ORIGINAL ARTICLE

Prevalence of Human Papillomavirus Genotypes in Preinvasive and Invasive Cervical Cancer-A UKM Study

Sharifa Ezat WP[1], Sharifah NA[2], Sayyidi Hamzi AR[2], Norin Rahayu S[3], Shamsul Azhar S[1], Syed Mohamed A[4]

[1]*Department of Community Health, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre*
[2]*Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre*
[3]*Faculty of Informative Technology and Quantitative Science, Universiti Teknologi MARA (UiTM)*
[4]*United Nations University-International Institute for Global Health (UNU-IIGH), Kuala Lumpur, Malaysia*

ABSTRAK

Satu kajian hirisan lintang telah dilaksanakan bertujuan mengenalpasti prevalens dan distribusi genotip virus papiloma manusia (HPV) di kalangan pesakit prakanser (neoplasia intraepitelial serviks gred 3 atau CIN 3) dan kanser serviks invasif (ICC), di Pusat Perubatan UKM (PPUKM). Blok tisu benaman parafin dari tahun 1999 ke 2007 dikenalpasti dari Jabatan Patologi, PPUKM. Sebanyak 80 rekod perubatan (20 CIN 3 dan 60 ICC) dengan informasi lengkap telah dikumpul untuk data klinikopatologi. Dari kanser invasif (n=60), karsinoma sel skuamus (SCC) terdiri dari 75% dan adenokarsinoma 25%. Purata umur pesakit ialah 52.0 ± 12.21 tahun dan wanita Cina merupakan etnik yang tertinggi (66.3%). Sebanyak 12 jenis genotip HPV telah dikenalpasti iaitu HPV 16, 33, 18, 39, 52, 45, 58, 59, 31, 35, 6 dan 11. Prevalens infeksi HPV ialah setinggi 92.5% dengan genotip 16 yang paling kerap ditemui (73.8%), diikuti dengan genotip 33 (30%) dan genotip 18 (22.5%). Sebanyak 31 kes (38.8%) menunjukkan kehadiran satu jenis genotip HPV sahaja, manakala 44 (53.8%) menunjukkan kehadiran genotip HPV berganda (dua genotip atau lebih). Pada ICC, HPV 16, 33, 18, 52 dan 39 merupakan lima jenis HPV yang paling kerap ditemui. Infeksi HPV yang tinggi dan infeksi berganda merupakan perkara utama ditemui pada lesi CIN 3 dan kanser serviks.

Kata kunci: virus papiloma manusia (HPV), kanser serviks, genotip HPV, distribusi HPV, prevalens HPV

ABSTRACT

A cross sectional study was done to determine the prevalence and distribution of human papillomavirus (HPV) genotypes in pre-invasive (cervical intraepithelial

Address for correspondence and reprint requests: Prof Dato' Dr Syed Mohamed Aljunid, United Nations University-International Institute of Global Health (UNU-IIGH), UKM Medical Centre, Jalan Yaacob Latiff, 56000 Cheras Kuala Lumpur. Tel: +603-9171 5394. Fax: +603- 9171 5402.

neoplasia, grade 3 or CIN 3) and invasive cervical cancer (ICC), in Universiti Kebangsaan Malaysia Medical Centre (UKMMC). A total of 80 paraffin-embedded tumour tissue blocks (20 CIN 3, 60 invasive cancers) between 1999 to 2007 were retrieved from the archives of the Department of Pathology. Patient's medical records were obtained from the Medical Records Office. Among invasive cancers (n=60), squamous cell carcinoma (SCC) account for 75% and adenocarcinoma 25%. The mean age of cases studied was 52.0 ± 12.2 years and Chinese was the predominant ethnicity (66.3%). Twelve HPV genotypes were identified, namely, HPV 16, 33, 18, 39, 52, 45, 58, 59, 31, 35, 6 and 11. The prevalence of HPV was 92.5% with types 16 being the most common (73.8%), followed by types 33 (30%) and 18 (22.5%). A total of 31 cases (38.8%) showed single HPV genotype, while 43 (53.8%) had multiple HPV (two genotypes or more) genotypes. In ICC, HPV 16, followed by types 33, 18, 52 and 39 were the top five common HPV genotypes detected. High prevalence of HPV and multiple HPV infections were major findings among patients with pre-invasive and invasive cervical cancer.

Key words: Human papillomavirus (HPV), cervical cancer, HPV genotype, HPV distribution, HPV prevalence

INTRODUCTION

Cervical cancer is the second most common cancer among women worldwide. The WHO (2002) reported that in 2002, over 471,000 new cases were diagnosed and 288,000 women died from cervical cancer worldwide. The incidence and mortality rate of cervical cancer were higher in developing countries (Sankaranarayanan et al. 2001), where no effective, systematic and organised screenings are carried out. It is recommended that for a screening method to be effective, it must be targeted on high risk women once or twice in their lifetime using a sensitive method (Sankaranarayanan et al. 2001) and a high enough coverage of more than 80% of targeted population.

The Ministry of Health (MOH) Malaysia reported cervical cancer as the second leading cause of death after breast cancer among women in year 2003 (Malaysian National Cancer Registry 2003) with an age-standardised incidence of 19.7 per 100,000 population. Its incidence rate by ethnicity was highest among Chinese women, intermediate among the Malays and lowest among Indians. In the third Malaysian National Health Morbidity Survey (NHMS 3) 2006, 43.7% women were reported to have undergone Pap smear examination with highest prevalence among Chinese women (50.5%), other Bumis (Sabahans, Sarawakians) (45.5%), Malays (43.6%), Indian (37.2%) and others (24.7%). The cost of screening and managing pre and invasive cervical cancer (ICC) in Malaysia is calculated to be RM 250 million per year (Wan Puteh et al. 2008).

The human papillomavirus (HPV) is considered a public health problem as the virus is sexually transmitted (Kjaer et al. 2001; Lehtinen et al. 2002; Jastreboff & Cymet 2002) and is a major risk factor for cervical cancer development worldwide (Reid & Lörincz 1996). The International Agency for Research in Cancer (IARC) in 1995 reported that HPV type 16 and 18 are carcinogenic to human. However, more than 100 types of HPV have been discovered (Krejci & Sanchez 2005; Jastreboff & Cymet

2002), of which approximately 15 are oncogenic. Another 30 types of HPV are associated with ano-genital tract infections (Krejci & Sanchez 2005) and type 18 is also found in inverted papilloma and papillary transitional cell carcinoma of the bladder (Chan et al. 1997). Cervical co-infection with multiple types of HPV was postulated to be a predictor of acquisition and persistence of HPV infections and subsequent development of cancer (Rousseau et al. 2001). The prevalence of HPV among normal population (Clifford et al. 2005) shows that it varies greatly between populations, from 1.4% in Spain to 25.6% in Nigeria.

HPV types within a species are related, e.g. the Alpha 7 species consists of HPV 18, 39, 45, 59, 68, 70 and a novel c85. Alpha 9 consist of types 16, 31, 33, 35, 52, 58 and 67. The Alpha 10 species consists of the low risk types 6, 11, 13, 44, 55, 74, PcPV and CCPV (De Villiers et al. 2004). HPV infections especially types 16 and 18 (Dillner et al. 1997) are necessary but not sufficient causes of cervical cancer (Yadav et al. 1997; Samoff et al. 2005). Other external cofactors or host factors are required for progression to cancer. There are many risk factors contributing to cervical cancer, these include multiple sexual partners; early sexual debut and activity at a younger age (Nor Hayati 2003); high parity (Hinkula et al. 2004); genital infection e.g. with Chlamydia trachomatis (Hakama et al. 2000; Samoff et al. 2005); herpes simplex virus (Lehtinen et al. 2002); chemical carcinogens (oral contraceptive drugs, cigarette smoking) and low socioeconomic status (Nolte & Walczak 2000). The development of cervical cancer is common in older women even though the HPV infections usually occur in the younger age group (Sellors et al. 2003). In the African region, the mean age of invasive cervical cancer (ICC) among patients was 33.9 years (±11.4 years) compared to Southern Europe which is 56.5 years (±14.3 years) (Bosch et al. 1995).

HPV genotypes have been found to be geographically determined. A previous local study showed that HR HPV genotypes were detected in 95% abnormal smears with HPV 16 being the most prevalent HPV genotype identified (Sharifah et al. 2009). In Europe (Clifford et al. 2005), the prevalence of HPV 16 among women was higher, compared with sub-Saharan Africa, while in Washington, type 16 dominated followed by type 18 (Schwartz et al. 2001), and this follows the general world trend.

The distribution of HPV types worldwide showed the same result as Schwartz et al. (2001), except for an earlier study from Indonesia in which HPV type 18 was reported to be the predominant type (Bosch et al. 1995). However, this study was later replicated using a bigger sample, and results showed that HPV positivity was as high as 96% (Schellekens et al. 2004) with types 16 (44%), 18 (39%) and 52 (14%) being the top three dominant types. A study by Chichareon et al. (1998) in Thailand showed that HPV type 16 was most commonly found in squamous cell carcinoma (SCC), followed by HPV types 18, 58, 52, 31, 33 and 39. Bosch and de Sanjosĕ (2003) also showed that the prevalence of HPV in SCC was around 80% and the five common HPV genotypes found are 16, 18, 45, 31 and 33.

From Hong Kong, HPV epidemiology demonstrated a high prevalence of type 52 (Cheung et al. 2008). In Iran, in addition to the predominant types 16/18, there was also a high prevalence of type 33 (Mortazavi et al. 2002). These studies showed that following infection with HPV type 16 and 18, some other types are also predominant and this may vary by regions. This inconsistency raises the question as to whether Malaysia has its

own particular HPV distribution that is not in trend with the rest of the world. The different HPV genotypes may translate into different risks of acquiring cervical cancers according to geographical distributions.

With the introduction of the HPV vaccines (quadivalent and bivalent), the protection against cervical cancer is tremendous. The quadrivalent vaccine that protects against types 6, 11, 16 and 18 has the potential to prevent vulval, vaginal and cervical cancers as well as genital warts. There is evidence of cross protection by the quadrivalent vaccine against similar genotypes in the same tree (Bosch et al. 2008). In a cross protection study by Brown (2007), the efficacy of the quadrivalent HPV vaccine against CIN 2, 3 or adenocarcinoma in situ associated with HPV types 6, 11, 16, 18 was 100%, but with increase in numbers of HPV types 31, 33, 34, 45, 51, 52, 56, 58, 59, the efficacy of the vaccine reduces to 38%.

Elucidation of HPV genotypes in Malaysian women has not been done on a major scale. This study aims to determine the prevalence and distribution of HPV genotypes in a cohort of Malaysian patients with preinvasive and invasive cervical cancer from Universiti Kebangsaan Malaysia Medical Centre (UKMMC)

MATERIALS AND METHODS

A total of 80 (20 CIN 3, 45 SCC and 15 adenocarcinoma) formalin-fixed, paraffin embedded tumour tissue (PET) blocks were retrieved from the Department of Pathology, Universiti Kebangsaan Malaysia Medical Centre (UKMMC) between 1999 to 2007. Patient consent was obtained through telephone interviews.

Five µm thick sections were cut from each case. Gloves and blades were changed during sectioning to avoid cross contamination between samples. DNA was extracted using DNeasy Blood and Tissue Kit (QIAGEN, Germany, Catalog No. 69506). Tissue sections were deparaffinized with xylene and alcohol according to the manufacturer's protocol. Samples were lysed using proteinase K. Lysates were loaded onto DNeasy spin columns. After two washings, pure DNA was eluted in low salt buffer. Presence of DNA was determined by 1% agarose gel electrophoresis. The optical density (OD) measurement was done to determine the purity and concentration of DNA within a ratio of 1.7 to 2.0. The extracted DNA was stored in -20 °C until RT-PCR.

HPV genotyping was carried out using SACACE HPV High Risk Typing Real-TM kit (SACACE, Italy, Catalog No. TV26-100FRT). This kit is an in-vitro Real Time amplification test for qualitative detection and genotyping of 12 HR-HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59). It contains four PCR-mix tubes; each of which contain primers directed against regions of three HPV genotypes with the β-Globin gene as an internal control. Multiplex amplification reaction was performed in a volume of 13 µl containing 8 µl of reaction mix (PCR-mix-1, PCR-buffer FRT, Hot Start DNA Polymerase) and 5 µl of DNA sample. Each reaction was run for 45 cycles, under the following conditions: 15min at 95ºC, 20 sec at 95ºC and 60 sec at 60ºC. Data obtained from Real-Time PCR is then keyed-in to a software (Microsoft® Excel HPV Typing Real-Time MX Results Matrix.xls) provided by the SACACE kit, and interpreted. Statistical analysis was performed using Microsoft® Excel 2007.

RESULTS

A total number of 80 PET samples were analyzed (20 CIN 3, 45 SCC and 15 adenocarcinoma). The mean age of patients was 52 years, ranging from 30 to 80 years (Table 1). The highest number

Table 1: Prevalence of HPV genotypes in CIN 3 and invasive cervical cancer by age group, (n=80)

Age group	n	(%)
30-39	12	15
40-49	19	23.8
50-59	26	32.5
≥ 60	23	28.8
Total	80	100

Table 2: Prevalence of HPV genotypes in CIN 3 and invasive cervical cancer, (n=80)

Ethnic	n	(%)
Malay	20	25
Chinese	53	66.3
Indian	4	5.0
Others	3	3.8
Total	80	100

of HPV positive cases were from the 50-59 year age group (32.5%) with the Chinese showing the highest prevalence (66.3%) followed by Malay (25%) and Indian (5%) (Table 2).

Of the 80 samples of preinvasive and invasive cervical cancer (ICC), 92.6% showed presence of HPV genotypes. Twelve different HPV genotypes were detected i.e 16, 18, 31, 33, 35, 39, 45, 52, 58, 59, 6 and 11. Among the CIN 3 cases, HPV 16 was the most prevalent (60%), followed by HPV 33 (35.0%), HPV 45 (25.0%), HPV 18 (15.0%) and HPV 39 (15.0%). HPV 16 (80.0%) was the most prevalent genotype in SCC of the cervix, followed by HPV 45 (54.5%), HPV 33 (31.1%), and HPV 52 (26.7%). The prevalent HPV genotypes in adenocarcinoma of the cervix were HPV 16 (73.3%), 18 (46.7%), 33 (20.0%), 39 (6.7%) and 59 (6.7%) (Table 3). The five most prevalent HPV genotypes in 80 samples of preinvasive and ICC were HPV 16 (73.8%), 33 (30.0%), 18 (22.5%), 39 (16.3%) and 52 (16.3%) (Figure 1). The five most prevalent HPV genotypes in ICC were 16 (78.3%), 33 (28.3%), 18 (25%), 52 (20%) and 39 (16.7%) (Figure 2).

Multiple infections of at least two HPV genotypes were noted in 53.8% of the cases. 38.8% showed presence of a single genotype while 31.3% had two genotypes, 12.5% had three genotypes, 5% had four genotypes and 5% had five genotypes. Multiple HPV genotypes were more prevalent (53.8%) than single HPV genotype (38.8%) (Table 4).

DISCUSSION

The higher prevalence of HPV in Chinese ethnicity among sampled patients diagnosed to have cervical cancer in this study was consistent with results from the Malaysian Cancer Registry (2003) which reported that the incidence are highest among the Chinese with 28.8 per 100,000, followed by Indians (22.4) and Malays (10.4). Even though samples were from UKMMC which is a public teaching hospital, with a high number of Malay patients, results showed that the highest percentage of cervical cancer was found in the Chinese patients (66.3%). Malays (25%) and Indians (5.0%) showed lower prevalence of cervical cancer in this study. This result reflects the findings of cervical cancer incidence at the National level. In a Muslim country, and coming from a Malay culture that strictly prohibits cohabitation or sex outside wedlock may have a role in the reduction of risky sexual behaviours among its youths. Findings from the National Health Morbidity Survey 3 (NHMS 3) 2006 showed that an estimated proportion of those who had genital discharge/ulcers and who subsequently came forward for treatment were Chinese (49.9%). The prevalence profile of those who had

Table 3: Distribution of HPV genotypes in CIN 3 and invasive cervical cancer, (n=80)

HPV genotypes	CIN 3		SCC		Adenocarcinoma		Total	
	n	(%)	n	(%)	n	(%)	n	(%)
HPV 16	12	(60.0)	36	(80.0)	11	(73.3)	59	(73.8)
HPV 18	3	(15.0)	8	(17.8)	7	(46.7)	18	(22.5)
HPV 31	1	(5.0)	0	(0)	0	(0)	1	(1.3)
HPV 33	7	(35.0)	14	(31.1)	3	(20.0)	24	(30.0)
HPV 35	0	(0)	1	(2.2)	0	(0)	1	(1.3)
HPV 39	3	(15.0)	9	(20.0)	1	(6.7)	13	(16.3)
HPV 45	5	(25.0)	6	(54.5)	0	(0)	11	(13.8)
HPV 52	1	(5.0)	12	(26.7)	0	(0)	13	(16.3)
HPV 58	2	(10.0)	1	(2.2)	0	(0)	3	(3.8)
HPV 59	0	(0)	1	(2.2)	1	(6.7)	2	(2.5)
HPV 6	0	(0)	1	(2.2)	0	(0)	1	(1.3)
HPV 11	0	(0)	1	(2.2)	0	(0)	1	(1.3)
Total HPV genotypes*	34		90		23		147	
No. of cases with HPV Infection	18	(90.0)	43	(95.6)	13	(86.7)	74	(92.5)
No. of cases without HPV infection	2	(10.0)	2	(4.4)	2	(13.3)	6	(7.5)
Total no. of cases	20		45		15		80	

*Samples with one or more HPV genotypes
CIN 3: Cervical Intraepithelial Neoplasia 3
SCC: Squamous Cell Carcinoma

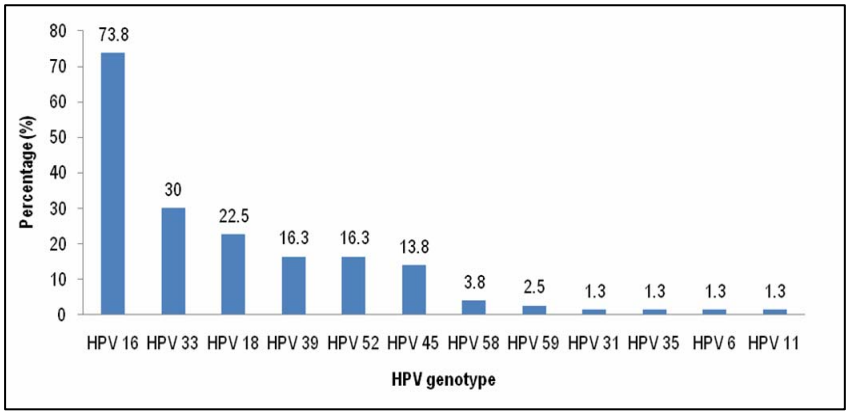

Figure 1: Prevalence of HPV genotypes in CIN 3 and invasive cervical cancer, (n=80)

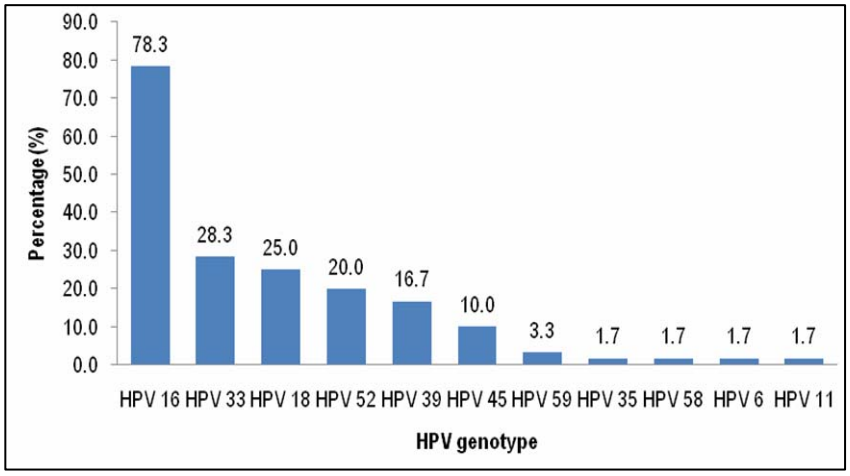

Figure 2: Prevalence of HPV genotypes in invasive cervical cancer, (n=60)

genital discharge/ulcer was highest among the Indians (3.5%) followed by Chinese (2.9%) and Malays (1.7%) (NHMS 3 2006).

Results of this study did not show any association between age and HPV positivity, and numbers of HPV types. In a study by Tang (2002), age was found to be inversely associated with HPV infections. Young females were more likely to harbour HPV, and oncogenic HPVs were found in patients below the age of 61 years old. Women in this study were from the middle age to elderly age groups with a mean age at 52 ± 12.2 years. In this study, 28.8% cases aged 60 and above, presented in the later stages of cancer. The Durex sexual survey (1999) and Sexual and Reproductive Health of Adolescents and Youths in Malaysia (2005), showed that sexual debut for women in this country was highest among the 17-18 year old. During adolescence, the knowledge of early sex and its' risk management is poor. NHMS 3 (2006) showed that the knowledge on symptoms of sexually transmitted illness was low, (50.9-53.8%) especially among the younger age group (below 19 years old) and the extreme age (above 70 years old). Findings from the NHMS 3 (2006) showed that females had their first sexual intercourse at the age of 22.8 years and initiation of sex does not vary much by ethnicity. However, this is an understatement as young adolescents do not declare sexual initiations to parents and guardians openly. This sexual novelty of a young woman with usually a more mature and experienced partner, puts her at risk of acquiring the infection through sexual intercourses and transmission to other partners later on. Data on the HPV pre-

Table 4: Prevalence of single and multiple HPV genotypes in CIN 3 and invasive cervical cancer, (n=80)

No. of HPV genotypes	n	(%)
Negative	6	7.4
Single	31	38.8
Two	25	31.3
Three	10	12.5
Four	4	5.0
Five	4	5.0
Total	80	100

valence and types of HPV among men is not readily available, and the cost effectiveness of HPV vaccination among men are still debated. The development of infection takes time, sometimes up to decades (Sankaranarayanan et al. 2001) with most clearing the infection within one year (Sellors et al. 2003). Women usually become infected when they are younger (Rousseau et al. 2001; Clifford et al. 2005), and some infections do not clear and progresses into dysplasia and frank cancer. HPV 16 infection (new infections) was associated with an increased risk of acquisition of other HPV types (Rousseau et al. 2001) and increases the risk of ICC (Dillner et al. 1997). By this time the women are usually in their older age group. From this study, we noted the presence of cancer among 'relatively younger' women i.e. less than 52 years old (mean age of cases in this study). Multiple infections play a role in oncogenesis and reduce cellular resistance, thus accelerating progression to cancer. In the 30-39 year age group, 41.7% had developed CIN 3, 75% had infection with type 16, 16.7% had infection with type 18 and 33.3% showed type 33. A high percentage of multiple infections was also noted in the younger age group.

Vinh-Hung et al. (2007) reported that SCC accounted for two thirds of the incidence of cervical cancer in the United States. Our results showed HPV types 16, 18, 33 and multiple infections dominated in SCC. Type 18 was also found at a high percentage in adenocarcinoma (46.7%). Both types 16 and 33 are from the Alpha 9 species, while type 18 is from the Alpha 7 species. Our results also showed that HPV positivity in ICC was highest in SCC (95.6%).

Lehtinen et al. (2002) in their study among SCC, showed that HPV type 16, 18 and 33 were associated with ICC especially types 33 and 16. According to Tang (2002) oncogenic HPV types are less infectious but cause more persistent infections than their lower risk counterparts, thus, repeated exposures are needed for infection to occur.

Clifford et al. (2005) reported that HPV 16 was twice as frequent as any other high-risk type in all regions except the sub-Saharan Africa where HPV 35 was equally common. The next common types were type 33 and 56 in Asia, 58 in South America and 31 in Europe. In our patients with CIN 3 and ICC, the prevalence of HPV 16 was found to be as high as 73.8%.

These current findings showed that types 16 and 18 play a dominant role in cervical carcinogenesis, both globally as well as in Malaysia. However, the high prevalence of HPV type 33 is a deviation against the normal trend except in Iran (Mortazavi et al. 2002), where the common HPV that predominate are types 16, 18 followed by 33. In the Iranian study, the prevalence of HPV in ICC was 85.5% which is nearly as high as the findings from our study which was 93.3%. Banura et al. (2008) reported that among the 12-24 year old sexually active women, the prevalence of HPV genotypes by decreasing order are 52, 51, 18 and 16. Other studies have also reported similar results (Clifford et al. 2005) in which type 33 and 56 are common among Asian women.

HPV 16/18 vaccines are estimated to provide about 67% protection against ICC in Asia (Bao et al. 2007). The difference in the prevalence of HPV types by different regions is shown in a mass study of women by Bao et al. (2007); by decreasing frequencies: HPV 16, 18, 58, 52, 33 in East Asia (China, Hong Kong, Taiwan, Korea, Japan); HPV 45, 52, 58 in South-East Asia; and HPV 45, 33 and 35 in South Central Asia (Bao et al. 2007). The difference in the HPV prevalence need to be considered before mass vaccination of women is carried

out, if the prevalence of HPV is not within the bivalent or quadrivalent vaccine efficacy coverage. However, results from our HPV genotyping suggests that a vaccine that covers both HPV types 16 and 18 will be able to cover 54.9% of this disease.

From our study of preinvasive and ICC, HPV type 16 was the most prevalent type detected (73.8%), followed by type 33 (30%) and type 18 (22.5%). With the different variations seen and different infectivity rates reported from various countries and regions, it is very important that local genotypes be properly determined and elucidated. The results of our study, reflect the prevalence and distribution of HPV genotypes in a cohort of Malaysian patients with preinvasive and ICC.

CONCLUSION

HPV infection is very high among high grade preinvasive lesions and ICC (92.5%) in Malaysian women. The five most prevalent HPV types are HPV 16, 33, 18, 39 and 52. Multiple HPV infections (53.8%) are relatively common compared to single infections. If the same HPV types represent the country's HPV infection trends among local high risk or cancer inflicted women, a vaccine that prevents infections from HPV types 16 and 18 will protect as high as 54.9% of women in this society.

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