Introduction

Incidence of cervical cancer in developing countries is common where its accounts for 15% of female cancers with a risk of 1.5% at age below 65 years old (Parkin and Bray, 2006). As for developed countries, cervical cancer accounts for only 3.6% of new cancers, with a cumulative risk of 0.8% (<65 years) (Parkin and Bray, 2006). Cancer of the cervix was the third most common cancer among women and fifth most common cancer in the entire general population of Malaysia (NCR, 2011). There are about 9 million women eligible to be screened [age 22-60 years] for cervical cancer and the presence of abnormal smears in routine cervical scrapings cytology accounts to 4-7%. Cervical cancer incidence rate increased after 30 years old and peaks at ages 65-69 years. Currently, though there is no national screening program available, around 30-40% coverage is achieved by opportunistic screening.

HPV infection is now a well-established causal agent in the development of a variety of epithelial lesions, which range in severity from benign warts to invasive cervical cancer (Carter et al., 2011). More than 90% of invasive cervical cancer specimens contain HPV-DNA with approximately 5% may be unrelated to HPV infection (Janicek and Averette, 2001; Bhatla et al., 2008). HPV prevalence among countries may vary considerably across studies. This is due to the insufficient degree of geographical coverage and sample size, diversity in the techniques used for HPV detection and different methods used for estimating HPV type-specific prevalence (Bosch et al., 2008; Giuliano et al., 2008). The adjusted global prevalence of HPV infection was 10.4% (Burchell et al., 2006). The overall HPV-DNA prevalence in invasive cervical cancer is 96%, based on pooled analysis of 12 studies (Clifford et al., 2006b). The prevalence of HPV types that cause cervical cancer are; HPV-16 (53.5%), -18 (17.2%), -45 (6.7%), -31 (2.9%), -33 (2.6%), and other high-risk types; -52, -58, -35, -59, -56, -39, -51, -73, -68 and -66 (15-20%) (Clifford et al., 2006a).

Complete data is not yet available on HPV burden in...
general population of Malaysia. However, according to the South-Eastern Asia HPV prevalence, about 6.2% of women are likely to harbour cervical HPV infection at any given time (Castellsagüé et al., 2007). A report issued by HPV Information Centre (WHO/ICO, 2007) showed that the HPV prevalence in Malaysia in cervical cancer cases (n=23) was 95.7% (95% CI: 78.1-99.9). Domingo and co-workers (2008) reported that, HPV-16 and -18 were the two most common HPV types in Malaysia (excluding East Malaysia) contributing 73.9% and 65.2% of total HPV prevalence, respectively. Whilst, HPV-31 contributes 13% followed by HPV-33 which accounts for 4.3% from a total of 23 cases of cervical cancer (Domingo et al., 2008).

The purpose of our study were: a) to ascertain the presence of HPV infection in routine cervical samples from women of North-Eastern region of West Malaysia; b) to determine the feasibility of detecting the presence of HPV-DNA in routine cervical sampling taken as for conventional Pap smear; c) to ascertain the number of ‘missed cases’ in which the cytology diagnosis is Within Normal Limits (WNL) or Unsatisfactory for Evaluation (USFE) while the cervical samples contain high risk HPV sub-types.

Materials and Methods

Cervical samples

The subjects were women volunteers who attended three main hospitals in North-Eastern region of West Malaysia [Hospital Kota Bharu (HKB), Hospital Universiti Sains Malaysia (HUSM) and Hospital Kuala Terengganu (HKT)] for routine cervical cancer screening. Conventional Pap smear was collected and immediately smeared on slide, alcohol-fixed, stained with Papanicolaou stain and read by cytologists according to the Bethesda System 2001 (TBS, 2001). Immediately afterwards, the same cervical broom was cut off and inserted into liquid solution (preservative solution) for HPV study. This study was approved by the local institutional human ethics review board.

HPV-DNA detection and typing

Genomic DNA was extracted from preservative solution using QIAamp DNA mini kit (QIAGEN, Hiden, Germany) according to the manufacturer’s instructions.

The HPV-DNA detection was carried out by two-tube nested PCR. The first PCR (using MY primer) assay was carried out containing 5U Taq DNA Polymerase (0.05U), 10X Buffer, 200mM dNTP, 1.5mM MgCl₂, (Fermentas) and 0.5 mM of each of primer. The nested-PCR (using GP+ Primer) master mix used was as above except 1 µl of first nested-PCR was added as a template and 3.0mM MgCl₂. The cycle conditions for first PCR were as follows: denaturation of the template DNA for 1 cycle of 95°C for 3 min, amplification of the target DNA for 30 cycles of 95°C for 30s, 53.1°C for 30 s and 72°C for 30s; and a final extension for 1 cycle of 72°C for 7 min. The cycle condition for nested PCR was similar as above except the annealing temperature was 42.3°C. PCR amplification was carried out in a DNA thermal-cycler (Eppendorf AG, Hamburg, Germany). The amplified DNA was visualized on ethidium bromide stained 1.5% agarose gel after electrophoresis. Positive controls and negative water blanks were included in each run for quality control. HPV testing and smear interpretation were blinded.

HPV positive samples were subjected to cycle-sequencing using BigDye terminator v3.1 cycle sequencing mix (Applied Biosystems). It was analyzed using BioEdit Sequence Alignment Editor version 7.0.5.3. Sequences were submitted to the NCBI nucleotide–nucleotide BLAST (blastn) website (online alignment analysis) (http://www.ncbi.nlm.nih.gov/BLAST/) to arrive at specific genotyping.

Classification

The HPV types were classified into three categories; high-risk oncogenic HPV (HR-HPV:-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, -82), low-risk oncogenic HPV (LR-HPV:-6, -11, -40, -43, -44, -54, and -70) and undefined HPV risk (either HR or LR).

Results

Participant characteristics

A total of 635 women were recruited into the study with mean±SD age of 43±10.5 years. They were stratified into four age groups, as follow: under 35 years (24.4%), between 35-44 years (30.9%), between 45-54 years (34.3%) and older than 55 years (10.4%). The ethnic grouping of the women studied were predominantly Malays (85%), followed by Chinese (10.4%), Indian (0.6%) and other ethnics (3.9%) comparable to the ethnic proportion of these three states in Malaysia.

HPV positivity and cytology diagnosis

HPV-DNA was detected in 24 of 635 cervical samples in the first PCR. Of 635 cervical samples tested, 25 samples showed visible band in nested PCR. A total of 28 samples were considered HPV positive by both PCR (Table 1). Overall, pathologic findings were observed in 11 (1.7%) cases: 8 (72.7%) with low-grade squamous intraepithelial lesions (LSIL) and 3 (27.3%) with invasive cervical cancer. Two (0.3%) cases of Atypical Squamous/Glandular Cell of Undetermined Significance (ASCUS/AGUS) were identified. Smear within normal limit (WNL) was detected in 588 (92.6%) samples with 34 (5.4%) cases of ‘unsatisfactory smear for evaluation (USFE)’.

Table 1. HPV Positivity in Different Lesions Diagnosed by Conventional Pap smears

<table>
<thead>
<tr>
<th>Cytology (No. of Cases)</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>WNL (588)</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>AS/AG-CUS (2)</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>LSIL (8)</td>
<td>3</td>
<td>37.5</td>
</tr>
<tr>
<td>ICC (3)</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>USFE (34)</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Total (635)</td>
<td>28</td>
<td>4.4</td>
</tr>
</tbody>
</table>

HPV DNA in Routine Cervical Scraping Samples in Malaysia

Seven PCR positive samples were weakly positive after gel electrophoresis and failed in sequencing. Of 28 nested-PCR positive samples, 21 exhibited 6 different genotypes; 85.7% (18/21) were HR-HPVs and 14.3% (3/21) were LR-HPVs. HPV infection was found in 2.1% (4/195) and 1.5% (5/337) among women with normal without and with inflammation on cytology respectively. Among women with microbes infection seen in cytology, 5.4% (3/56) was HPV positive. HPVs was also found in 5.9% (2/34) of smears unsatisfactory for evaluation. Additionally, 37.5% (3/8) and 50% (1/2) were HPVs positive respectively among women with Low-grade Squamous Intraepithelial Lesion and Atypical Squamous/Glandular Cell of Undetermined Significance. All Invasive Cervical Cancer cases were positive for HPVs (100%). HPV-16 was by far the most prevalent genotype 57.1% (12/21) of all HPVs, representing 1.9% (12/635) of total samples, followed by HPV-58 (19.0%; 4/21); and HPV-6 (9.5%; 2/21). There were equal incidences of HPV-18, 33 and 61 (4.8%; 1/21). Type-distribution of HPVs in different cytological diagnosis of conventional Pap smear is shown in Figure 1. Thirteen out of 635 cases (2.0%) were found to be abnormal during classification of the conventional Pap smear into normal [cytology diagnosis is WNL] and abnormal cases [cytology diagnosis is AS/AG-CUS, LGSIL or higher].

Discussion

This is a first large scale HPV study on women population conducted on women populations of the North-Eastern region of West Malaysia, who are predominatly Malays unlike the central and west coast region of the country. Indian women had the highest incidence rate for cervical cancer followed by Chinese and Malay (NCR, 2011) these states. We noted that among the women with normal cytology, 3.0% harboured HPV-DNA, of which 91.7% were HR-HPV. Conventional Pap smear ‘missed’ to detect high-risk type of HPVs in a small proportion of samples. Our present HPV burden in normal population was comparable with various countries in Europe (1.4-9.2%) (Clifford et al., 2006b). However, it was lower than those generally observed in France (20.2%) (Casalegno et al., 2011), USA (27%) (Evans et al., 2006) and worldwide (10.5%) (Clifford, et al, 2006a).

There were only two cases of ASCUS/AGUS in this study and HPVs (HPV-16) was noted in the AGUS sample not in the ASCUS sample. HPV in ASCUS samples had been reported worldwide in variable proportions; 33% by Boulanger et al. (2004); 42% by Clifford et al. (2006a) and 89.5% by Evans et al. (2006). This could mean that we are more stringent in making diagnoses of ASCUS and AGUS. Lower HPV prevalence in ASCUS/AGUS had previously been reported in United States (4.1%) (Stoler et al., 2011). In our study, 37.5% of cases diagnosed as LSIL in cytology had HPVs. The presence of HPVs in such lesions seen by other researchers is also variable; ranging from 50% to above 90% (Clifford, et al, 2006a; Evans, et al, 2006). As in other studies, we noted high-risk HPV in all cases (albeit a small number) of invasive cervical cancer confirming an established fact that there is a strong relationship of HPVs and cervical cancer.
However, in Hanoi, Vu et al. (2012) reported only 91.3% HPV infection of their cervical cancer cases. Such low number we believe could be due to technical issue rather that the real prevalence of HPV in cervical cancer.

Our HPV type distributions in various lesions, based on the nested PCR plus sequencing were heterogeneous and in agreement with previous studies elsewhere in Asia and Europe (Speich et al., 2004). HPV-16 is known to be the most prevalent genotype in Southeast Asia and the rest of the world, regardless of the cytological status except in Indonesia and the Philippines (Clifford, et al., 2006a; Domingo, et al., 2008). Our HPV16 prevalence (57.1% of all HPVs) is lower than the values reported by Domingo and co-workers (2008); 73.9%. Interestingly, we found that HPV58 is the second (19.0%) most prevalent types followed by HPV6 (9.5%) and HPV18, 33 and 61 (4.8% each).

Domingo and co-workers (2008) reported that the second most prevalent types of HPV in Malaysia were HPV18 (65.2%) followed by HPV31 (13.0%) and HPV33 (4.3%). The biased result might be due to the samples they studied. Our samples came from women who attended gynae clinics for screening whilst Domingo’s study involved samples of cervical cancer. Furthermore they did not include populations from the East Coast of West Malaysia. Due to the low number of cases of high- and low-grade lesions obtained in samples recruited, we were unable to determine the real HPV type specific prevalence and type-distribution in respective lesion. In 2009, one study from Malaysia reported the prevalence of HPV in abnormal cervical lesions as 94.7% (Sharifah et al., 2009). In our study all the cervical cancer cases were HPV positive.

These findings highlight the presence of various high-risk HPV types in different cervical lesions. It may estimate the theoretical fraction of cervical cancer which could be prevented. Until recently, cytology-based screening programmes (using Pap smears) were the main tool to prevent cervical cancer. Well-organized programmes established in developed countries are able to detect and identify precancerous lesions at the early stages as they can easily be treated thus preventing up to 80% of cervical cancers. The programmes incorporate HPV testing in their routine cervical cancer screening. However, many countries which have high prevalence of cervical cancer are developing nations thus including HPV testing in the screening program not feasible. Having said that if HPV testing in cervical scraping samples. A special thanks to the women who participated in this study. This study was funded by research grant (Grant No: 06-02-05-1027 PR0024/09-01 and 1001/PPSP/812097) of the Ministry of Science, Technology and Innovation, Malaysia.

Acknowledgements

The authors would like to thank the Director General of Health (Malaysia), Deputy Director General of Health (Research & Technical Support, Ministry of Health Malaysia) and the Director of the Institute for Medical Research for the permission to publish this scientific paper. We also thank all staffs from Institute for Molecular Medicine (INFORMM) and Pathology Department, HUSM Kubang Kerian who helped in the study; especially the gynecologists of the 3 hospitals - Professor Nik Zaki Mahmood of HUSM, Dr Mohd Zulkifli Mohd Kasim of HKT and Dr Zainal Abidin Hanafi of HKB for providing cervical scraping samples. A special thanks to the women who participated in this study. This study was funded by research grant (Grant No: 06-02-05-1027 PR0024/09-01 and 1001/PPSP/812097) of the Ministry of Science, Technology and Innovation, Malaysia.

References


National Cancer Registry (NCR), Ministry of Health Malaysia (2011) Malaysia Cancer Statistics Report 2007 - Data and Figure.


