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Original Article

Trend of Human Papillomavirus Genotypes in Cervical Neoplasia Observed in a Newly Developing Township in Yangon, Myanmar

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Persistent infection with oncogenic types of human papillomavirus (HPV) is the most important risk factor associated with cervical cancer. This study detected the oncogenic HPV genotypes in cervical neoplasia in relation to clinicopathological findings using a cross-sectional descriptive method in 2011 and 2012. Cervical swabs and colposcopy-directed cervical biopsy tissues were collected from 108 women (median age 45 years; range 20–78) showing cervical cytological changes at Sanpya General Hospital, Yangon, Myanmar. HPV DNA testing and genotyping were performed by polymerase chain reaction and restriction fragment length polymorphism. HPV was identified in women with cervical intraepithelial neoplasia (CIN) 1 (44.4%), CIN2 (63.2%), CIN3 (70.6%), and squamous cell carcinoma (SCC) (74.1%). The association between cervical neoplasia and HPV positivity was highly significant ($p = 0.008$). Most patients infected with HPV were between 40–49 years of age, and the youngest were in the 20– to 29–year-old age group. The most common genotype was HPV 16 (65.6%) with the following distribution: 70% in CIN1, 41.7% in CIN2, 91.7% in CIN3, and 60% in SCC. HPV-31 was the second-most frequent (21.9%): 30% in CIN1, 33.3% in CIN2, 8.3% in CIN3, and 15% in SCC. The third-most frequent-genotype was HPV-18 (7.8%): 8.3% in CIN1, and 20% in SCC. Another genotype was HPV-58 (4.7%): 16.7% in CIN1 and 5% in SCC. The majority of CIN/SCC cases were associated with HPV genotypes 16, 31, 18, and 58. If oncogenic HPV genotypes are positive, the possibility of cervical neoplasia can be predicted. Knowledge of the HPV genotypes distribution can predict the effectiveness of the currently used HPV vaccine.

Key words: human papillomavirus, genotyping, Myanmar

Human papillomavirus (HPV) infection is the most common sexually transmitted disease worldwide [1], with the prevalence of high-risk (HR)-HPV in asymptomatic women varying from 2% to 44% [2]. Most HPV infections are transient: 54% resolve spontaneously in one year [3] and 91% in two years [4]. However, 10% to 60% of women positive for HPV will have the same genotype one year later [1].

Worldwide, cervical cancer is the third-most common cancer in women and accounts for 9% of all

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female cancers [5]. In Myanmar, the estimated cervical cancer incidence was 26.4% in 2008 [6]. Cervical cancer ranks as the second-most frequent cancer among women in Myanmar. Data are not yet available on the HPV burden in the general population of Myanmar. However, in South-eastern Asia, about 8.4% of women in the general population are estimated to harbor cervical HPV infection and 72.6% of invasive cervical cancers (ISCCs) are attributed to HPV 16 or 18 [7].

The risk of mild cervical changes leading to severe cervical changes, *i.e.*, cervical intraepithelial neoplasia 3 (CIN3), is higher when HR-HPV types, especially HPV genotype 16, are detected [8]. The possibility for the regression of CIN2 cervical changes caused by HPV genotype 16 is lower than that for cervical changes caused by other HR-HPV types [9]. Cervical intraepithelial changes regress when infection with HR-HPV resolves spontaneously [10].

HR-HPV-positive women even without cytological changes have a 210-fold higher risk of developing CIN3 in 6 years as compared with HR-HPV-negative women [11]. Only 9 women per 10,000 will have CIN3 if they are HR-HPV-negative with normal cytological findings [12]. HPV-16 is more likely to persist and is more aggressive compared with other types [13]. Although the prevalence of HR-HPV has changed recently, HPV genotypes 16 and 18 remain the most prevalent all over the world. HPV types -31, -33, and -45 compete to be the third-most prevalent in most countries, though an increasing prevalence of HPV type 58 has been observed recently (mostly in East Asia). However, HPV types -16, -18, and -45 are the only types that are more common in their invasive forms than as precancerous lesions [14].

HPV typing has important prognostic and therapeutic value. Information on the HPV type distribution in CIN and ISCC is crucial to predicting the future impact of HPV16/18 vaccines and screening programs and to establish appropriate post-vaccinal virologic surveillance. The aim of this study was to determine the prevalence of HPV infection and of oncogenic HPV genotypes-16, -18, -31, -33, -35, -52, and -58 in women with cervical neoplasia in relation to clinicopathological findings.

Materials and Methods

A cross-sectional descriptive study was carried out in women with cervical neoplasia in relation to clinicopathological findings in 2011 and 2012. After informed consent was obtained, cervical swabs and colposcopy-directed cervical biopsy tissues were collected from a total of 108 women (median age 45 years; range 20-78) with cervical neoplasia (CIN1, CIN2, CIN3 or squamous cell cancer) who received HPV DNA testing and genotyping at Sanpya General Hospital, Thingangyun, Yangon. Cervical cells and biopsy tissues were collected in phosphate buffered saline and stored at −20℃.

DNA extraction. For DNA extraction, the samples were suspended in 300μL of proteinase K and incubated at 50℃ for 2h and then treated with 100μL of 5M sodium chloride (NaCl). After centrifugation, the supernatant was treated with 900μL of ethanol. DNA precipitates were collected by centrifugation at 12,000rpm for 10min and washed with 400μL of 70% ethanol. DNA was dissolved in 100μL of Tris with ethylene-diamine-tetra-acetic acid (TE).

HR-HPV testing. HPV-DNA testing was performed using the polymerase chain reaction (PCR) method. Consensus sequence primer pairs within the E6 and E7 open reading frames, *i.e.*, forward primer (*pU-1M*): 5'-TGTCAAAAACCGTTGTGTCC-3' and reverse primer (*pU-2R*): 5'-GAGCTGTCGCTTAA TTGCTC-3') (oligo@sigma genosys-PCR, Japan), were used to amplify HR-HPV (HPV-16, -18, -31, -33, -35, -52b, -58) [8]. The reaction mixture contained 0.15μL of taq polymerase (Applied Biosystems, Roche, CA, USA), 2μL 10Xbuffer, 3.2μL dNTPs, 0.4μL of forward and reverse primers, 12.85μL distilled water and 1μL DNA. The samples were subjected to 35 cycles of amplification using an ASTEC thermal cycler (ASTEC Co. Ltd, Fukuoka, Japan). Each cycle included denaturation, annealing and extension steps. Detection of the PCR products was performed by electrophoresis on 6% polyacrylamide gel (PAGE) at 200V for 30 min and by silver staining.

HPV genotyping. In PCR-positive cases, HPV genotyping was analyzed by the restriction fragment length polymorphism method. HPV genotypes were determined by PAGE and silver staining of the digest of the PCR products with the restriction enzymes,

Ava II (HPV-16, HPV-18 and HPV-33), *Rsa* I (HPV-31), *Bgl* II (HPV-52b), *Acc* I (HPV-58) and *Ava* I (HPV-35) (Wako, Osaka, Japan). Enzymatic digestion was performed under the conditions recommended by the manufacturer [8].

Statistical analysis. Data analysis was done by using Microsoft Office Excel 2007 and the Statistical Package for Social Sciences (SPSS-16), *i.e.*, the SPSS full version free download (http://en.softonic.com/s/spss-16-full-version-free-download/) accessed December, 2013.

Results

Among 108 women tested for HR-HPV, 64 (59.3%) were positive as revealed by PCR bands around 240 bp～260 bp (Fig. 1). Analysis of the women positive for HR-HPV is shown in Table 1. The association between cervical neoplasia and HR-HPV positivity was highly significant ($p = 0.008$). HPV genotyping was analyzed by the restriction fragment length polymorphism method. Restriction analysis using *AvaII* revealed 42 samples having 2 fragment bands at 155 bp and 80 bp were of the HPV-16 genotype, and 5 samples having 2 fragment bands at 170 bp and 90 bp were HPV-18. Analysis using *RsaI* of 14 samples that had a broad band overlapping the 2 fragments at 119 bp and 114 bp showed them to be HPV-31. Analysis using *AccI* of 3 samples that had a broad overlapping band in the 126 bp and 118 bp regions showed them to be HPV-58. The most common genotype was HPV genotype 16 (65.6%). In this study, HPV genotypes -33, -35 and -52 were not detected. The majority of CIN/squamous cell carcinoma (SCC) cases were associated with HPV genotypes -16, -31, -18, and -58 (Table 2).

The proportions of HR-HPV in cervical neoplasia by age groups are shown in Table 3. In this study, 26.6% of the women having cervical neoplasia infected with HR-HPV were in the 30- to 39-year age

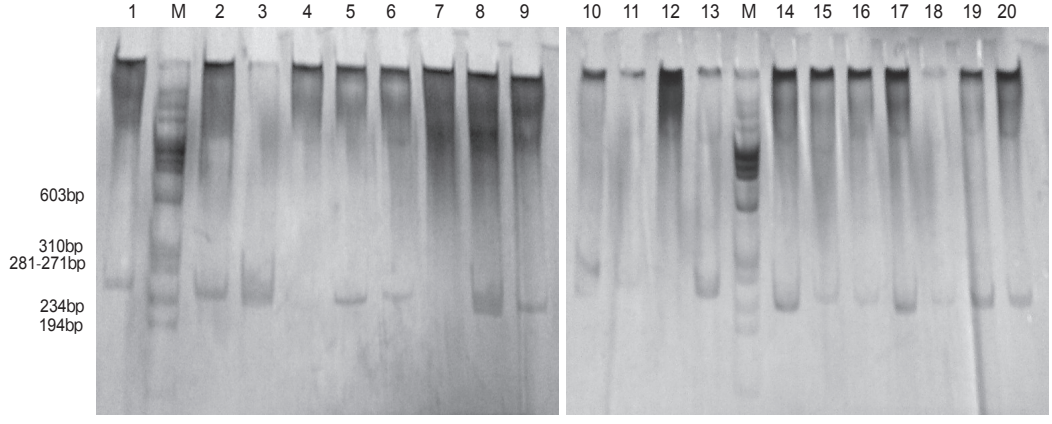

Fig. 1 Amplification of HPV using *pU1M/pU2R* primers showing lane M, molecular marker: ΦX174/HaeIII digest; lane 1 - positive control, lane 11 - negative control, lanes 2, 3, 5, 6, 8, 9, 13, 14 to 20 - positive HPV DNA, lanes 4, 7, 10, 12- negative HPV DNA, 6% PAGE, 200 V, 30 min with silver staining.

Table 1 Proportion of high-risk human papillomavirus infection in cervical neoplasia

HPV PCR	Histopathology				Total
	CIN I	CIN II	CIN III/CIS	SCC	
Positive	20 (44.4%)	12 (63.2%)	12 (70.6%)	20 (74.1%)	64 (59.3%)
Negative	25 (55.6%)	7 (36.8%)	5 (29.4%)	7 (25.9%)	44 (40.7%)
Total (count) (% within histopathology)	45 (100.0%)	19 (100.0%)	17 (100.0%)	27 (100.0%)	108 (100.0%)

group, 25% were aged 40–49 years, and 29.7% were aged 50–59 years. The youngest woman infected with HR-HPV was 20 years old, and the oldest was 78 years old in this study. Of the cases of HPV–16, 31% were among women aged 30–39 years, 31% were among 50– to 59–year-old women, and 28.6% were among 40– to 49–year-old women (Fig. 2A). A large percentage of HPV–18 cases (40%) were in women aged =/> 70 years and the same percentage (20%) was found among women aged 20–29 years, 40–49 years, and 50–59 years (Fig. 2B). As for HPV–31, 28.6% of all cases were found in women aged 30–39 years, and the same percentage was found among women aged 50–59 years (Fig. 2C). HPV–58 was detected in women aged 60–69 years (66.7% of HPV–58 cases) and women aged 50–59 years (33.3% of HPV–58 cases) (Fig. 2D). Therefore, HPV–16 was detected in 90.6% of women with HPV positive cervical neoplasia aged between 30 to 59 years. HPV–31 was detected in 78.6% of women with HPV positive cervical neoplasia aged between 30 to 59 years. HPV–18 and HPV–58 were more commonly found in older aged group as 40% of HPV–18 cases and 66.7% of HPV –58 cases were found in women aged 60 years and above. There was no significant difference in the prevalence of HPV according to the clinical stage of cervical cancer ($X^2 = 0.78$, $p = 0.37$).

Discussion

Data on the geographical distribution of HPV types in high-grade squamous intraepithelial lesion (HSIL) and ISCC are crucial for estimating the impact of HPV vaccines on cervical cancer and cervical screening programs. A meta-analysis of the HPV distribution in Asia reported a mean prevalence of 14% in women with normal cytology (NC) results and little variation between regions in Asia [16]. In Southeastern Asia, about 8.4% of women in the general population are estimated to harbor cervical HPV infection, and 72.6% of ISCC, 33.3% of HSIL, and 14.2% of low-grade squamous intraepithelial lesion (LSIL) cases are attributed to HPV–16 or –18 [7].

The prevalence and distribution of HPV genotypes

Table 2 Proportion of high-risk human papillomavirus genotypes in cervical neoplasia

HPV genotypes	Histopathology				Total
	CIN I	CIN II	CIN III/CIS	SCC	
HPV-16	14 (70.0%)	5 (41.7%)	11 (91.7%)	12 (60.0%)	42 (65.6%)
HPV-18	0 (.0%)	1 (8.3%)	0 (.0%)	4 (20.0%)	5 (7.8%)
HPV-31	6 (30.0%)	4 (33.3%)	1 (8.3%)	3 (15.0%)	14 (21.9%)
HPV-58	0 (.0%)	2 (16.7%)	0 (.0%)	1 (5.0%)	3 (4.7%)
Total (count) (% within histopathology)	20 (100.0%)	12 (100.0%)	12 (100.0%)	20 (100.0%)	64 (100.0%)

Table 3 Proportion of high-risk human papillomavirus in cervical neoplasia by age groups

HPV PCR	Age Group (Years)						Total
	20–29	30–39	40–49	50–59	60–69	=/>70	
Positive	4 (6.2%)	17 (26.6%)	16 (25.0%)	19 (29.7%)	4 (6.2%)	4 (6.2%)	64 (100.0%)
Negative	3 (6.8%)	10 (22.7%)	21 (47.7%)	9 (20.5%)	1 (2.3%)	0 (0%)	44 (100.0%)
Total (count) (% within HPV PCR)	7 (6.5%)	27 (25.0%)	37 (34.3%)	28 (25.9%)	5 (4.6%)	4 (3.7%)	108 (100.0%)

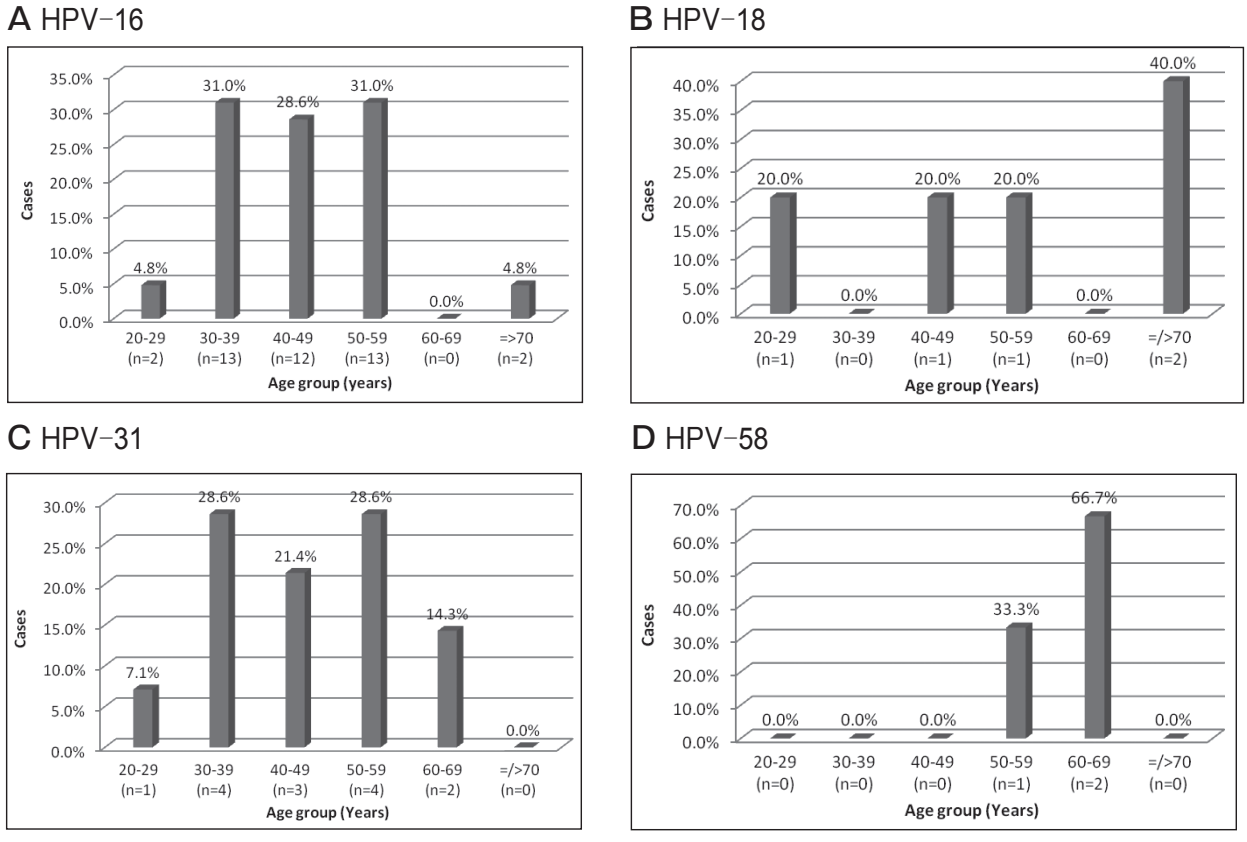

Fig. 2 Proportion of human papillomavirus genotypes **A**, HPV-16 **B**, HPV-18 **C**, HPV-31 **D**, HPV-58 in cervical neoplasia by age group.

varies greatly worldwide, and these differences might be related to the complex geographical and biological interplay between different HPV types and host immunogenetic factors [17]. With the exception of Eastern Africa, China, Japan and Taiwan [18-19], HPV-16 is the most prevalent type in all parts of the world. A meta-analysis done in 2011 found that 46.5% of HSIL cases harbored HPV-16 and 8.9% harbored HPV-18 and in ISCC, 53.2% of cases harbored HPV-16 and 13.2% harbored HPV-18. The next 5 most common types in decreasing frequency were HPV-31, -58, -33, -45, and -52 [20].

Martin *et al.* [21] reported that 96.3% of HSIL cases were positive for HPV, and the most frequent type was HPV-16 (51%), followed by -52 (14%) and -31 (11%). Jariene *et al.* [22] revealed that 59% of women with cervical neoplasia were positive for HR-HPV, and 61.1% of CIN1/CIN 1-2 cases and 74.2% of CIN2/CIN2-3/CIN3/carcinoma in situ cases were positive for HPV types-16, -18 or -45. In Myanmar, Mu Mu Shwe *et al.* [23] reported that 49% of women with abnormal cervical cytology and 5% with NC were positive for HR-HPV. They were identified in 35.5% of inflammatory smear cases, 60% of atypical squamous cells of undetermined significance (ASCUS), 86.7% of LSIL, 50% of HSIL and 100% of SCC. The detection of HPV genotypes showed the following: 60.4% of HPV positive cases were HPV-16, followed by HPV-31 (14.6%), HPV -18 (12.5%), and HPV-58 (12.5%).

In the present study, 59.3% of women with cervical neoplasia treated at Sanpya General Hospital located in the emerging urban area of Yangon city, Myanmar, were positive for HR-HPV which was detected in 44.4% of CIN1 cases, 63.2% of CIN2, 70.6% of CIN3, and 74.1% of SCC (Table 1). Analysis of the studies done in Myanmar demonstrated that the highest prevalence of HR-HPV was in SCC cases. This study was consistent with the other studies mentioned above. The association between cervical

neoplasia and HPV positivity was highly significant ($p = 0.008$). Thus if a patient is found positive for an HR-HPV genotype, the possibility of cervical neoplasia can be predicted. HPV testing improves the accuracy of colposcopy in the detection of CIN in women with ASCUS and LSIL cytological changes [24].

In the present study, 65.6% of HR-HPV cases were HPV-16, followed by HPV-31 (21.9%), HPV -18 (7.8%) and HPV-58 (4.7%). Among the cervical cancer cases, HPV-16 and -18 were most commonly detected. Analysis of both studies conducted in Myanmar showed that HPV-16 was the most common genotype (60.4% vs. 65.6%). HPV-31 was the second-most prevalent genotype in both studies (14.6% vs. 21.9%). HPV-18 was the third-most prevalent genotype (12.5% vs. 7.8%), and HPV-58 was found with low prevalence (12.5% vs. 4.7%) in the 2 studies. An interesting finding in the present study was that HPV-16 and -31 were detected in all CIN1, CIN2, CIN3, and SCC cases.

There were no significant differences among HPV genotypes in this newly developing area, *i.e*, in the periphery of Yangon, Myanmar, compared with the previous study [23]. The most prevalent genotype was HPV-16 followed by HPV-31, HPV-18 and HPV-58. Most of the studies have shown that HPV-16 was the most prevalent genotype associated with cervical neoplaisa [20, 21, 22]. One limitation of por study was the use of a methodology involving consensus primers that could detect seven genotypes of HPV-DNA (HPV-16, -18, -31, -33, -35, -52b, and -58). Thus restriction enzymes could differentiate those seven genotypes in this study. More advanced molecular technology such as molecular array and sequencing methods should be performed.

Recently, multiple types of HPV infections have been analyzed because, with the development of anti-HPV vaccines that do not cover all genotypes, the distribution of infection due to types not covered by vaccines could be affected. The elimination of one HPV type could affect the natural history of the remaining genotypes. Therefore, it is becoming imperative to obtain solid knowledge of genotype HPV distribution. Multiple types of HPV have been observed in up to 50% of NC and LSIL cases in Northwest Spain [25]. It is still not clear whether co-infection with several types increases the risk of progression. It seems that, as lesions progress from low to high grade, HR oncogenic types may persist, whereas less oncogenic types are eliminated [26]. A limitation of our study was the use of a methodology that could detect only seven HPV genotypes. If more advanced molecular methods that can detect multiple types were performed, multiple HPV infections could be analyzed.

According to our data, the prevalence rates of HR-HPV in women in different age groups with cervical neoplasia (30-39 yrs, 40-49 yrs, and 50-59 yrs) did not differ significantly ($X^2 = 1.16$, $p = 0.28$). This finding was consistent with Jariene K *et al.* [22]. In the present study, the youngest woman suffering from ISCC who was HR-HPV positive was 22 years old, and the oldest was 76 years old. 90.6% of HPV-16 positive cases and 78.6% of HPV-31 positive cases were found in women aged between 30 to 59 years. HPV-18 and HPV-58 were more commonly found in older age groups, with 40% of HPV-18 cases and 66.7% of HPV-58 cases found in women 60 years old and older.

The first peak of HR-HPV prevalence was observed in women in their early 20s up to 25 years [2], but these infections were mostly transient [27]. Severe cervical intraepithelial changes are also observed in teenagers and women up to 25 years of age just after the acquisition of HR-HPV infection [28]. According to the data reported, the peak incidence of HPV infection occurs within 5 to 10 years after the beginning of sexual activity. The prevalence of HPV decreases with age, but its persistence increases [29]. According to our data, there was no significant difference in the prevalence of HPV in women of different age groups. This may be because the present study was focused on the prevalence of HR-HPV in women with CIN/SCC, and not in the general population.

The methods commonly used to treat precancerous cervical lesions with or without HPV infection include cryosurgery (freezing that destroys tissue), the loop electrosurgical excision procedure (LEEP, which is the removal of cervical tissue using a hot wire loop), surgical conization (surgery with a scalpel, a laser, or both to remove a cone-shaped piece of tissue from the cervix and cervical canal), and laser vaporization conization (the use of a laser to destroy cervical tissue).

If a woman with NC is infected with HPV that can lead to cancer, frequent Pap tests are needed to watch for signs of abnormal cell changes in the genital area. Abnormal cell changes in the cervix are a warning sign of possible cervical cancer. In cases of LSIL and HSIL determined by cytology, colposcopy-directed biopsy should be performed to obtain an accurate diagnosis. Regarding the management of HPV-positive and HPV-negative patients with cervical cancer, HPV-infected individuals who develop cervical cancer generally receive the same treatment as patients whose tumors do not harbor HPV infections, according to the type and stage of their tumors. However, people who are diagnosed with HPV-positive oropharyngeal cancer may be treated differently than people with oropharyngeal cancers that are HPV-negative. Recent research has shown that patients with HPV-positive oropharyngeal tumors have a better prognosis and may do just as well on less intense treatment. An ongoing clinical trial is investigating this question.

Prophylactic HPV vaccines have been developed against HPV-16 and HPV-18 based on virus-like particles (VLPs) and which induce high titer of neutralizing antibodies; they could potentially prevent 70% of cervical cancers worldwide. These vaccines are more than 90% effective at preventing type 16/18 associated CIN, although their overall efficacy against all types (CIN2/3) in all vaccine recipients irrespective of current infection is considerably less [30]. The rationale for prophylactic HPV vaccination is based on the necessity of HPV infection in cervical carcinogenesis, so that by preventing this primary event, secondary changes which result in cytological abnormalities will also be prevented. The vaccine is intended to prevent pathological change in the cervix because the infection itself is asymptomatic and does not cause acute damage. This concept of preventing a viral infection in order to prevent a subsequent cancer, which might otherwise not occur for 25 years, is novel.

In Myanmar, only an opportunistic cervical screening program is present. There is no HPV vaccination program. Also, HPV-DNA testing could not be performed as a method of early detection of cervical cancer in clinical practice. Our study identified the prevalent HPV genotypes associated with different cervical neoplasia types so we could assess the usefulness of HPV vaccination in Myanmar. Before the start of a vaccination program, we should collect more evidence-based data about the prevalent HPV genotypes associated with cervical neoplasia in Myanmar.

The authors suggest that a strategy of primary prevention is needed to reduce the morbidity associated with the management of abnormal cytology. Such a strategy would also offer a potential means of prevention in countries without any cervical screening, including many underdeveloped countries with high rates of cervical cancer. However, screening is needed because of the limitations of the current HPV vaccines, both in their lack of a therapeutic effect (*i.e.*, their inability to protect women with an ongoing neoplastic process) and in the limited number of HPV types they can prevent (thus allowing some 25–30% of cervical cancer cases related to HPV types other than -16 and -18 to develop). A combination of both screening and vaccination is reasonable in a setting where screening is already developed and reasonably efficient.

The development of polyvalent vaccines (including some 5–8 HPV types) will extend protection to be effective against more than 90% of the oncogenic HPV types, and when such vaccines are achieved, vaccination alone would be sufficient. At present, no vaccination can provide total protection. Patients with cervical neoplasia infected with HPV prior to vaccination will not be protected and will still be at risk of infection with other non-vaccine HPV types.

In conclusion, this study detected the oncogenic HPV genotypes-16, -31, -18 and -58 in the majority of CIN and cervical cancer cases in Myanmar. If the oncogenic HPV genotypes are positive, the possibility of cervical neoplasia can be predicted. Knowledge of the distribution of HPV genotypes can make it possible to predict the effectiveness of the currently used HPV vaccine. As HPV genotypes -16 and -18 are the vaccine-preventable genotypes that are associated with cervical cancer, primary prevention of cervical cancer by HPV vaccination in the adolescent age group should be promoted in Myanmar, as it would reduce the incidence of cervical cancer and the associated mortality and morbidity.

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