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ImmunohistochemicalOverexpression of p16INK4a is Predictive of High Risk Human Papilloma Virus infection in VIA Positive Cervical Lesions

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Abstract

Background: Cervical cancer is a major health problem worldwide. Epidemiological studies have clearly established High-Risk (HR) Human Papilloma Virus(HR-HPV) as a central cause of invasive cervical cancer. Expression of E6 and E7 oncogenes of HR-HPV affecting the Rb-p16 pathway, leads to p16INK4a protein upregulation. It is widely reported that, immunohistochemical overexpression of p16 indicates persistent infection by HR-HPV in a high percentage in cervical premalignant and malignant lesions. **Objective:** To evaluate the predictive value of p16 to identify the HR-HPV infected cervical premalignant and malignant lesions. **Methods:** This descriptive cross-sectional study was carried out in the Pathology Department of DMC with collaboration of BSMMU and BAU, Mymansingh, during July 2016 to June 2018.Total 40 VIA positive and clinically suspected premalignant and malignant patient's attendant at DMC included study population. **Results:** The mean age was 43 years. The most common symptoms were per vaginal whitish discharge (95%), lower abdominal pain (87%), irregular p/v bleeding (60%) and post coital bleeding (7.5%). By colposcopic examinations, the diagnoses were CIN I in 57.5%, carcinoma cervix in 30%, CIN II in 10 % and CIN III in 2.5%. Chronic cervicitis 10(25.0%), CIN I 12(30.0%), CIN II 5(12.5%), CIN III 1(2.5%), invasive squamous cell carcinoma 11(27.5%) and adenocarcinoma in 1(2.5%) case. Out of 40 cases, p16 was intensely positive (>50% cells) in total 22 cases including all cases of invasive cancers. Among 22 p16 positive cases, 12 cases were positive for HR-HPV (16, 18). In addition, p16 showed sensitivity 73.3%, specificity 100%, accuracy 80%, PPV 100% and NPV 55.6%. **Conclusion:** The results of this study confirmed that, immunohistochemical overexpression of p16 is associated with HR-HPV infected cervical lesions. Therefore, along with the conventional screening methods, p16 can be used as a useful biomarker in identifying HR-HPV infected cervical premalignant and malignant lesions.

Key word: HR-HPV, p16, cervical, CIN, Malignant, Pre-Malignant

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INTRODUCTION

Cervix uteri cancer is the 4th most common cancer among women worldwide and the 7th overall, with an estimated 527,624 new cases and 265,672 deaths.[1] Invasive cervical cancer, most of which are squamous cell carcinomas, preceded by precancerous lesions designated as cervical intraepithelial neoplasia (CIN). Among them, only a trivial percentage of low grade lesions (LSIL) progress to high grade lesions (HSIL) and invasive cervical cancer. About 10% of CIN I usually progress to CIN III and from CIN III, only 12% progress to invasive cancer.[2]Human papillomavirus (HPV) infection plays an important role in cervical carcinogenesis.[3] In fact, HPV infection has been detected in almost all pre-neoplastic or neoplastic lesions of the cervix. The E6 and E7 genes of High Risk (HR)-HPV specifically bind and inactivate p53 tumor suppressor gene and retinoblastoma (Rb) protein. This phenomenon leads to disruption of cell cycle leading to increased cell proliferation and eventually carcinoma.[4] The HPV subtypes have been classified into three categories according to the risk of malignant transformation- high risk, intermediate risk, and low risk.[5] HPV 16 and 18 are high risk (HR) HPVs and are the most clinically important HPV subtypes. HPV 31, 33, 35, 51, 52 and 58 are of intermediate risk HPV and associated with high-grade squamous intraepithelial lesion (CIN II, CIN III) and cervical cancer. HPV 6 and 11 are classified as low risk types and are usually associated with benign hyperplastic lesions.[6-7] Persistent infection with oncogenic, HR-HPV genotypes is strongly associated with the development of cervical cancer.[8] The risk of developing cervical squamous cell carcinoma is about 400 times higher following infection with HPV-16 and about 250 times higher following infection with HPV-18 compared to the risk in uninfected women.[9] Recently, along with cancer screening programs, various biotechnological modalities are available to identify precancerous lesions of the cervix.[10] Among different methods, the histopathological examination of colposcopy-guided biopsy specimen is considered as the "Gold Standard". However, this procedure confines only to the interpretation of morphology. It provides little or no information regarding the risk of persistent, progression or regression of dysplasia. Moreover, the diagnosis is complicated by inter-observer variability.[11] For example, truly neoplastic lesions are sometimes misclassified as 'negative for dysplasia' due to various reactive and metaplastic changes, inadequate and fragmented samples. This limits the efficiency of screening programs and emphasizes the need for the specific biomarkers for identification of truly dysplastic cells.[12] p16INK4A (p16) is a Cycline D Depended Kinases (CDK 4, 6) inhibitor. It is a tumor suppressor protein that decelerates cell cycle by inactivating CDK, which phosphorylates retinoblastoma protein. Functional inactivation of Rb by HPV oncogene E7, strongly affects the P16 functions. It results in an enhanced expression of P16. Following HPV infections, marked elevation of p16 protein in dysplastic cells are noted, which is not seen in normal cervical epithelium.[13] This overexpression of p16 can be detected immune histochemically, which helps to

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identify HPV infected cells. Therefore, the evaluation of p16 protein could improve conventional histopathological diagnosis of HPV infected preneopastic diseases.[14] In Bangladesh, cervical cancer remains as the 2nd most leading cause of female cancers, mostly among the women aged 15 to 44 years. About 11,956 new cervical cancer cases are diagnosed annually in Bangladesh.[15] In our community early age of marriage and conception, multiple pregnancies as well as other factors play important roles in HPV induced various cervical premaliganant and malignant lesions. So, new and effective method for detecting HPV should be integrated with the conventional systems to lower the rate of cervical cancer. Therefore, the present study is designed to assess the predictive value of p16 immunostain in diagnosis of HR-HPV induced premalignant and malignant cervical lesions.

MATERIALS& METHODS

This descriptive cross-sectional study was carried out in the Pathology Department of DMC with collaboration of BangabandhuSreikhMujib Medical University (BSMMU), Microbiology Department and Bangladesh Agricultural University (BAU), Mymansingh, during July 2016 to June 2018. A total 40 VIA positive and clinically suspected premalignant and malignant patient's attendant at DMC included as study population.Ethical clearance was taken from institutional ethical committee of DMC, Dhaka.40 VIA positive patients with complaints of lower abdominal pain, irregular per vaginal bleeding, dyspareunia and post coital bleeding were selected from GOPD. These patients were advised for colposcopic examination by the gynecologist. After taking detail history with attention to age, age of first pregnancy, parity, history of contraception, sign & symptoms, 40 patients were selected. Colposcopic examinations were done by gynecologist. Colposcopic biopsy specimens were sent to the Department of Pathology, DMCH, for histopathological examination. Some portion of biopsy samples were preserved at -4°C for HR-HPV DNA detection. HR-HPV (16, 18) DNA were detected by PCR. p16 IHC were preformed from the paraffin blocks. The sections were stained with p16 antibody was done manually following the avidin-biotin-peroxidase staining method. All the sections that showed either strong nuclear or cytoplasmic stains were considered positive. To determine the score of p16 expression, a four-semi quantitative class was used. PCR utilizing the consensus primer, directed at relatively conserved regions of specific HPV genomes, allowed amplification of specific HR-HPV genotypes in a single reaction. The present study aimed to detect HR-HPV namely HPV 16 and 18.Statistical analyses were done by using the SPSS 22.0. Results were presented in tables, figures and diagrams. The sensitivity, specificity, accuracy, positive predictive values and negative predictive values were calculated. A p value<0.05 was considered as significant at 95% confidence interval.

RESULTS

Table I: Distribution of patients according to age (N=40)

Age (years)	Frequency (n)	Percentage (%)
≤30	5	12.5

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31 - 40	18	45.0
41 - 50	5	12.5
51 - 60	7	17.5
>60	5	12.5
Mean age ±SD	43.20±12.20	
Range	27.00-68.00	

Table I shown age distribution of the patients. Highest number of cases 18(45.0%) were in fourth decade and their mean age was found to be 43.20±12.20, ranging from 27 to 68 years.

Table II: Distribution of patients according to symptoms (N=40)

Symptoms	Frequency (n)	Percentage (%)
Per vaginal discharge	38	95.0
Backache/ lower abdominal pain	35	87.0
Irregular p/v bleeding	24	60.0
Post coital bleeding	3	7.5

Table II shown sign symptoms of study population. Most common symptoms observed were per vaginal whitish discharge in 38(95%), lower abdominal pain in 35(87%) and irregular p/v bleeding in 24(60%).

Table III: Distribution of patients according to colposcopic examination (N=40)

ColposcopicExamination	Frequency (n)	Percentage (%)
CIN I	23	57.5
CIN II	4	10.0
CIN III	1	2.5
CaCx	12	30.0

CIN I–Cervical intraepthelialneoplasia I, CIN II–Cervical intraepthelialneoplasia II, CIN III–Cervical intraepthelialneoplasia III, CaCx- Cancer cervix

Table III demonstrated the findings of colposcopic examinations of total 40 cases. Common diagnoses were CIN I in 23(57.5%) cases, carcinoma cervix in 12(30%) cases, CIN II in 4(10%) cases and 1(2.5%) case represented CIN

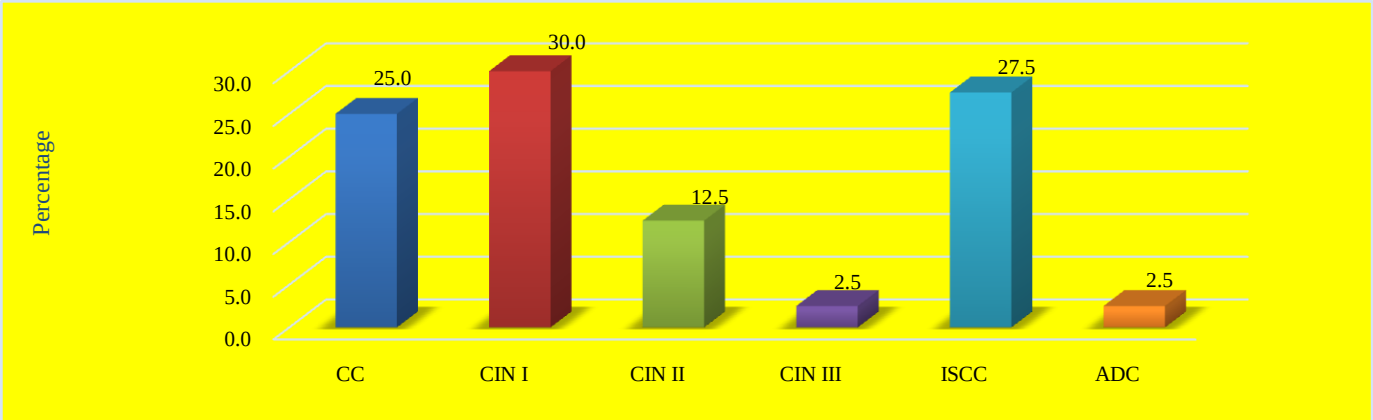

Figure 1: Distribution of patients by histopathological diagnoses (N=40)

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The bar diagram reveals the histopathological diagnoses of 40 patients. It shows that the common diagnoses were CIN I in 12(30.0%), invasive squamous cell carcinoma in 11(27.5%), chronic cervicitis in 10(25.0%), CIN II in 5(12.5%).

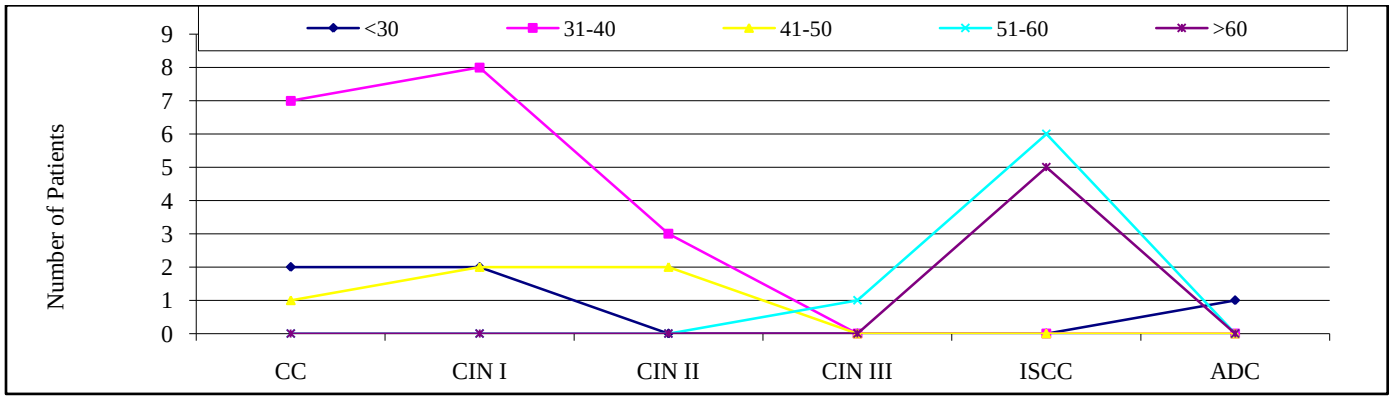

Figure-2:Histopathological diagnoses of patients with age groups.

The line chart shows histopathological diagnoses of the patients with age groups. It reveals CIN I and chronic cervicitis were highest in 4th decade. Majority of CIN II and CIN III cases were in 5th decade and most of the malignancy were observed in 6th decade. No HSIL or carcinoma was found before 4th decade and no CIN I was found after 5th decade.

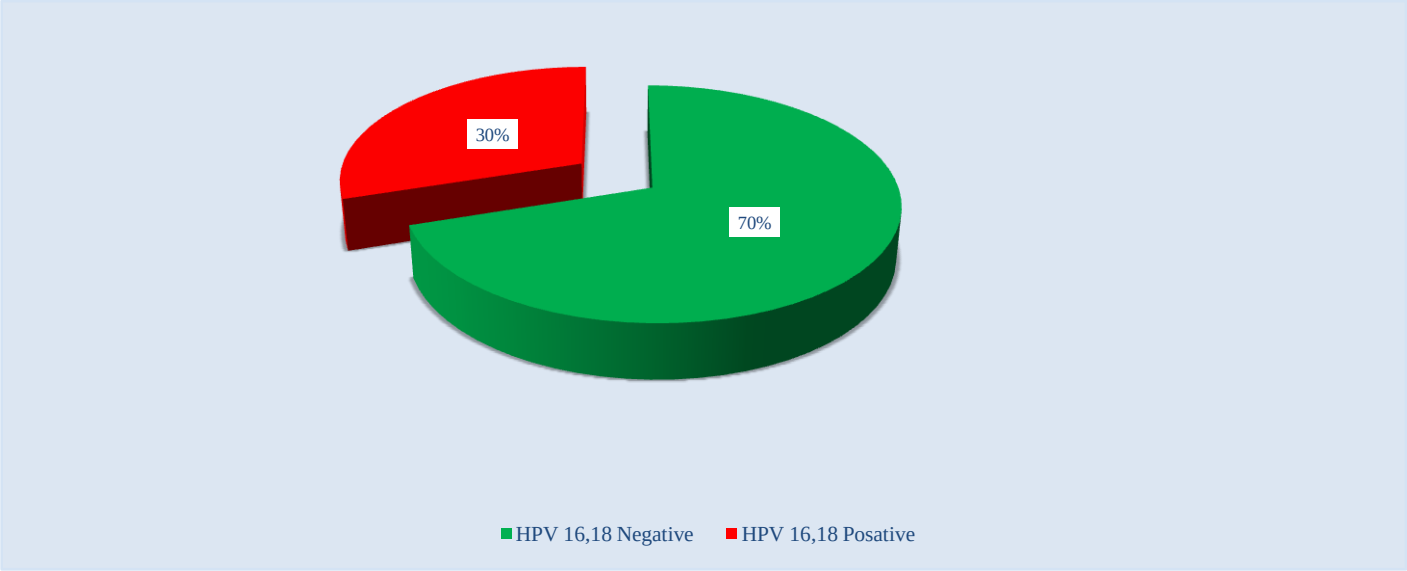

Figure 3: Distribution HR-HPV (16, 18) of the patients (N=40)

HR-HPV- High Risk Human Papilloma Virus
Figure 3 illustrates the distribution of HR-HPV (16, 18) in total 40 cases. Among 40 cases, 12(30.0%) cases were positive for HR-HPV and remaining 28(70.0%) cases were negative.

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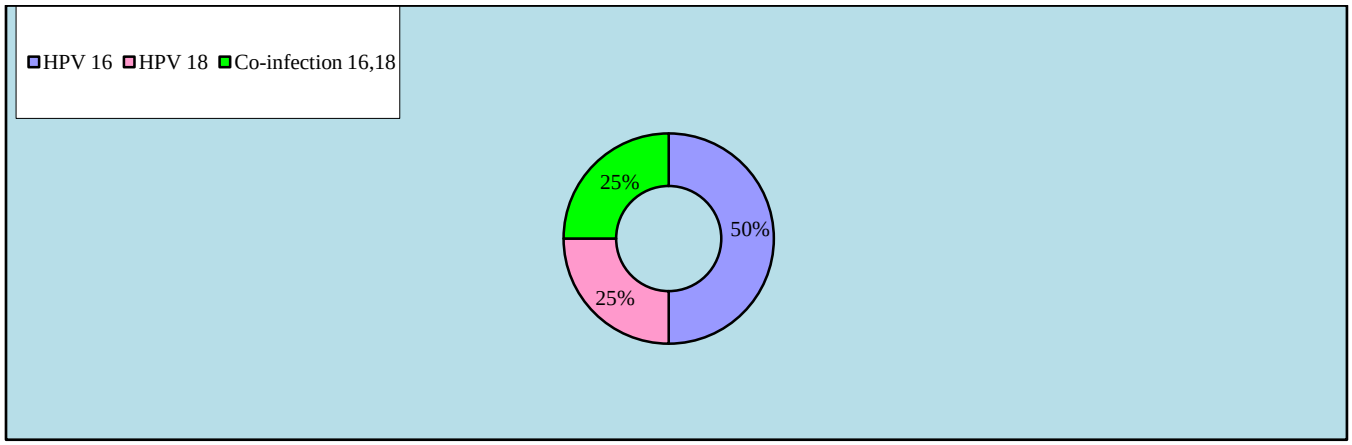

Figure 4: Distribution of specific types of HR-HPV (16, 18) infection (n=12)

This pie chart illustrates the distribution of specific types of HPV (16/ 18/ 16+18) in total 12 HR-HPV cases. HPV 16 was positive in 6 (50.0%) cases, HPV 18 was positive in 3 (25.0%) cases, co-infection (16+18) was positive in 3 (25.0%) cases.

Table IV demonstrates distribution of HR-HPV (16, 18) in different histopathological diagnoses. Out of 40 cases, HPV 16 was detected in 6(15.0%) cases, HPV 18 was detected in 3(7.5%) and co-infections (16+18) were found in 3 (7.5%) cases. In about 28(70.0%) cases, HR-HPV (16, 18) could not be detected.

Table IV: Distribution of HPV (16, 18) infections with different histopathological diagnoses (N=40)

HR- HPV (16, 18)	Histopathological diagnosis						Total
	CC	CIN I	CIN II	CIN III	ISCC	ADC	
HPV 16	0	1	1	1	3	0	6 (15.0%)
HPV 18	0	1	0	0	2	0	3 (7.5%)
Co-infection (16+18)	0	0	1	0	2	0	3 (7.5%)
Negative	10	10	3	0	4	1	28 (70.0%)
Base	10	12	5	1	11	1	40

Table IV shown distribution of p16 score with cervical lesions in study subjects. Among 40 cases, p16 is intensely positive in total 22 cases. All 12 cases of cervical cancer including 11 (100%) cases of ISCC and 1 (100%) case of ADC were intensely positive. Apart from that, 6 (50%) cases of CIN I, 3 (60%) cases of CIN II and 1(100%) case of CIN III expressed intense p16 positivity. All the cases of chronic cervicitis were scored negative (no stain) and 1/12 case of CIN I expressed weak p16 positivity, 5/12 cases of CIN I and 2/5 cases of CIN II were moderately p16 positive.

Table V: Distribution of p16 score with cervical lesions (N=40)

Dx	p16 score					Total	p16 positive
	Neg <5%	Weak + ve 5 - 25%	Mod + ve 26 - 50%	Intense + ve 50%	US		
CC	8	0	0	0	2	10	0(0.0)
CIN I	0	1	5	6	0	12	6(50.0%)
CIN II	0	0	2	3	0	5	3(60.0%)

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CIN III	0	0	0	1	0	1	1(100.0%)
ISCC	0	0	0	11	0	11	11(100.0%)
ADC	0	0	0	1	0	1	1(100.0%)
Total	8	1	7	22	2	40	22

Table VI: Comparison of p16 score with HPV: (N=40)

HR-HPV (16, 18)	p16 score		p value
	Positive	Negative	
Positive	12 (40.0)	0 (0.0)	0.017[s]
Negative	18 (60.0)	10 (100.0)	

Kappa=0.250

According to p16 score a total 30(22 true positive+ 08 false positive) cases were p16 positive. Out of which 12(40.0%) and 18(60.0%) were HR-HPV positive and negative respectively. Similarly, according to p16 score 10 negative cases were negative for HR-HPV DNA as well. The difference was statistically significant.

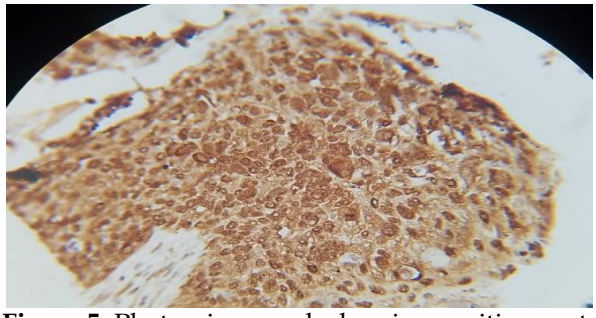

Figure 5: Photomicrograph showing positive control of p16 (Invasive squamous cell carcinoma of cervix, 40x)

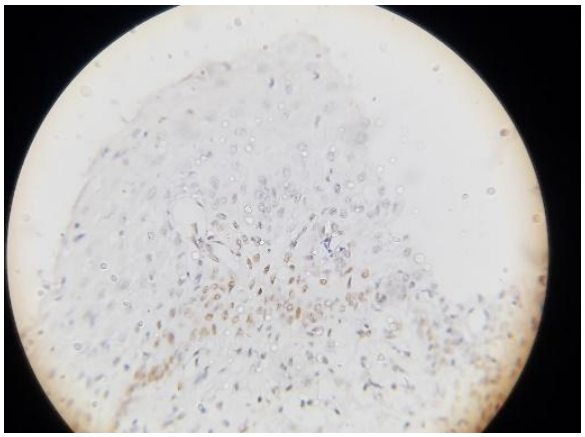

Figure 6: Photomicrograph showing negative control of p16 (cervix, 40x)

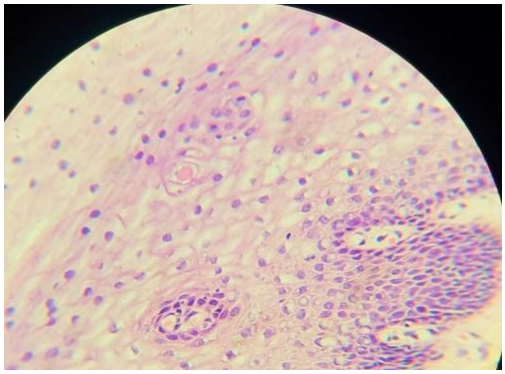

Figure 7: Photomicrograph showing a case of CIN 1 (case no. 09; H & E X 40x)

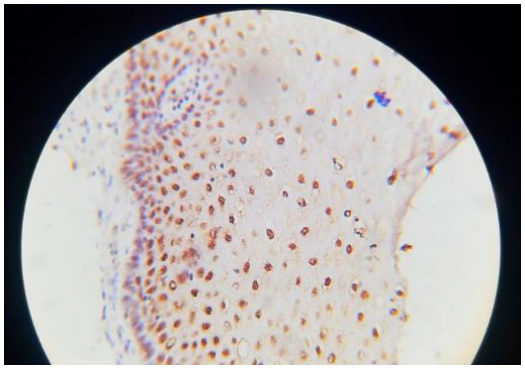

Figure 7A: Photomicrograph showing cytoplasmic and nuclaear positivity of p16 immunostain in more than 50% cells (case no. 09; p16 IHC X 40x)PCR –HPV 18 positive

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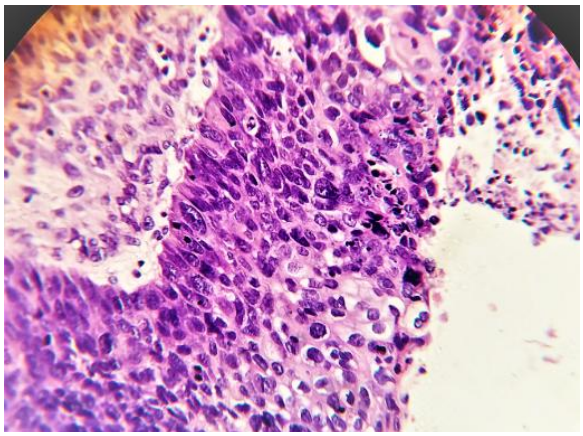

Figure 6: Photomicrograph showing a case of CIN 3(case no. 04; H & E X 100x)

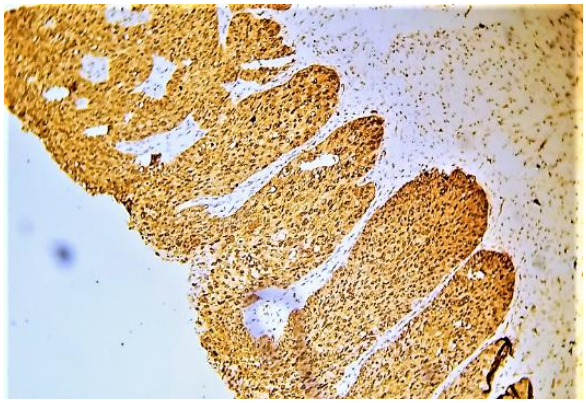

Figure 7A: Photomicrograph showing nuclear and cytoplasmic p16 immunostain in more than 50% cells (case no. 04; p16 immunostain X 100x)PCR –HPV 16 positive.

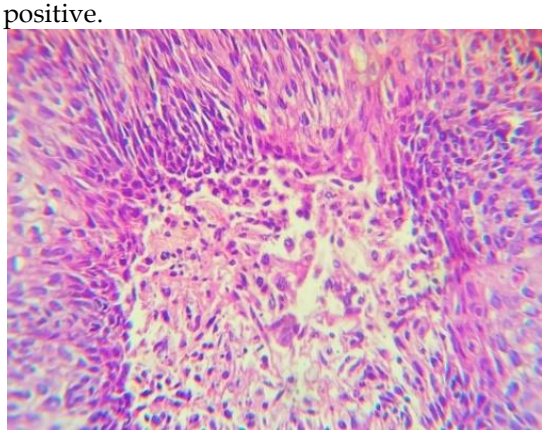

Figure 8: Photomicrograph showing a case of ISCC- I (case no. 23; H & E X 40x)

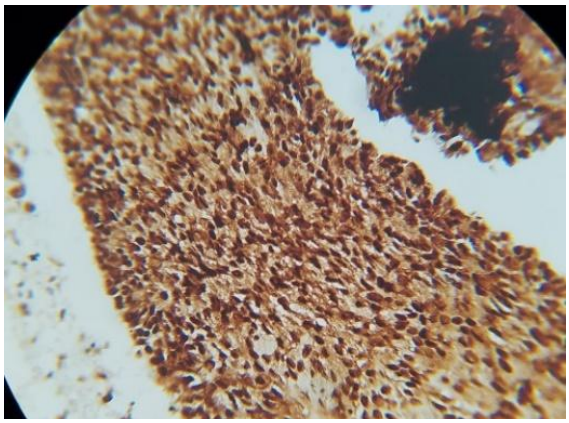

Figure 8A: Photomicrograph showing diffuse over expression of p16 immunostain in more than 50% cells (case no. 23; p16 IHC X 40x)PCR –HPV 18 positive.

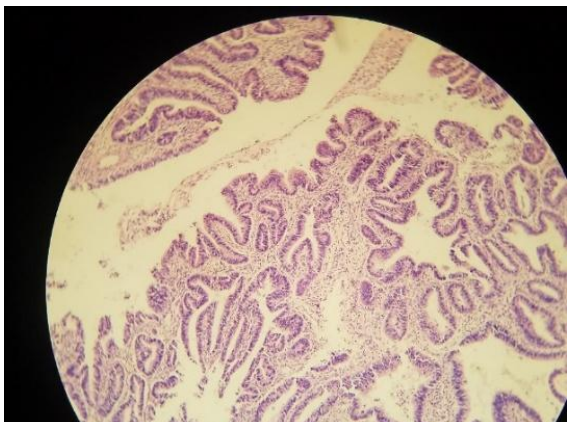

Figure 10: Photomicrograph showing a case of ADC-I (case no. 10; H & E X 40x)

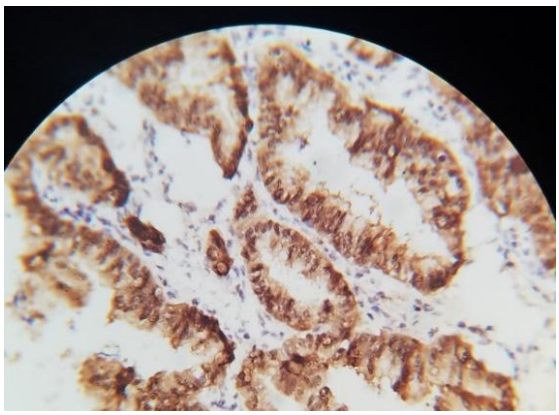

Figure 10 A: Photomicrograph showing diffuse over expression of p16 immunostain in more than 50% cells (case no. 10; p16 IHC X 40x)PCR –HPV 16, 18- negative

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DISCUSSION

Cytology, colposcopy and histopathology are three most important and complementary methods to study uterine cervix and its lesions. Different findings can show variations among results and can be explained by the experiences of the pathologists as well as the resolution of the colposcopy guided biopsy. Despite the success of several screening programmes, questions have arisen concerning the reliability of conventional cervical cytology and histology. These concerns have appeared the need for improved screening technologies like tumor associated antigen markers as an adjunct to conventional Pap testing. One such potential biomarker is p16 tumor suppressor gene.[16] Recent biological studies have revealed that p16 expression is markedly influenced by the status of RB expression.[17] This immunohistochemical p16 overexpression has been demonstrated in cervical cancers due to functional inactivation of RB by HR-HPV.[18] This descriptive cross-sectional study was carried out with an aim to evaluate the predictive value of p16 indicating the presence of HR-HPV in different cervical premalignant and malignant lesions. In this present study, it was observed that the age of majority of the cases (45.0%) belonged between 31-40 years. The mean age of the study subjects was 43.20±12.20 years. Almost similar observations regarding the peak age incidence were also observed by authors.[19] In this study, it was observed that among 40 cases, maximum 72.5% cases were married during the age between 16 to 20 years, 22.5% cases were married at less than 15 years of age and 5% cases were married above the age of 20 years. The mean marital age was 17.33±2.23 years. Similarly, in Bangladesh Karim et al.[20] observed the mean marital age was 16.7±2.7 years. Regarding the sign and symptoms, the common symptoms were per vaginal discharge, recorded in 95.0% cases, lower abdominal pain in 87.0% cases, irregular per vaginal bleeding in 60.0% and post coital bleeding in 7.5% cases. In a study, Begum et al.[21] observed abnormal intermenstrual bleeding in 12.19%, post-coital bleeding in 24.3%, and excessive vaginal discharge in 56.0% which are comparable with the current study. The majority of histopathological diagnoses of forty VIA positive cases, CIN I were 30.0%, invasive squamous cell carcinoma was found in 27.5% cases and chronic cervicitis were diagnosed in 25.0% cases. However, Begum et al.[21] found majority of colposcopy directed biopsy of 164 VIA positive cases represented 13.4% normal findings, 55.48% inflammation and 17.07% cases CIN I. The above mentioned data differs from the results of current study which could be explained by the smaller sample size of the subjects.In the present study, the common histopathological diagnoses were CIN I and chronic cervicitis cases. They were highest in 4th decade, CIN II and CIN III were in 5th decade and most of the cancer cases were observed in 6th decade. No case of HSIL or carcinoma was found before 4th decade and no CIN I was found after 5th decade. This finding is consistent with that of Karim et al.[20] Regarding the distribution of HR-HPV in different cervical lesions, it was observed that out of 40 patients, 12 (30%) cases were positive for HR-HPV. HPV 16 was found in 6 (15.0%) cases, HPV 18 in 3 (7.5%) cases and co-infections (16+18) in 3 (7.5%) cases. In about 28 (17%) cases were negative for HR-HPV (16, 18). According to the ICO Information Center on HPV and Cancer in 2017, HPV 16 and 18

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were most prevalent in cervical precancerous and lesions with HPV 16 being 15.4% and HPV 18 being 7.7%, in Bangladesh.[15] These data clearly corresponds with the current study where HPV 16 were mostly found. It was observed in our study that among 40 cases, monoclonal anti-p16 (p16) antibody was intensely positive (nuclear or cytoplasmic stain in more than 50% cells) in total 22 (54%) cases. Among them, all 12 (100%) cases of cervical cancer including 11 cases of ISCC and 1 case of ADC case were intensely positive. Apart from that, 6 (50%) out of 12 cases of CIN I, 3 (60%) out of 5 cases of CIN II and 1(100%) case of CIN III expressed intense positivity. Moreover, 6 among 12 cases of CIN I and 2 among 5 cases of CIN II were moderately positive (nuclear or cytoplasmic stain in less than 26%-50% cells). All the cases of chronic cervicitis were scored negative (nuclear or cytoplasmic stain in less than 5% cells). Zouheir et al.[22] and Hamdani et al.[23] reported p16 protein overexpression in 72.9% and 92.45% of cervical cancer specimens respectively. Calil et al.[24] reported that nearly 100% of high grade lesions and invasive cancers showed strong overexpression of p16 protein, while non-dysplastic lesions remained negative. Many other studies revealed diffuse p16 expression throughout the height of squamous epithelium in 80-100% of CIN II, almost all CIN III and nearly in all invasive cervical cancers.[25-26] These data indicate that p16 protein expression can be used to improve the histopathological diagnosis of precancerous cervical lesions.Benevolo et al.[13] investigated in parallel, p16 expression and HPV infection in 100 cervical biopsies (17 normal tissues, 54 CIN I, 10 CIN II, 11 CIN III, 8 ISCC). The obtained results demonstrated that none of the 17 normal cervical tissues presented p16 positivity, whereas starting from CIN I (31%) to CIN II (90%), CIN3 (100%) and carcinomas (100%), a constant and significant increase of protein overexpression ($P<0.05$) was observed. The findings from all the above investigators are comparable to our results. In the current study, it was evident that out of 5 cases, 2 CIN II cases were positive for p16 immunostaining which enlightens upon an important aspect.Nuno and Garcia, in[27] described the Lower Anogenital Squamous Terminology (LAST) recommendations in their study. Among 5 work groups (WG), the WG-4 had dealt with use of biomarkers in HPV detection such as p16 IHC. The recommendations designate that p16 is recommended to confirm a diagnosis of a HSIL when a diagnosis of CIN II is given based on H & E morphology. If a 'CIN II' specimen is p16 positive, it will be classified as 'HSIL'. If p16 is negative, it will be classified as 'LSIL'. In a similar study done by Waxman et al.[28] explained additional recommendation of using p16 to facilitate diagnosis when a potential high-grade lesion is often very similar to some benign mimics. These includes such as reactive squamous metaplasia, atrophy, reparative epithelial changes, placental implantation site, cautery artifact, radiation or even tangential sectioning. It is worth mentioning that the above stated findings definitely explain the importance of p16 as a biomarker for distinguishing among high grade and low grade lesion.The current study revealed p16 positivity in 6 cases among 12 CIN I cases. It has been suggested the possible usefulness of p16 protein as a marker to identify the HR-HPV associated LSIL cases. Most low-grade lesions spontaneously disappear 6–12 months after appearance, probably due to immunological intervention.[29]Arbyn et al.[30] reported that CIN1 cases with diffuse p16

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staining have a significantly greater tendency to progress to a high-grade lesion than p16-negative cases. Therefore, p16 staining appears to be useful in the diagnosis of HR-HPV infected LSIL cases, as the lesions would progress to HSIL or cancer.In this present study, among 10 cases of chrnic cervicitis, none of the cases was positive for p16 and no HR-HPV could be detected as well. This finding correlates with the findings of Stanculescu et al.[10] because p16 did not show any reaction with normal epithelial or mesenchymal cells. In our study, the comparison between p16 with cervical lesions showed that, 22 cases were true positive, 08 cases were false negative and 10 cases were true negative. No false positive cases were found. Lu-Lu Yuet al.[31] observed that there were no p16-negative cases in the HR-HPV positive group. These data clearly correlate with the present study.Regarding the validity test of p16 for predictive of HR-HPV infection cervical premalignant and malignant lesions, the present revealed sensitivity 73.3%, specificity 100.0%, accuracy 80.0%, PPV 100.0% and NPV 55.6%. Benevoloet al.[13] mentioned in their study that p16 overexpression consistently showed elevated sensitivity 84.0% and specificity 98.0% in detecting HR-HPV infection with a high PPV 97.0% and NPV 86.0%. All the above mentioned findings clearly correlate with current study.The over expressions of p16 were noted in all the 12 cancer cases in this study. Among them, 7 cases were HR-HPV (16, 18) positive and 4 cases were negative. In 1 case of CIN III, p16 showed intense positivity and it was positive for HR-HPV as well. In total of 5 cases of CIN II, 3 cases showed p16 positivity and among them, 2 cases were HR-HPV positive. Out of 10 cases of CIN I, 6 cases were intensely positive for p16. Among them, 2 cases were found positive for HR-HPV. All the cases of chronic cervicitis were negative for p16 and HR-HPV.The reasons of failure to detect more HR-HPV in CIN II and invasive cancer cases could be due to false negative results of HR-HPV, loss of a sub genomic region of the HPV DNA, by a very low HPV copy number that might be below the limit of detection by PCR method or by the presence of a novel or unknown HPV subtype.[32] In addition, the HPV negative cell line C33A is p16 positive; this clearly indicates that a non-HPV dependent p16 expression pathway may also exist.[33] The present study demonstrates that p16 could be a useful biomarker in detecting HR-HPV infected cervical lesions. The World Health Organization (WHO) approved the use of cell markers like p16 and the dual test p16/ Ki 67 in the clinical practices.[10] In an over populated country like Bangladesh, where women are usually married in their teens, have a high parity and unaware of the importance of cervical cancer screening system it is important to improve the conventional screening techniques. Therefore, integration of p16 with the conventional screening methods would be helpful in early detection of cervical premalignant and malignant lesions.

CONCLUSION

The study showed overexpression of p16 in 22 cases of premalignant and malignant lesions out of 40 cases. It also revealed that, all 12 HR-HPV (16, 18) infected cervical lesions stained intensely by p16 IHC. Moreover, the study revealed that p16 had a high sensitivity and optimum specificity in predictive of HR-HPV infection cervical premalignant and malignant lesions. Therefore, it can be inferred that overexpression of p16 demonstrates the

possibilities of potential use as a diagnostic marker for HR-HPV infected cervical lesions.

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