HPV: Obvious but Not Necessary Cause of Cervical Cancer

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BACKGROUND/AIMS
Cervical cancer is one of the most common cancers worldwide. Human papilomavirus (HPV) has been proposed to be one of the main players in the development of cervical cancer. The aim of this study was to investigate the association of HPV DNA and cervical cytology.

MATERIAL and METHODS
A total of 201 women undergoing routine gynaecological check up in Hospital were recruited. HPV genotyping and cervical Pap test were analysed.

RESULTS
Overall, 42% (85/201) of women analysed were tested positive for at least one of the HPV types tested. HPV DNA prevalence was the highest in women younger than 30 years old (59%, 50/85). Eighty four percent (71/85) of the HPV positive women had low to high grade cytological anomalies.

CONCLUSION
The presence of HPV DNA was strongly associated with the cytological anomalies, especially with specific HPV types. This study showed that detection of multiple HPV types is rather important in understanding the possible crosstalk among HPVs during the initiation and progression of cervical lesions. In conclusion, HPV still remains to be the most prevalent marker for cervical cancer and thus regular check up should be evaluated as a preventative policy for cervical cancer.

Keywords: Cervical cancer, cytological pathology, HPV

INTRODUCTION

Cervical cancer is the third most common cancer affecting almost half a million women worldwide. Human papilomavirus (HPV) poses a substantial risk for the development of cervical cancer and genital HPV infections are associated with more than 99% of all cervical cancers (1, 2). HPVs are small, non-enveloped viruses with double stranded circular DNA compromising almost 8000 nucleotide base pairs (3, 4). Up to date, over 180 different types of HPVs have been completely sequenced (5, 6) and all these HPVs are shown to infect the epithelial cells usually either the cutaneous or the mucosal surfaces. Neoplastic changes are mainly caused by HPVs with high oncogenic potential (high risk, HR-HPV) (7). Women with HPV16 (61%) and HPV18 (10%) were shown to have 200-fold higher risks for cervical cancer (3, 8). The prevalence of other HPV types are less observed in cervical cancer cases, in such HPV45 was observed in 6%, HPV31 in 4%, HPV52 in 3%, HPV35 in 2% and HPV58 in 2% of cervical cancer cases (9). HPVs, mainly HR-HPVs, have also been observed in women with intermediate grade cervical cytological abnormalities that may progress to cervical cancer, such as atypical squamous cells of undetermined significance (ASCUS) and low-grade squamous epithelial lesions (LSIL) (2, 10, 11).

The association between the oncogenic HPV types and the subsequent development of cervical cancer has introduced the detection of HPV DNA as an alternative or supplementary screening and early detection strategy (12). Therefore, with a well-designed screening programme involving HPV genotyping and cytological analysis, unnecessary operations, such

as colposcopies, can be prevented. Moreover, with an established policy, early detection of cervical cancer will improve the mortality and morbidity. Hence, in this study we aimed to assess the prevalence of HPV infection in relation to cytological diagnosis in women undergoing routine gynaecological check up. In this way, the association between the presence of HPV DNA and development of cervical abnormalities can be predicted and appropriate treatment can be applied in advance.

MATERIAL and METHODS

Study Population: Physical Examination and Specimen Collection
Ethical approval conforming to the provisions of the Declaration of Helsinki was granted and written consensus was obtained from each patient. Women between the ages of 19-57 years old undergoing routine gynaecological check-up in Hospital were recruited into this prospective study investigating the correlation between the prevalence of HPV DNA and the cytological findings. Each participant had a pelvic examination at enrolment and exfoliated cervical cells were obtained for cytological analysis and for HPV DNA testing. Informed consent was received from each patient.

Cytological Assessment
The cervical cells were spread on microscope slide and fixed for the cytological assessment. The samples were stained with Papanicolaou (Pap) stain (BD Sure Path liquid based Pap Test; Papnicolou's solution, Merck) within the Laboratory of Pathology, Hospital. Pap smear samples were analysed by trained cytopathologists and the cytological smears were classified following 2014 Bethesda system (13, 14). In brief, the samples were classified as; no epithelial cell anomaly, atypical squamous cell of undetermined significance (ASC-US), atypical squamous cells with high grade squamous intraepithelial lesion (ASC-H), low grade squamous intraepithelial lesion (LSIL) and high grade squamous intraepithelial lesion (HSIL). The presence of fungal or bacterial infection as well as inflammation was also reported.

HPV DNA Testing
HPV DNA testing of all the participants was performed on cervical cell samples at the Medical Genetics and Cancer Diagnosis-Research Centre, Hospital. DNA was extracted using Gene-All® Ribo_spin vRD™ kit (Gene All, Gambio) and DNA was tested using Seeplex® HPV4A ACE Screening kit (Seeplex, Seegene) following manufacturer's instructions. This kit uses the principle of "Dual Priming Oligonucleotide (DPO) technology" maximising PCR specificity and sensitivity by fundamentally blocking non-specific priming. Internal control and positive control provided within the Seeplex® HPV4A ACE Screening kit were included for each reaction. DNA was tested for the simultaneous genotyping of the high-risk HPV types, HPV16 and HPV18, and screening of 16 additional high-risk HPV types (HRC) including

HPV26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82 and low risk HPV types, HPV6/11. The presence of HPV DNA was assessed by observing the band with appropriate product size on etihidium bromide stained (2%/1xtris borate EDTA) agarose gel electrophoresis. The presence of HPV16 was detected with a band at 588bp, HPV18 at 230bp, HRC at 465bp, and HPV6/11 at 302bp with an internal control at 1000bp.

Statistical Analysis
The prevalence of HPV DNA was correlated according to the women's age. Two-tailed student's t-test was used for the analysis of distribution of high-grade cytological findings among HPV positive and negative women. A $p < 0.05$ was considered statistically significant. All statistical analysis was conducted using GrapPad prims v6 (California, USA.)

RESULTS

HPV DNA Detection
The study population was composed of 201 women aged between 19 and 57 years old with the median age of 30. Of these women, 45% (85/201) were tested positive for at least one of the HPV types tested. HPV DNA prevalence was the highest in women younger than 30 years old (59%, 50/85) though these values were not statistically significant compared to women older than the age of 30 years old ($p > 0.05$, Table 1). Only six women had two sexual partners and two of these women were tested positive for HPV.

Overall, the presence of HPV16 was detected in 10.9% (22/201) of the women tested, HPV18 in 2.9% (6/201), HPV6/11 in 10.9% (22/201) and at least one of the HRC group of HPVs in 28.9% (58/201; Table 2).

Among HPV positive women 92% (185/201) were tested positive for one HPV type (excluding the analysis of HRC) whereas the rest of the women had multiple HPV types detected. All of these women were positive for at least one type of high-risk HPV and 65% (11/17) have multiple high-risk HPVs detected. Thirty six percent (8/22) of HPV16 positive women were also screened positive for HRC and 18% (4/22) for HPV6/11.

Cytological Diagnosis
Cytological assessments of cervical cells revealed that 14% (12/85) of the HPV positive women did not have any cytological abnormalities (Table 3). The rest of the women have at least one kind of abnormal cervical cytology ranging from epithelial cell

Main Points:

- Detection of multiple HPV types is rather important.
- There is a possible crosstalk among HPVs during the initiation and progression of cervical lesions.
- HPV still remains to be the most prevalent marker for cervical cancer.

TABLE I. HPV DNA positive according to age groups				
	HPV DNA positive		**HPV DNA negative**	
Age groups	Number of women	% (Percentage)	Number of women	% (Percentage)
<30	50	59	41	35
30-40	22	26	41	35
41-55	12	14	31	26
>55	1	1	3	3
Total	85		116	
HPV: human papilomavirus				

anomalies to LSIL and HSIL. Overall, abnormal cytological outcome was observed to be more common in HPV positive women (90%, 76/85, of women) compared to HPV negative women (76%, 89/116, Table 3). In HPV positive women low grade cervical abnormalities, such as LSIL (22%) were detected. Additionally, bacterial vajinozis within the vaginal flora (3%, 5/85), fungal infection morphologically similar to candida (6%, 5/85) and inflammation (23%, 20/85), were also detected in HPV positive women. Thirty six percent (32/85) of HPV positive women were diagnosed to have epithelial cell anomalies, 17% (15/85) with ASCUS, 1% (1/85) with ASC-H, 22% (1/85) with LSIL, 8% (6/85) with HSIL (Table 3).

Association of HPV Types with Cytological Diagnosis
Overall, 23% (5/22) of the HPV16 positive women did not present any cytological anomalies and the rest had at least one aberrant cytological diagnosis ranging from low, intermediate and high-grade cervical anomalies. The findings of cytological diagnoses are summarised in table 4. The low-grade cervical anomalies of HPV16 positive women included bacterial vajinozis, fungal infection, warts, inflammation as well as epithelial cell abnormalities. Intermediate and high-grade cervical lesions for HPV16 positive women included ASCUS (18%, 4/22), LSIL (18%, 4/22) and HSIL (9%, 2/22) (Figure 1). The number of women tested positive for HPV18 is low; however, half these women were diagnosed for ASCUS, 33% (2/6) for LSIL and 17% (1/6) for HSIL. The highest detection of HSIL (26%, 5/19) was reported in HPV6/11 positive women. Presence of warts and ASCUS were detected in 26% (5/19) of the HPV6/11 positive women (Table 4, Figure 1). For the HRC positive women, epithelial cell abnormalities were detected in 38% (22/58) followed by 24% (14/58) LSIL and 21% (12/58) ASCUS.

The risk of having LSIL was considerably increased ($p < 0.05$) when the women were HPV16 positive as well as HRC positive, in such cytological diagnosis for half of the HPV16 and HRC positive women revealed that they have LSIL. Although the rate of

TABLE 2. Distribution of HPV types			
HPV type	Number of women	Overall percentage (%)	Percentage among HPV positive women (%)
HPV16	22	10.9	29
HPV18	6	2.9	7
HPV6/11	19	10.9	22
HRC	58	28.9	68
HPV: human papillomavirus; HRC: high-risk HPV types			

| TABLE 3. Association of HPV with cytological diagnosis				
Cytological diagnosis	Number of women (Total HPV positive: 85)	% (Percentage)	Number of women (Total HPV negative: 116)	% (Percentage)
Negative for intraepithelial lesion or malignancy	12	14	27	23
Bacterial Vaginosis	5	3	2	3
Fungal infection	5	6	2	2
Non-specific Inflammation	20	23	22	19
ASCUS	15	17	17	15
ASC-H	1	1	0	0
LSIL	19	22	5	4
HSIL	6	8	0	0
ASCUS: atypical squamous cells of undetermined significance; ASC-H: atypical squamous cells-cannot exclude high-grade squamous intraepithelial lesion; LSIL: low grade squamous intraepithelial lesion; HSIL: high grade squamous intraepithelial lesion				

TABLE 4. Association of HPV prevalence with cytological outcome				
Cytological diagnosis	Percentage of women with HPV16	Percentage of women with HPV18	Percentage of women with HPV6/11	Percentage of women with HRC
No abnormalities	23	0	16	14
Epithelial cell abnormalities	27	2	21	38
Bacterial vajinozis	5	0	5	3
Fungal infection	5	0	5	2
Warts	14	0	26	7
Inflammation	23	33	32	3
ASCUS	18	50	26	21
ASC-H	0	0	0	2
LSIL	18	33	21	24
HSIL	9	17	26	2
HPV: human papillomavirus; HRC: high-risk HPV types; ASCUS: atypical squamous cells of undetermined significance; ASC-H: atypical squamous cells-cannot exclude high-grade squamous intraepithelial lesion; LSIL: low grade squamous intraepithelial lesion; HSIL: high grade squamous intraepithelial lesion				
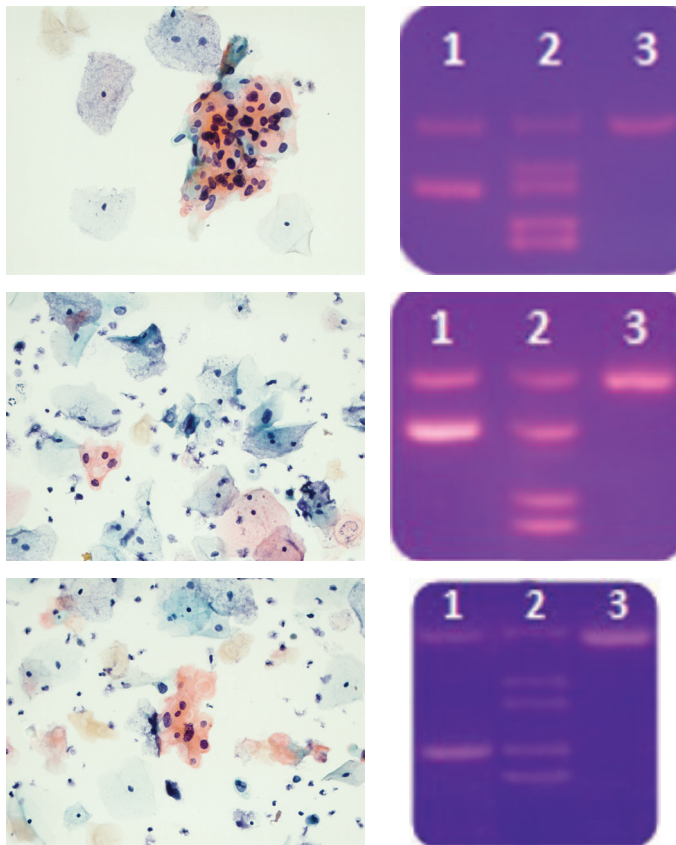
FIGURE I. Diagram showing the pathology and HPV genotyping results
Results of pathological analysis are shown in (a) and the HPV DNA genotyping is shown in (b). Lane I represents the patient's DNA, lane 2 represents the marker with a band at I000bp for internal control, 588bp for HPVI6, 302bp for HPV6/II and 230bp for HPVI8. Lane 3 represents the negative control with only a band at I000bp for the internal control. a) Results of a patient diagnosed for i. ASCUS following pathological analysis and ii. HPV6/II following HPV DNA genotyping at lane I. b) Results of a patient diagnosed for i. LSIL following pathological analysis and ii. HPVI6 following HPV DNA genotyping at lane I. c) Results of a patient diagnosed for i. HSIL following pathological analysis and ii. HRC following HPV DNA genotyping at lane I. Abbreviations: Aypical squamous cells of undetermined significance (ASCUS), low grade squamous intraepithelial lesion (LSIL) and high grade squamous intraepithelial lesion (HSIL).

having HSIL is supposed to be higher when women are positive for more than one HR-HPVs, this study showed that the rates were almost similar in developing HSIL for woman tested HPVI6 and HRC positive.

DISCUSSION
High risk HPV types are well known to cause cervical cancer and HPV DNA is detected in approximately 99.7% of cervical cancer tissue specimens (I). Therefore, HPV genotyping is important to investigate the natural history of the infections and whether the infection of HPV is involved in the initiation and progression of cervical cancer lesions (I5, I6). With the use of HPV DNA detection strategies, early detection and screening provided tremendous improvement for cervical cancer diagnosis (I7). In this study, we present the type specific prevalence of HPVs providing a picture of the frequency of genital infections caused by these mucosal HPVs and estimating the risk of cervical cancer development.

HPV DNA was detected in 42% of 20I women. Worldwide, the prevalence of HPV infection among women was estimated to range between 2% and 44% (I8). In general, high risk HPV types are observed at a higher rate in women younger than 30 years (I9, 20) which is in agreement with this study, though there are some controversies showing increased prevalence of HPV in the older age group (2I), in such high risk HPVs (HPVI8 and HPV5I) were associated with HSIL (22). All these variabilities among different outcomes including the present study could be due to different techniques used or population differences such as analysis of both normal and abnormal cytologies that may lower the rates of HPV prevalence.

Twelve percent (I4/85) of the HPV positive women had normal cytology which is in accordance with the current literature. In confirmation for the carcinogenic role of the high-risk HPV types, cytological diagnosis of the women including low, intermediate and high-grade cervical anomalies were compared to women with normal cytology. The prevalence of epithelial cell anomalies, LSIL and HSIL were shown to be statistically significant in HR-HPV positive women compared to HPV negative women (p<0.05). The prevalence of the HRC types followed by HPVI6 was higher in women with epithelial cell anomalies. The prevalence of HPVI6 and HPVI8 was also relatively high in women diagnosed for LSIL and ASCUS as also reported previously (23). The prevalence of different HPV type differs depending on the ethnic group, such as in Botswana women HPV was associated most with HSIL (24). Similar to our findings, HPV positive women were shown to have increased cytological anomalies of LSIL (50%), HSIL (100%) and invasive cervical cancer (II, I9). In this study, HSIL in addition to genital warts and inflammation were mostly detected in HPV6/II positive women. HPV6 and HPVII have been mostly associated with genital warts and condyloma acuminatum, whereas HPVI6 and HPVI8 to be the main cause of cervical cancer development (25-27).

Among HPV positive women, 20% to 40% have been reported to harbor at least two HPV types (8, I2, 28-30). The idea of the presence of one HPV type increasing the likelihood of attaining another HPV type is still controversial. Although it is suspected that presence of multiple HPV types increases the risk of the persistency of HPV and cervical lesions, it remains to be one of the debates due to the unknown biological activity of these HPVs and the viral load (3I, 32). In this study, 8% (I7/20I) of the women were infected with multiple HPV types and in this study, women tested positive for more than one HPV type did not have an increased rate of having cytological anomalies. Since coinfections are shown to be more frequent among women with cytological abnormalities or impaired immune response (33-35), development of cytological anomalies could be due to the patient history profile as well.

One of the other factors affecting the prevalence of genital HPV infection is the number of sexual partners. In this study only 2 of 6 women with two sexual partners were tested positive for HPV; hence having more than one partner did not seem to have the main reason of HPV infection. Although the main factors increasing the HPV infections accounts for multiple sexual partners, other factors increases the rate of HPV infection including the presence of other sexually transmitted infections, smoking, immunosupression, use of oral contraceptives, hormonal thera-

py, age of the first sexual intercourse and sexual behaviours of the male partner (36-42).

One of the main limitations of this study involves the cross-sectional design and the lack of analysis of temporal relationship between the HPV detection and the cytological outcome that may have developed at a later stage. Therefore, prospective studies play a crucial role in understanding the association of the presence of HPV DNA, the persistency of the infection and the risk of cervical lesion formation. The types of HPVs analysed in this study is also limited since genotyping is performed for the high-risk HPV types HPV16 and HPV18 and screening is performed for the other high risk HPV types including HPV26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82 and low risk HPV types HPV6 and HPV11.

In conclusion, detection of multiple HPV types is rather important in understanding the possible crosstalk among HPVs during the initiation and progression of cervical lesions. Our study provides information on the association and distribution of different HPV types with cytological assessment enabling better outcome measures and early detection and screening for cervical cancer. Overall, this study highlights the importance of identifying the prevalence of HPV types that may define the causality of individual HPV types with cervical cancer and form the basis for secondary prevention measures for cervical cancer and the need of longitudinal studies on the natural history of HPV. In the light of the literature and as it is seen with our results, HPV still remains to be the most prevalent marker for cervical cancer and thus regular check up should be evaluated as a preventative policy for cervical cancer.

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