

**PREVALENCE AND MOLECULAR
VARIANCE OF HUMAN PAPILLOMAVIRUS
AMONG WOMEN IN KOTA KINABALU,
SABAH, MALAYSIA**

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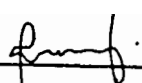


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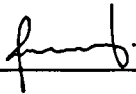
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ABSTRACT

Human papillomavirus (HPV) is regarded as one of the influential causes of cervical cancer incidences worldwide. Information concerning the geographical distribution of HPV genotypes is now considered as crucial in order to determine the efficacy of HPV vaccine protection against infections of diverse types of HPV. Studies of HPV genotype distribution along with its association with various risk factors and the nature of its carcinogenicity based on the established lineages are still at a juvenile stage in Malaysia with a lack of data particularly among the population in Sabah. A total of 240 samples were successfully collected from female volunteers who were recruited from Sabah Women and Children Hospital as well as UMS Polyclinics Kingfisher in a period of 12 months. HPV was detected by means of polymerase chain reaction (PCR) amplification of L1 gene from HPV viral genome using primer pair MY09/MY11. Then, HPV genotyping was performed by subjecting the PCR products to an established restriction fragment length polymorphism (RFLP) procedure which was able to detect 49 mucosal HPV genotypes. All data concerning participants' sociodemographic, clinical, and behavioural characteristics were acquired from the questionnaires which were provided to the women along with informed consent forms. The results of Pap smear test were obtained from gynaecologists in charge. Approximately 13 primer pairs were designed based on the regions of LCR and E6 gene of each HPV genotype detected. PCR amplification was conducted using the designed primers and all PCR products were purified and sequenced. Analyses of sequence variation of each HPV isolate was performed by comparing the acquired sequences to the prototype sequences obtained from GenBank. Meanwhile, multiple alignment was performed on all acquired sequences and their prototypes, and neighbour-joining (NJ) phylogenetic trees were generated along with 1000 bootstrap replicates in order to evaluate their reliability. The prevalence of HPV infection among the 240 women residing in Kota Kinabalu was 9.6%. A total of 13 HPV genotypes were discovered whereby HPV-56 (high-risk) and HPV-70 (probable high-risk) were among the most prevalent types identified. HPV-56 was also found to associate with low-grade neoplasia (LSIL). Univariate and multivariable models were used to analyse the strength of relationship between risk factors and HPV infection. The results of the analyses indicated a significant association between HPV infection and two risk factors: education (OR: 0.13, 95% CI: 0.03-0.62) and employment (OR: 4.94, 95% CI: 1.58-15.40). The LCR regions and E6 genes of the 13 HPV genotypes were then amplified and sequenced. The analyses of sequence variation and construction of phylogenetic trees indicated that at least four highly oncogenic lineages were found among high-risk HPV variants identified in the study namely HPV-16 A4 (Asian), HPV-33 A1, HPV-56 B, and HPV-58 A, respectively. These four lineages are highly associated with incremental risk of severe cytological lesions of the cervix and development of cervical cancer. The outcomes of the study served as a significant baseline information crucial for the women population of Sabah. The high prevalence of HPV-56 among women in Sabah indicated that a new strategy of HPV vaccination with next generation of vaccine targeting HPV-56 will be necessary in the future.



ABSTRAK

KELAZIMAN DAN VARIANS MOLEKUL PAPILLOMAVIRUS MANUSIA DALAM KALANGAN WANITA DI KOTA KINABALU, SABAH, MALAYSIA

Papillomavirus manusia (HPV) dianggap sebagai penyebab utama kejadian kanser serviks di seluruh dunia. Maklumat mengenai taburan geografi genotip HPV kini dianggap sebagai penting untuk menentukan keberkesanan perlindungan vaksin HPV terhadap jangkitan pelbagai jenis HPV. Kajian taburan genotip HPV dan hubungannya dengan pelbagai faktor risiko serta sifat kekarsinogenannya berdasarkan keturunan yang telah didirikan masih berada di tahap juvenil di Malaysia dengan kekurangan data terutamanya dalam kalangan penduduk di Sabah. Sejumlah 240 sampel berjaya dikumpulkan daripada sukarelawan wanita yang direkrut dari Hospital Wanita dan Kanak-kanak Sabah serta UMS Polyclinics Kingfisher dalam tempoh 12 bulan. HPV dikesan dengan cara tindak balas berantai polimerase (PCR) bagi mengamplifikasi gen L1 daripada genom virus HPV dengan menggunakan pasangan primer MY09/MY11. Kemudiannya, penjenisan HPV dilakukan dengan mendedahkan produk PCR kepada prosedur polimorfisme pemotongan panjang cebisan (RFLP) yang mampu mengesan 49 genotip HPV mukosa. Semua data mengenai ciri sosiodemografi, klinikal, dan tingkah laku peserta diperolehi daripada soal selidik yang diberikan kepada para wanita bersama dengan borang makluman persetujuan. Hasil ujian Pap smear diperolehi dari pakar ginekologi yang bertanggungjawab. Kira-kira 13 pasangan primer telah direka berdasarkan kawasan LCR dan gen E6 bagi setiap genotip HPV yang dikesan. Amplifikasi PCR dilakukan dengan menggunakan primer yang telah direka dan semua produk PCR telah dituliskan dan dijujuk. Analisis variasi jujukan setiap pencilan HPV dilakukan dengan membandingkan jujukan yang diperolehi dengan jujukan prototaip yang diperolehi dari GenBank. Sementara itu, penjajaran berganda dilakukan atas semua jujukan yang diperolehi serta prototaipnya, dan pokok filogenetik neighbour-joining (NJ) dijana bersama 1000 replika butstrap bagi menilai kebolehpercayaan pokok-pokok tersebut. Taburan jangkitan HPV dalam kalangan 240 wanita yang tinggal di Kota Kinabalu adalah 9.6%. Sebanyak 13 genotip HPV ditemui di mana HPV-56 (berisiko tinggi) dan HPV-70 (kemungkinan berisiko tinggi) adalah antara jenis yang paling lazim dikenal pasti. HPV-56 juga didapati bersekutu dengan neoplasia gred rendah (LSIL). Model univariat dan multivariable digunakan untuk menganalisis kekuatan hubungan antara faktor risiko dan jangkitan HPV. Keputusan analisis menunjukkan hubungan yang signifikan antara jangkitan HPV dan dua faktor risiko: pendidikan (OR: 0.13, 95% CI: 0.03-0.62) dan pekerjaan (OR: 4.94, 95% CI: 1.58-15.40). Kawasan LCR dan gen E6 daripada 13 genotip HPV kemudiannya diamplifikasi dan dijujuk. Analisis variasi jujukan dan pembinaan pokok filogenetik menunjukkan sekurang-kurangnya empat garis keturunan onkogenik didapati dalam kalangan varian HPV berisiko tinggi yang dikenalpasti dalam kajian iaitu HPV-16 A4 (Asia), HPV-33 A1, HPV-56 B, dan HPV-58 A. Empat keturunan ini amat dikaitkan dengan peningkatan risiko lesi sitologi yang teruk pada pangkal rahim serta perkembangan kanser pangkal rahim. Hasil kajian ini merupakan maklumat asas yang penting bagi penduduk wanita di Sabah. Kelaziman HPV-56 yang tinggi dalam kalangan wanita di Sabah menunjukkan bahawa strategi baru untuk vaksinasi HPV dengan vaksin generasi seterusnya yang mensasarkan HPV-56 akan diperlukan pada masa akan datang.

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LIST OF ABBREVIATIONS

µl	Microliter
ADC	Adenocarcinoma
AGCUS	Atypical Grandular Cells of Undetermined Significance
ALTS	ASCUS-LSIL Triage Study
ASCUS	Atypical Squamous Cells of Undetermined Significance
ASR	Age-standardized incidence rate
BLASTn	Basic Local Alignment Search Tool for nucleotide
bp	Base pair
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
EtBr	Ethidium bromide
GBD	Global Burden of Disease
hTERT	Human telomerase reverse transcriptase
HDI	Human Development Index
HPV	Human papillomavirus
HSIL	High-grade Squamous Intraepithelial Lesion
IARC	International Agency for Research on Cancer
ICC	Invasive cervical cancer
LCR	Long control region
LSIL	Low-grade Squamous Intraepithelial Lesion
NCBI	National Center for Biotechnology Information
NILM	Normal for Intraepithelial Lesion or Malignancy
NJ	Neighbor-Joining
ng	Nanogram
OR	Odds ratio
ORF	Open reading frame
PATRICIA	Papilloma Trial against Cancer in young Adults
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RE	Restriction endonuclease
RFLP	Restriction fragment length polymorphism
SCC	Squamous cell carcinoma



SD	Standard deviation
SDI	Socio-demographic index
SI	Sexual intercourse
STD	Sexually transmitted disease
STI	Sexually transmitted infection
T_a	Annealing temperature
T_m	Melting temperature
TF	Transcription factor
URR	Upstream regulatory region
UV	Ultraviolet
VLP	Virus-like particle

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CHAPTER 1

INTRODUCTION

1.1 Research background

Human papillomavirus (HPV) is a type of infectious agent which is widely known to cause diseases in humans ranging from skin warts to neoplasia and cancer. It is also regarded as a common sexually transmitted infection (STI) particularly in women wherein HPV is associated with various genital illnesses including cervical cancer. Following the discovery of its infection in approximately 99.7% of global cervical cancer cases, HPV is considered as one of the prerequisites which can lead to the progression of cervical cancer (Walboomers *et al.*, 1999; Rajaram *et al.*, 2012).

There are approximately 198 genotypes of HPV currently identified and classified according to their carcinogenic properties (Bzhalava *et al.*, 2015). Studies of geographical prevalence and distribution of HPV genotypes across the world are now considered important in order to obtain information concerning the nature of HPV among diverse human population. Furthermore, investigation into the relationship between HPV infection and certain factors involving socio-demographic background, clinical records, and lifestyle behaviour is deemed necessary in determining the risk of acquiring persistent HPV infection and developing cervical cancer (Ribeiro *et al.*, 2015). Relevant data gained from these studies can help to improve the current preventive strategies involving prophylactic HPV vaccines and cervical cancer screening procedures.

With regard to the recent advances in biotechnological techniques and based on the fact that propagating HPV in tissue culture is impractical, molecular markers are currently designed and developed based on specific molecular mechanisms involved in HPV-driven carcinogenesis such as HPV Long Control Region (LCR) and E6 gene, therefore allowing meticulous analyses of its carcinogenic capacity and association with cancer at the molecular level. While HPV shares a similarity to other biological entities whereby its DNA is also susceptible to genetic evolution in every



genome replication that occur, low mutation rates are still maintained due to the high degree of viral proof reading (Burk et al., 2013). A genotype of HPV is defined by 10% difference in nucleotide bases. Therefore, it takes at least millions of years for such evolution to occur. However, a genetic change of less than 10% frequently occurs between the isolates of the same genotype and this is referred to as a subtype or a variant. Despite their genetic relatedness, HPV variants have distinct pathogenic differences and there are currently many efforts to identify and sequence the genome of these variants due to value of their medical significance and the knowledge possessed that can bring insights to the fundamental elements of HPV-induced cancer (Burk *et al.*, 2013).

The main focus of the study was the population of local women residing in Kota Kinabalu, the capital city of Sabah where the population number is the highest in the state. This city was selected for the study due to the population size and its role as a focal point for diverse ethnic groups to converge from various regions in Sabah. Hospitals and clinics in suburban and rural areas tend to refer their patients to the hospitals in the city where high-tech medical utilities and high number of specialists are available. The women involved in the study were Malaysian citizens who were born and raised in Sabah or have been living in the state for a very long time (≥ 10 years). Information regarding HPV gathered from the selected population was used as baseline data to compare with the information obtained from the populations in other states of Malaysia as well as in other countries worldwide.

1.2 Problem statements

Cervical cancer is currently the fourth most common cancer among females in the world and approximately more than one third of global cervical cancer cases occurred in Southeast Asian countries (Domercant *et al.*, 2015; Bruni *et al.*, 2017). According to the latest Malaysian national cancer registry report for 2007 – 2011, cervical cancer was listed as the third among ten most prominent cancers in Malaysian women. Both Sabah and Sarawak are regarded among the highest contributors of new cases in comparison to other states in West Malaysia due to their high age-standardized incidence rate (ASR) in cervical cancer (Azizah *et al.*, 2016). Despite high incidence rate of cervical cancer among the women in Sabah, public data regarding HPV

infection and its genotype distribution among the population were yet to be established.

Although HPV infection can lead to cervical cancer, it is not enough to induce cancer progression all by itself without the aid of a certain critical element which is the persistency of the infection (Riberio *et al.*, 2015). Acquisition of persistent infection among women is reported to be influenced by several risk factors involving socio-demographic status, lifestyle behaviours, and clinical history. In respect to the personal background of each individual, age, ethnicity, marital status, education, and employment have been among the significant risk factors to HPV infection. Furthermore, age at sexual debut, total number of lifetime partner(s), rate of sexual intercourse, condom usage, and oral contraceptive usage, and smoking are also regarded as the influencing variables for lifestyle behaviours. In addition, a record of other sexually transmitted disease(s) (STD) and illnesses such as diabetes as well as HPV vaccine uptake in the medical history may influence the vulnerability of an individual to HPV infection (Castellsagué *et al.*, 2002; He *et al.*, 2016).

HPV research studies in Malaysia are largely focused on the impact and cost-effectiveness of HPV vaccination on the Malaysian population, Malaysians' knowledge and attitudes toward HPV, Pap smear, and cervical cancer, and HPV prevalence which mainly targeted the population of West Malaysia (Zaridah, 2014). However, the information regarding association of HPV infection with the aforementioned potential risk factors among the women in Kota Kinabalu is limited.

Identification of HPV variants are conducted by sequencing and examining the mutations occurred in HPV genome sequences, and comparing them to the existing sequences. Among the genome sequences, HPV LCR and E6 gene were selected for this study due to their involvement in viral transcription and inactivation of tumour suppressor gene p53, respectively (Choi and Park, 2015). Polymorphisms in these two sequences can cause the disruption of transcription factor (TF) binding sites and amino acid alteration respectively, which in turn can lead to persistent HPV infection and cancer development (Sichero *et al.*, 2012). Currently, the information regarding HPV variants in Malaysia, particularly in Sabah, has yet to be publicly established.

1.3 Hypotheses

The important role of HPV as an etiological agent to cervical cancer and its presence in virtually all cervical cancer cases (Rajaram *et al.*, 2012) indicated that an increase in cervical cancer incidences is strongly associated with the increase of HPV prevalence. Therefore, it can be speculated that the prevalence of HPV in Kota Kinabalu is influenced by cervical cancer incidences recorded in the state. If the cancer incidences reported among women population in Kota Kinabalu are higher than the populations of the states in West Malaysia, the prevalence of HPV among women in Kota Kinabalu is expected to be higher than that of West Malaysia as well.

Several risk factors involving socio-demographic background, clinical history, and sexual lifestyle have been previously demonstrated to display significant association with HPV infection in different types of population in the world (Castellsagué *et al.*, 2002). Hence, it is surmised that these potential risk factors may also indicate significant association with HPV infection in the population of Sabah. While the information regarding HPV variants in Malaysia is not publicly established yet, various studies targeting the populations of Southeast Asian countries such as Thailand, Indonesia, and the Philippines revealed that the majority of HPV variants found in these regions shared similar genomic structures or lineage/sublineages which are highly involved in increased risk of cervical cancer such as sublineage A4 (Asian) among the HPV-16 variants (Yamada *et al.*, 1997; Chopjitt *et al.*, 2009). Therefore, since Malaysia is also located within the same region among these Southeast Asian countries, it is hypothesized that these oncogenic lineage/sublineages are also highly prevalent among women in Sabah.

1.4 Research objectives

Among the objectives established in this study were:

1. To determine the prevalence of HPV and its genotype distribution among women in Kota Kinabalu, Sabah;

2. To determine the significant association between HPV infection and potential risk factors involving socio-demographic data, clinical history, and lifestyle behaviours;
3. To characterize sequence variation within long control region (LCR) and E6 sequences of HPV variants found among women in Kota Kinabalu, Sabah;
4. To identify the lineage/sublineage of HPV variants found among women in Kota Kinabalu, Sabah based on the construction of phylogenetic trees for long control region (LCR) and E6 sequences of HPV.

1.5 Significance of study

This prevalence study encompassing local women will be able to provide an estimate of the current status of HPV infection in Kota Kinabalu, Sabah where there is a shortage of information concerning HPV. An investigation into the distribution of HPV genotypes in Kota Kinabalu will also be able to determine the most prominent pathogenic genotype exist within the population, therefore, providing significant information that can contribute to the improvement of HPV vaccine.

The knowledge obtained from the understanding of the association between various risk factors and acquisition of persistent HPV infection will be able to help in preventing the spread of the infectious disease. This matter can be achieved by pinpointing the significant variables and coming up with possible solutions to overcome or improve them, including the effort of increasing public awareness regarding the influence of such risk factors to the people's health and daily lifestyle.

Furthermore, the molecular study of LCR and E6 sequences among the variants of HPV will be able to provide a baseline information that can open the door to more research opportunities and new endeavours in developing effective treatments for HPV-related cancers.

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