RESEARCH ARTICLE

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Evaluation of *HPV 16* and *HPV 18* Oncoprotein Expression as Alternative Diagnostic Tools in Cervical Lesions

Nadiah Ahmad Sabri[1,2], Shazana Hilda Shamsuddin[1], Anani Aila Mat Zin[1,2]*

Abstract

Objective: The study aimed to evaluate E6 and E7 oncoproteins of *HPV16* and *HPV18* expression in formalin - fixed paraffin embedded (FFPE) tissue in different grades of the cervical lesion and evaluate the potential use of *E6* and *E7* oncoproteins derived from HPV 16 and 18 as diagnostic protein biomarkers for triaging cervical lesions. **Methodology:** A total of 102 FFPE cervical tissues were collected from 2 tertiary hospitals and immunohistochemical reactivity staining of E6 and E7 oncoproteins of *HPV16* and *HPV18* were evaluated using immunoreactive scoring (IRS) system and analysed statistically. **Result:** The result showed an increased oncoprotein expression with the progression of cervical lesions. There is a statistically significant association between histology grade and *HPV16/18-E6* expression ($p = 0.028$). However, there are no significant association of histological grade to *HPV16-E7* immunoreactivity score ($p = 0.264$) and *HPV18-E7* ($p=0.080$). **Conclusion:** The immunohistochemical expression of HPV oncoproteins is a potential alternative diagnostic tool applicable in a low-resource laboratory setting. The advantage of the histochemical evaluation is that this method is simpler to apply and less expensive in comparison to in situ mRNA hybridization. Nevertheless, our study also found that antibodies against HPV that are commercially available suffer quite substantial specificity issues such as background staining and inconsistency between different batches. Hence, the utilization of antibody-based staining warrants stringent quality control.

Keywords: *HPV16- HPV18- E6- E7-* immunohistochemistry- cervical lesion

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Introduction

Worldwide, cervical carcinoma is the fourth most common cancer in women after breast, colorectum & lung [1]. Malaysia ranked the highest incidence of cervical cancer at the age of 50-65 years [2]. Over the years, the incidence rate of cervical carcinoma has dropped largely due to efficient screening programmes and the subsequent eradication of its precursors. Cervical cancer is preventable if the precursor lesion is diagnosed at the early stage, due to the relatively slow progression to cervical carcinoma. Vaccination against the established causative agent is high-risk Human Papilloma Virus (HR-HPV) also offer protective measures against HPV-related outcomes. However, low compliance rates in cervical screening and inefficient HPV vaccination programmes may hamper the mission of eliminating this cancer as a public health problem.

The cervical cancer screening programme heavily relies on cytological smear tests, also known as the Papanicolau test, both locally and worldwide since the 1960s. Advancement in the technique has generated a liquid-based cytological test. Although these tests are highly specific, it has low sensitivity in detecting cervical cancer precursors [3]. Inter-observer variability is substantial due to reliance on the subjective interpretation of the cytology as well as potential error in sampling. These are the drawbacks when using cytological tests as primary screening.

In contrast, molecular HPV testing enhances the detection sensitivity but it suffers from low specificity and low positive predictive value [3]. The utilisation of molecular HPV testing reduces the frequency of screening episodes from every 3 to every 5 years. Hence, reducing the cost burden of frequent cytological smear tests on the healthcare system. However, it cannot differentiate between patients having transient or persistent HPV infections, whereby persistent infection has a higher risk for neoplastic progression. Unfortunately, the test is also quite expensive and may not be available in the low-resource healthcare setting.

At present moment, the most prominent validated biomarkers for HPV detection are nucleic acid-based including arrays of HPV DNA and mRNA transcriptional genes of *E6* and *E7* [4]. Protein-based biomarkers are limited, mostly targeting LI major capsid protein of HPV

*[1]Department of Pathology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia. [2]Department of Pathology, Hospital of Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia. *For Correspondence: ailakb@usm.my*

16, 18 and other high-risk HPV. These show that more validated biomarkers are imperative to improve efficacy in detection.

Increasingly studies have shown that *E6* and *E7* oncoproteins of high-risk HPV play major roles in tumour progression notably in persistent HPV infection [5]. To promote cervical cancer abnormalities, the virus must become integrated into the host genomic DNA. *E7* contributes to oncogenesis by binding and interfering with retinoblastoma (RB) protein, targeting them for degradation. Meanwhile, *E6* inhibits p53, causing the inactivation of apoptosis. *E6* and *E7* co-expression in HPV- infected cells establish an optimal environment for sustained proliferative signalling, causing evasion of most anti-tumorigenic checkpoints to allow the cells through uncontrollable cell division [5]. Taking these into consideration, this study will be investigating HPV oncoproteins immuno-expression in cervical tissues of varying grades and evaluating the potential use of *E6* and *E7* oncoproteins derived from HPV 16 and 18 as diagnostic protein biomarkers for triaging cervical lesions.

Of note, the majority of the protein-based assays are antibody-dependent techniques including immunostaining and enzyme-linked immunosorbent assay (ELISA). For decades, antibodies have been widely used as the research reagent in most the bioassays because of the specificity and sensitivity binding towards antigens. In the limited resource laboratory setting without any molecular diagnostic facilities, antibody-based histochemical assay might be useful as an alternative diagnostic tool of determining HPV association of cervical lesions especially malignancy.

Materials and Methods

Selection of samples

This is a cross-sectional study conducted in HUSM and HRPZII involving cases of cervical lesions diagnosed from 1st June 2014 until 31st May 2022. Selected cervical tissues were previously diagnosed histologically based on the grading of cervical lesions which includes non-neoplastic cervical lesion (n=10), low-grade squamous intraepithelial lesion (n=30), high-grade squamous intraepithelial lesion (n=32), adenocarcinoma in situ (n=2), squamous cell carcinoma (n=14), adenocarcinoma (n=10) and adenosquamous carcinoma (n=4). A number of these cervical lesions had a confirmed HPV-DNA status using the PCR method (Abbott m200sp). Simple demographic information and clinicopathological report were retrospectively retrieved from the computerized database. The specimens with missing or inadequate tissue for histochemical staining are excluded from the data pool.

Immunohistochemical staining

Briefly, selected FFPE tissues were sections to 4 µm thickness. Slides were deparaffinized in xylene for 5 min and rehydrated in a descending ethanol gradient at room temperature. Heat-induced antigen retrieval was performed at 97˚C in pH 9.0 Tris EDTA for E7 and at 121˚C in pH 6.0 citrate buffer for E6, respectively. Slides were incubated with primary antibodies Santa Cruz® *HPV16 E7* sc-6981 (1:25), Santa Cruz® *HPV18 E7* sc-365035 (1:25) and Bioss® *HPV16-E6 + HPV18-E6* bs-1719R (1:100) overnight at 4 ˚C. This is followed by incubation with mouse LINKER (Dako) and horseradish peroxidase (HRP)-conjugated secondary antibody (Dako) for 20 minutes at room temperature. Expression was visualized using DAB Substrate solution as chromogen and counterstained with haematoxylin solution. The sections were then dehydrated in ascending concentrations of ethanol, cleared in xylene and mounted. Cervical cancer tissues with confirmed HPV-DNA positivity status from the PCR testing were used as the positive control in which intense reaction was observed. Normal thyroid tissue that was not expressing HPV was used as a negative control.

Interpretation of E6 and E7 stained slides were analysed by two researchers for epithelial components immunoreactivity. Brown-coloured cytoplasm with or without nuclear staining was identified and graded as follows: 0 (no reaction); 1 (mild reaction); 2 (moderate reaction) and 3 (intense reaction). The positive cell percentages were graded as follows: 0 (0%); 1 (<10%); 2 (10-50%); 3 (51-80%) and 4 (>80%). The immunoreactive score ranging from 0 to 12 was finally obtained by multiplying the product of the staining intensity (0-3) and the positive cell percentage (0-4). According to the immunoreactive score (IRS), the staining results were defined as follows: negative (score 0-1); mild (score 2-3); moderate (score 4-8) and strongly positive (score 9-12) [6].

Statistical analysis

Categorical data were analysed using Pearson Chi-square or Fisher's Exact test. While numerical data were reported as median and means ± standard deviation. P values of less than 0.05 were considered significant.

Results

Demographic data

A total of 102 cervical lesion tissues were graded into four categories. Majority of the cases fall under high grade precursor lesion (33.3%), followed by low grade precursor lesion (29.4%), malignancy (27.5%) and non-neoplastic lesion (9.8%).

In our study, the age ranged from 25 to 91 years old, with overall mean age of 45 years old ± 12.7 (SD). The median age for non-neoplastic lesion was 39 years old. Meanwhile, the median age for low grade precursor, high grade precursor and malignancy increased concordantly; 39, 48 and 51 respectively.

There are 42/102 (41.2%) cases of this study that were *HPV16*-DNA confirmed positive using PCR test, while another 11/102 (10.8%) cases were HPV18-DNA confirmed positive (Figure 1). Almost half of the cases, 49/102, (48%) have no known HPV status as the center where the samples is obtained did not have in-house HPV-DNA testing available. Most of the low-grade precursor (19/30) and high-grade precursor (19/34) lesions were *HPV16*-DNA confirmed positive. Meanwhile, the majority of malignancy cases (26/28) had unknown HPV status.

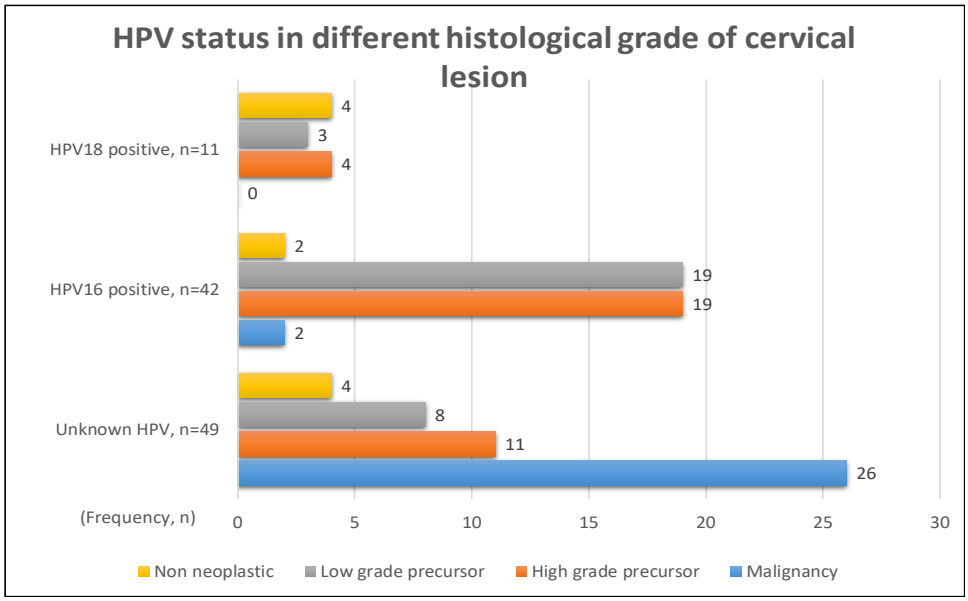

Figure 1. HPV-DNA Confirmed (by PCR) Status of Cervical Lesion

Expression of HPV16-E7, HPV18-E7 and HPV16/18-E6 in different histological grade of cervical lesions

In our study, we used *HPV16-E7* and *HPV18-E7* antibodies to be tested immunohistochemically on 102 FFPE cervical tissues of different histological grades. However, for *HPV16/18-E6* antibody, the immunohistochemical staining was carried out in 54/102 samples as there were a few technical hurdles and budget limitations. During the staining optimisation procedure, we did encounter antibody batch variability issues, causing inconsistency in the resulting staining. These are among the few limitations inherent in antibodies. Hence, only result of staining done using similar batch of antibodies was included in this study.

There was a gradual increase in staining expression of the oncoproteins as the histological grade increased, however it was not solidly consistent. Apart from that, non-specific staining was also observed at the non-neoplastic superficial most keratin layer and necrotic areas. There was also background stromal staining noted, more apparent with HPV16/18-E6 immunostaining. The staining was evaluated qualitative and quantitatively using immunoreactive score (IRS).

As presented in Table 1, for overall total cervical lesions (regardless of HPV-DNA status), the most frequent *HPV16-E7* expression was negative (40.0%) in the non-neoplastic category. Meanwhile, in low-grade precursor, the most frequent *HPV16-E7* expression was mild (46.7%). Subsequently, in high-grade precursor, the most frequent *HPV16-E7* expression was moderate (38.2%). Amidst, strongly positive *HPV16-E7* expression was only seen in high-grade precursor and malignancy. There is no significant association of histological grade to *HPV16-E7* expression ($p = 0.264$).

For *HPV18-E7*, the most frequently seen in non-neoplastic, low-grade precursor and high-grade precursor cervical lesion was negative expression; 50.0%, 53.3% and 52.9% respectively. Meanwhile, in malignancy,

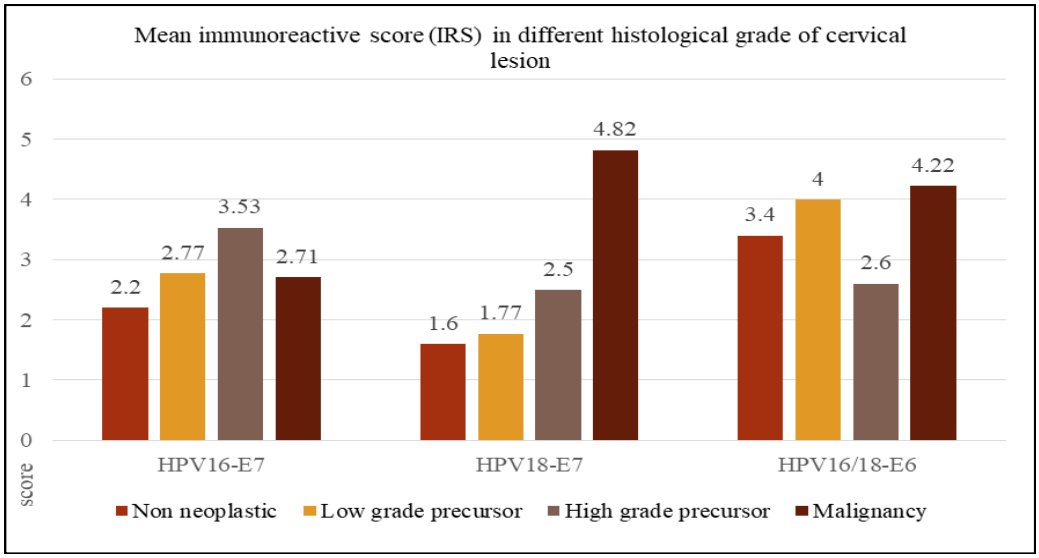

Figure 2. Expression of HPV Oncoproteins in the Total Cervical Lesion

Table 1. Expression of *HPV* Oncoproteins in the Total Cervical Lesion.

Histology grade	HPV16-E7 n, (%)						HPV16/18-E6 n, (%)					
	Negative (0-1)	Mild (2-3)	Moderate (4-8)	Strongly Positive (9-12)	P-value		Negative (0-1)	Mild (2-3)	Moderate (4-8)	Strongly Positive (9-12)	P-value	
Non-neoplastic	4 (40.0)	3(30.0)	3(30.0)	0(0.0)	0.264a		1(20.0)	0(0.0)	4(80.0)	0(0.0)	0.028a	
Low grade precursor	8 (26.7)	14(46.7)	8(26.7)	0(0.0)			0(0.0)	8(50.0)	6(37.5)	2(12.5)		
High grade precursor	7 (20.6)	12(35.3)	13(38.2)	2(5.9)			5(33.3)	5(33.3)	5(33.3)	0(0.0)		
Malignancy	13 (46.4)	5(17.0)	8(28.6)	2(7.1)			2(11.1)	4(22.2)	12(66.7)	0(0.0)		
Total	32 (31.4)	34(33.3)	32(31.4)	4(3.9)	102(100)		8(14.8)	17 (31.5)	27(50.0)	2(3.7)	54 (100.0)	

there is as much negative (28.6%) as strongly positive (28.6%) *HPV18-E7* expression seen. Again, strongly positive *HPV18-E7* expression was only seen in high-grade precursor and malignancy. There is no significant association of histological grade to *HPV18-E7* expression ($p = 0.080$).

There is a high percentage (80.0%) cases show of moderate expression of *HPV16/18-E6* in the non-neoplastic cervical lesion. Meanwhile, the *HPV16/18-E6* show mild positivity expression in low-grade precursor (50.0%) of cases. High-grade precursor lesions had similar percentages of negative (33.3%), mild (33.3%) and moderate (33.3%) *HPV16/18-E6* expression. Most of the malignant cases show moderate (66.7%) HPV16/18-E6 expression. In total, the *HPV16/18-E6* predominantly expressed in pathological lesions (low- grade, high-grade and malignant cases) compared to non- neoplastic/ pathological lesion. There is a significant association between histology grade and *HPV16/18-E6* expression ($p = 0.028$).

Figure 2 depicted that, there was an increasing trend of mean HPV18-E7 IRS in increasing histological grade of the cervical lesion. This is concordant with a previous study (Shi et al., 2018) which stated that increased E7 positivity was consistent with the increased severity of pathological grade.

For *HPV16-E7*, an apparent reduction of mean IRS was seen in the malignancy categories. This is because, despite some malignancy cases having the most intense *HPV16-E7* staining, few cases did not show any stain at all. A similar result of inconsistency was also reported [6]. *HPV16/18-E6* immunostaining show variable IRS with the lowest mean seen in the high-grade precursor. In a few cases, *HPV16/18-E6* staining was also seen in the normal and glycogenated squamous epithelial layer.

Discussion

Upon transmission of HPV infection to the cervical mucosa, the progression of low-grade precursor lesion to high-grade precursor and malignancy may take up to 20 years. In fact, 90% of HPV infections are resolved by themself in the immunocompetent population [5]. The initial age of sexual exposure would also affect the age of developing neoplastic intraepithelial lesions.

Our epidemiological data of age and cervical lesion incidence was in parallel with few meta-analytic data reflecting the population [7, 8]. The finding in our study, that most lesion including highest incidence within the age group of 30-49 years old further support the rationale of targeting this age group for cervical cancer screening, specifically for HPV testing.

As seen in Figure 3, the majority of the overall cases, which 70.0% of non-neoplastic, 66.7% of low-grade precursor, 67.6% of high-grade precursor and 42.9% of malignancy cases occur at the age between 30-49 years old, which was the target age group for HPV screening test according to Guidelines for Primary HPV Testing for Cervical Cancer Screening in Malaysia, 2019 [9]. Only low-grade precursor lesions were diagnosed within the age group of less than 30 years old. Those within the age

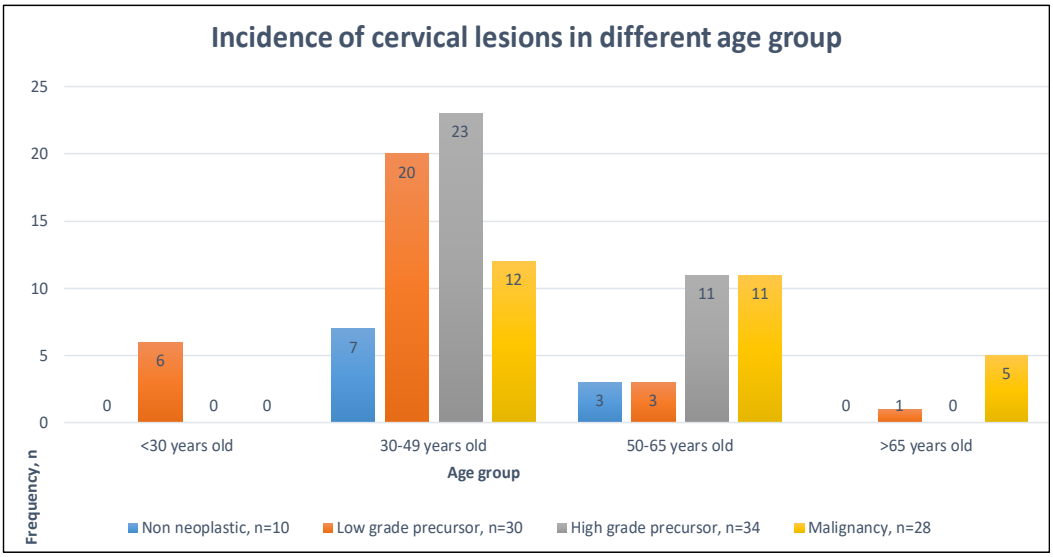

Figure 3. Group of age based on cervical lesions

Table 2. Expression of *HPV16-E7* Oncoprotein in *HPV16-DNA* Confirmed Cervical Lesion

Histology grade	HPV16-E7 n, (%)				
	Negative (0-1)	Mild (2-3)	Moderate (4-8)	Strong (9-12)	P-value
Non-neoplastic	1 (50.0)	0 (0.0)	1 (50.0)	0 (0.0)	0.001a
Low-grade precursor	4(21.1)	11(57.9)	4 (21.1)	0 (0.0)	
High-grade precursor	0 (0.0)	9 (47.4)	9 (47.4)	1 (5.3)	
Malignancy	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)	
Total	5 (11.0)	20 (47.6)	14 (33.3)	3 (7.1)	42(100)

group of 50-65 years old were more likely to be diagnosed as high-grade precursor lesion or malignancy. While those who were more than 65 years old had the highest probability of malignancy. A significant association between age group and histological grading of cervical lesion was noted (p = 0.001, Fisher's Exact test).

Worldwide, the most common high-risk HPV types in cervical cancer were types 16 (57%), 18 (16%), 58 (5%), 33 (5%), 45 (5%), 31 (4%), 52 (3%), and 35 (2%) (15). This is concurrent with Malaysian prevalence data, which found that in cervical squamous cell carcinoma, *HPV 16* was the commonest (76.6%) genotype detected,

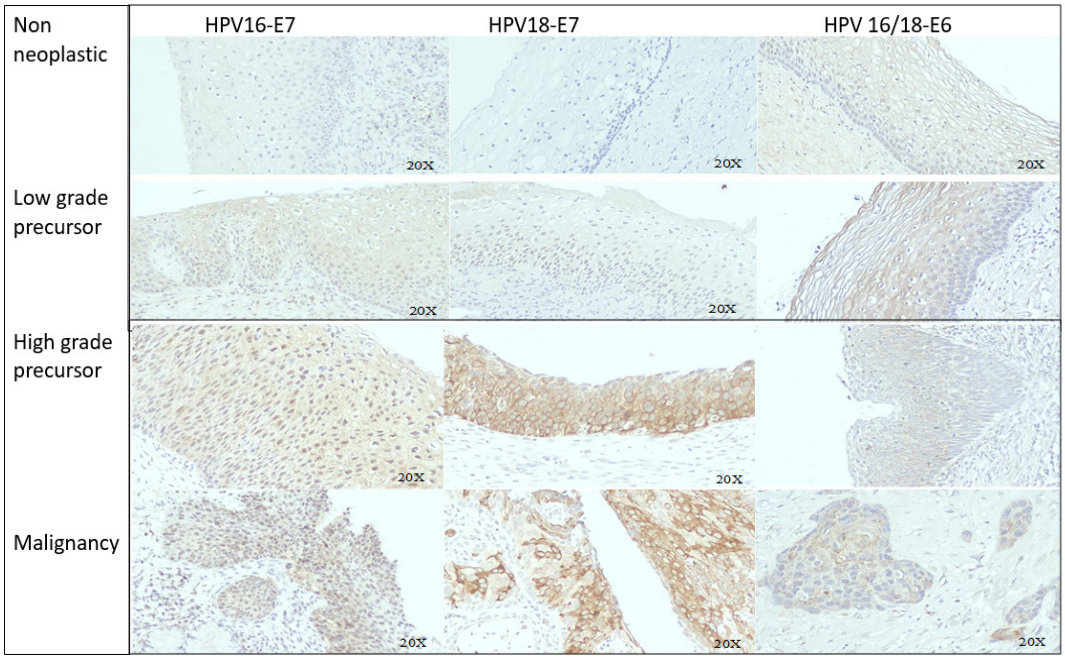

Figure 4. Immunohistochemical Staining Characteristic of HPV Oncoproteins in the Cervical Lesion.

followed by *HPV 18* (35.0%) [10]. In contrast, *HPV 18* (51.8%) was the commonest genotype detected in cervical adenocarcinoma followed by HPV 16 (48.2%) [10]. Overall, HPV 16 and HPV 18 genotypes were the commonest, detected in almost 70% of total cervical cancer cases.

Expression of HPV16-E7, HPV18-E7 and HPV16/18-E6 in different histological grade of cervical lesions

As of now, surrogate protein biomarker p16 immunohistochemical staining had been used to diagnose HPV-associated cervical carcinoma in biopsies. However, p16 is not a direct viral protein, instead was transcripted and upregulated during the inactivation of pRb by E7 viral oncoprotein, upon viral nuclear integration [5]. Thus, a direct method detecting HPV-derived proteins such as E6 and *E7* in clinical specimens is desirable to evaluate HPV infection. Few molecular studies targeting *E6/E7* mRNA using histological samples had been showing promising and comparable results with PCR methods as a screening biomarker [4, 11, 12].

Principally, the transformation of cervical cancer occurs when HPV viral genome is integrated into the host genome, within the nucleus. Interestingly, our results showed that the immunostaining of *HPV16-E7*, *HPV18-E7* and *HPV16/18-E6* was observed mainly at the intracytoplasmic region of neoplastic cervical epithelium, with focal areas showing selective nuclear staining (Figure 4). Previous studies characterizing the *E6* and *E7* expression in cervical tissues reported the staining to be cytoplasmic [13, 14]. while others reported the staining was more localized to the nuclear [1] . On the other hand, [15] consider brownish nuclear with or without cytoplasmic staining as positive. Our study, we decided to choose both cytoplasmic and/or nuclear staining as a positive stain following the methods by Rodrigues LC et al. [15].

The molecular test is currently deemed the gold standard for HPV genotyping in cervical lesions. In our study, a comparison of *HPV16-E7* immunohistochemical staining was tested against *HPV16*-DNA confirmed positive cervical lesions. The staining was evaluated qualitatively and quantitatively using an immunoreactive score (IRS). As seen in Table 2, the low-grade precursor mainly shows mild HPV16-E7 expression (57.9%). There was a similar percentage of mild (47.4%) and moderate (47.4%) *HPV16-E7* expression in the high-grade precursor. However, within the two samples of malignant cervical lesion, only strongly positive *HPV16-E7* expression (2/2 - 100.0%) was noted. There was a significant association seen between the histology grade of HPV16-DNA positive cervical lesions with *HPV16-E7* expression (p=0.001). The result was concordant with the previous study in which the *HPV16-E7* positive expression rate increases as a histological grade of *HPV16* positive cervical lesion increases [14]. Another study also mentions a significant positive correlation between *HPV16* E7 expression with histological grade [13].

In our study, the resulting mean of *HPV18-E7* IRS is linearly raised following increasing grade of cervical lesion. This is concordant with study [16] which stated increased *E7* positivity is consistent with the increased severity of pathological stages. For HPV16-E7 expression, we found out that a few malignant cases were unexpectedly negative or mildly stained. Similar result of inconsistency was also reported [6].

Rodrigues LC et al. [15] found a significant association between *HPV16/18-E6* expression with high grade squamous intraepithelial lesion (HSIL). On the other hand, our study resulted in relatively lower mean IRS of *HPV16/18-E6* in high grade precursor. However, a significant association was found between overall *HPV16/18 E6* expression with cervical lesion histological grade.

A wide variation of E6 and E7 of *HPV16* and *HPV18* oncoproteins immunostaining patterns were noted in our samples. Few significant associations were identified between the histological grade of the cervical lesion and IRS categories, some were concordant with previous studies [14, 16, 6, 13]. All in all, immunohistochemistry application in detecting HPV in tissue samples was feasible, especially in basic equipped laboratories. In this study, we have prepared a repeatable, accessible, immunohistochemical method of detecting HPV in FFPE cervical lesion

In Conclusion, we reported an evaluation of oncoprotein *E6* and *E7* of *HPV16* and HPV18 expression on FFPE cervical tissue through the histochemical method. A reliable immunohistochemistry protocol was developed for application in various cervical lesions. Specific recognition of *E6* and *E7* expressing cervical cells were feasibly demonstrated visually and directly on histology slides.

The advantage of the histochemical evaluation is that this method is simpler to apply and less expensive in comparison to in situ mRNA hybridization. This study widened the range of analysis methods for screening and diagnosis of cervical specimens. *E6* and *E7* of *HPV 16* and *HPV18* can be considered as a potential ideal biomarker candidate for the detection of precancerous cervical lesions. However, our study also found that antibodies against HPV that are commercially available suffer quite substantial specificity issues. Hence, the utilisation of antibody-based staining warrants stringent quality control.

The findings from this study show that immunohistochemical staining can be used as an alternative diagnostic tool in low-resource pathology laboratory settings.

Limitation

There was a limitation in terms of the availability of molecularly tested cervical lesions. A confirmed HPV-DNA status of tissue samples would allow comprehensive analysis to an overall comparison of histochemical application in HPV detection versus gold standard molecular test. Apart from that immunohistochemical assay is still dependent on antibody clones, which may have different sensitivity and specificity per batch. Standardization is an important key point in establishing significant findings. We also believe, a larger sample size in general may lead to a more conclusive statistical analysis and result.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

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Conflict Of Interest

We have no conflicts of interest to disclose.

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