 18 E2:245 IgA and IgG antibodies seemed to have specificity for cervical cancer and thus have the potential to be used as markers for the disease. The higher prevalence of anti-E2:245 antibodies in cervical cancer patients suggest a role of the E2 protein in cervical carcinogenesis.

The increasing association of HPV 16 and 18 with enhanced severity of the cervical lesions suggest that these high risk oncogenic viruses have a role in the development and progression of cervical cancer. However since molecular and serological evidence have revealed that HPV 16 and 18 infections are widespread among healthy Malaysian females, it can therefore be deduced that infection with HPV 16 and 18 per se is important but may not represent a sufficient etiological component for cervical cancer development.

## ABSTRAK

Beberapa jenis virus papiloma manusia (HPV) telah dikaitkan dengan karsinoma serviks di seluruh dunia. Penggunaan pelbagai teknik molekul telah mengesan dan megenalpasti jenis-jenis HPV yang hadir di dalam lesi servikal pesakit-pesakit di Malaysia. Ekspresi protin HPV dan gerakbalas imun terhadap protin-protin HPV telah dikaji dengan menggunakan teknik-teknik imunohistokimia dan serologi.

DNA dari 40 sampel karsinoma seviks, 30 sampel kikisan serviks normal dan 30 sampel bukan serviks telah dikaji untuk mengesan kehadiran DNA HPV dengan menggunakan teknik rantaian polimeras (PCR). Hibridisasi blot Southern ke atas amplikon HPV DNA telah mengenalpasti kehadiran HPV 16 dalam 57.5% dan HPV 18 dalam 57.5% dari spesimen-spesimen kanser serviks tetapi tidak dalam sampel-sampel bukan serviks. Virus-virus ini juga telah dikesan dalam 26% dari kikisan serviks normal.

Sejumlah 74 tisu parafin yang terdiri dari beberapa gred lesi serviks telah dikaji 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 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% dari kes-kes adenokarsinoma dan karsinoma sel skuamus. HPV 16 telah dikenalpasti dalam sebanyak 25% dari CIN 1, 67% dari dari lesi CIN 2, 55% dari lesi CIN 3/CIS, 80% dari adenokarsinoma dan 67% dari karsinoma sel skuamus. HPV 18 pula telah dikesan dalam 67% dari lesi CIN 1, 53% dari lesi CIN 2, 85% dari lesi CIN 3/CIS, 80% dari adenokarsinoma dan 61% dari karsinoma sel skuamus. Pengesanan DNA HPV

meningkat dari 25% di dalam lesi gred rendah (CIN 1) kepada 77% di dalam lesi gred tinggi (CIN 2 dan CIN 3/CIS).

Gabungan data menunjukkan bahawa kadar pengesanan DNA HPV 16 (57.9%) tidak banyak berbeza dari kadar pengesanan DNA HPV 18 (64%) dalam kesemua lesi serviks yang dikaji. Bagi karsinoma sel skuamus, DNA HPV 16 dan HPV 18 telah dikesan dengan kadar yang sama. Walaubagaimanpun, DNA HPV 18 (80%) telah lebih kerap dikesan dalam adenokarsinoma berbanding dengan DNA HPV 16 (20%). Infeksi bercampur dan kehadiran kedua-dua jenis HPV telah dikesan dalam kesemua gred lesi serviks yang dikaji. Kecuali adenokarsinoma, infeksi bercampur adalah lebih kerap berlaku berbanding dengan infeksi tunggal dengan mana-mana satu jenis HPV.

Analisis imunohistokimia telah mengesan ekspresi protein E6 dari HPV 18 dan protein L1 dari HPV 16. Kadar ekspresi protein E6 didapati meningkat dengan keterukan lesi iaitu dari 53.3% untuk CIN 2 kepada 90% untuk CIN 3/CIS, 100% untuk adenokarsinoma dan 83.3% untuk karsinoma sel skuamus. Protein tersebut tidak dapat dikesan dalam lesi gred rendah tetapi meningkat kepada 75.7% dalam lesi gred tinggi dan terus dikesan dengan kadar yang tinggi dalam karsinoma. Ini menunjukkan bahawa aktiviti transformasi oleh protein E6 adalah tinggi dalam lesi-lesi yang teruk.

Daripada 74 lesi serviksl yang dikaji, 52.7% telah menunjukkan kehadiran protein L1 dari HPV 16. Protein ini dikesan dalam 26.6% dari lesi CIN 2, 50% dari lesi CIN 3/CIS, 80% dari adenokarsinoma dan 70% dari karsinoma sel skuamus. Kadar pengesanan protein L1 meningkat dengan keterukan lesi. Ini membuktikan bahawa tidak semua gen L1 yang terhapus semasa proses integrasi DNA HPV ke dalam genom perumah.

Gerak balas serologi terhadap HPV 16 dan HPV 18 dalam serum yang diperolehi dari 80 orang pesakit karsinoma serviks, 32 orang wanita hamil yang sihat dan 30 orang pelajar universiti telah dikajiselidik dengan menggunakan teknik ELISA dengan menggunakan enam jenis peptida sintetik. Peptida-peptida sintetik tersebut telah dibuat berasaskan jujukan gen dari HPV 16 dan HPV 18. Antibodi-antibodi HPV telah dikesan dalam ketiga-tiga kumpulan wanita yang dikaji. Kajian menunjukkan bahawa kehadiran antibodi-antibodi terhadap protein L1:13., L1:30, L2:49 dan E7:5 dari HPV 16 tidak spesifik kepada penyakit karsinoma serviks dan dengan itu tidak sesuai untuk dijadikan penanda bagi penyakit tersebut. Data yang diperolehi menunjukkan bahawa walaupun antibodi terhadap protein E7:5 dari HPV 16 telah dapat dikesan dalam serum wanita-wanita sihat, protein tersebut telah dihasilkan dengan kuantiti yang lebih banyak dalam pesakit-pesakit karsinoma serviks. Ini membuktikan bahawa aktiviti transformasi oleh protein E7 adalah tinggi dalam pesakit-pesakit karsinoma serviks. Kajian mendapati bahawa antibodi IgA terhadap protein E2:245 dari HPV 16 dan antibodi IgA dan IgG terhadap protein E2:245 dari HPV 18 adalah spesifik kepada karsinoma servikss dan dengan itu berpotensi untuk dijadikan penanda bagi penyakit tersebut. Kehadiran antibodi-antibodi terhadap protein E2:245 dengan kadar yang lebih tinggi di kalangan pesakit-pesakit karsinoma serviks mencadangkan penglibatan protein E2 dalam pembentukan karsinoma tersebut.

Peningkatan kadar pengesanan HPV 16 dan HPV 18 dengan keterukan lesi prekursor dan karsinoma serviks mungkin menunjukkan penglibatan virus onkogen risiko tinggi ini dalam pembentukan dan perkembangan kanser serviks. Adalah wajar jika disimpulkan bahawa jangkitan oleh HPV 16 dan HPV 18 adalah penting tetapi ianya bukan faktor tunggal dalam pembentukan karsinoma serviks kerana penemuan

kajian molekul dan serologi telah menunjukkan bahawa jangkitan oleh HPV 16 dan HPV 18 adalah berleluasa dikalangan wanita-wanita di Malaysia.

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## CHAPTER 3 - MATERIALS AND METHODS

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| % | : | percentage |
| μ | : | micron |
| °C | : | degree centigrade |
| μg | : | microgram |
| μl | : | microlitre |
| bp | : | base pair |
| CIN | : | cervical intraepithelial neoplasia |
| CIS | : | carcinoma in situ |
| cm | : | centimeter |
| dATP | : | deoxyadenosine triphosphate |
| dCTP | : | deoxycytidine triphosphate |
| $ddH_2O$ | : | deionized distilled water |
| dGTP | : | deoxyguanosine triphosphate |
| DIG | : | digoxigenin |
| DNA | : | deoxyribonuclease |
| dNTP | : | deoxynucleotide triphosphate |
| dTTP | : | deoxythymidine triphosphate |
| EDTA | : | ethylenediamine tetraacetic acid |
| ELISA | : | enzyme-linked immunosorbent acid |
| g | : | gram |
| HEPES | : | hydroxyethylpiperazine ethanesulfonic acid |
| kb | : | kilobase |
| M | : | molar |
| mg | : | milligram |
| ml | : | millilitre |
| MOPs | : | 3-N-morpholino-propanesulfonic acid |
| NBT | : | 4-nitroblue tetrazolium chloride |
| ng | : | nanogram |
| PBS | : | phosphate buffered saline |
| RNase | : | ribonuclease |

| | | |
|---|---|---|
| rpm | : | revolution per minute |
| SCC | : | squamous cell carcinoma |
| SDS | : | sodium dodecyl sulphate |
| TBE | : | tris-borate-EDTA |
| UV | : | ultraviolet |
| w/v | : | weight per volume |
| WHO | : | World Health Organization |
| $CO_2$ | : | carbon dioxide |
| FCS | : | fetal calf serum |
| OD | : | optical density |
| nm | : | nanometer |
| PCR | : | polymerase chain reaction |
| pmole | : | picomole |
| U | : | units |
| ADC | : | adenocarcinoma |
| CS | : | lamb serum |
| PBS-T | : | phosphate buffered saline-tween |
| PCR-SB | : | polymerase chain reaction-Southern blot hybridization |
| ISH | : | in situ hybridization |
| ORF | : | open reading frame |
| FIGO | : | Federation of Gynaecology and Obstetrics |
| HPV | : | human papillomavirus |
| E | : | early region |
| L | : | late region |
| LCR | : | long control region |
| kDa | : | kilodalton |
| BPV | : | bovine papillomavirus |
| RNA | : | ribonucleic acid |
| MHC | : | major histocompatibility complex |
| NK | : | natural killer cells |
| Tm | : | melting point |

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