Bangladesh J Obstet Gynaecol, 2019; Vol. 34(2): 79-86

Clinical Utility of Biomarkers in Diagnosis and Management of High Grade Cervical Intraepithelial Neoplasia (HSIL)

JANNATUL FERDOUS[1], SABERA KHATUN[2], ASHRAFUNNESSA[3], SHIULY CHOWDHURY[4], FERDOUSY BEGUM[5], LATIFA AKTER[6], JAWAD MAHRUJ KHAN[7]

Abstract:

Objective of the study: *The increasing inter- and intra-observer variability in the diagnosis of CIN by histopathology has led to the advent of different biomarkers. The aim of this study is to evaluate Ki-67 and p16 ^{INK4a} marker in differentiating CIN lesions and the clinical utility of it in the management of high grade squamous intraepithelial lesions (HSIL).*

Materials and methods: *This cross-sectional study has been carried out among the biopsy proved cases of CIN2 and CIN3 in which P16 and Ki-67 immunostain was performed in the Colposcopy clinic, Bangabandhu Sheikh Mujib Medical University (BSMMU) and Department of Pathology, BSMMU respectively from 14-3-17 to 30-05-18. Thirty-two cases were incorporated in the study by purposive sampling. Data was analyzed using SPSS version 17.*

Results: *Among 32 cases of HSIL, 25 were diagnosed as CIN 2 (78.13 %) and 7 cases as CIN 3 (21.87 %) by histopathology. All the cases were subjected to immunohistochemistry with P16 and Ki 67. Regarding the status of P16, it was found positive only in 32% cases of CIN 2 but in 100% cases of CIN 3. Similarly, Ki67 was found positive in 36 % of CIN2 cases but in all cases of CIN 3 lesions. Thus immunostain confirmed one third histopathologically diagnosed CIN 2 cases. On the other hand, all cases of CIN 3 were confirmed as HSIL with the immunostain. All the cases of CIN 2 were reviewed for consensus opinion. Only 8 (32%) cases of CIN 2 were both immunostain positive , the review diagnosis by consensus opinion was also CIN 2 in those cases. One case (4%) of CIN 2 was P16 negative but Ki 67 positive, which ultimately revealed as immature squamous metaplasia. Sixteen cases of CIN 2 were negative for both P16 and Ki 67. Among these, majority cases (52%) , consensus opinion revealed as CIN 1 and 12% cases as immature squamous metaplasia. Therefore, an HSIL diagnosed by histopathology could be false positive in a significant number of cases and overtreatment of a false positive CIN 2 lesion can affect future reproductive status, also have some psychological consequences and economic burden in the health sector.*

Conclusion: *By using the biomarkers, overtreatment and undertreatment of CIN can be avoided. All cases of CIN 2 should be carefully dealt with either review of the slides by consensus opinion or by doing immunohistochemistry if facility is available.*

Key words: *High-grade cervical intraepithelial neoplasia (HSIL), Biomarker, Histopathology.*

Introduction:

Cervical cancer, once the second most common cancer in women in both incidence and mortality, is now no longer than 11th in incidence and 13th in mortality in the United States, with similar reductions seen in countries with well-established cervical

1. Prof. Jannatul Ferdous, Professor, Gynae Oncology, BSMMU, Dhaka.
2. Prof. Sabera Khatun, Chairman, Gynae Oncology, BSMMU, Dhaka.
3. Prof Ashrafunnessa, Professor, Gynae Oncology, BSMMU, Dhaka.
4. Dr. Shiuly Chowdhury, Associate Professor, Obs & Gynae, BSMMU, Dhaka.
5. Dr.Ferdousy Begum, Associate Professor, Pathology, BSMMU, Dhaka.
6. Dr. Latifa Akter, Medical Officer, Gynae Oncology, BSMMU, Dhaka.
7. Jawad Mahruj Khan, 4th year student, Department of Bio-engineering, University of Riverside, California.

Address of Correspondence: Prof. Jannatul Ferdous, Professor, Gynae Oncology, BSMMU, Dhaka.

cancer screening and management programs[1]. This unparalled success in cancer prevention has come in large part from the ability to detect and treat the precursor lesion to cervical cancer. Most important has been the partnership between cervical cytology screening and treatment of colposcopically detected high-grade neoplasia[1].

Cervical cancer accounted for approximately 7.8% of all gynaecological patients and 70% of all gynaecological malignancies admitted in BSMMU in the year 2007[2]. More than 80% are diagnosed with this eminently preventable cancer in clinically advanced, inoperable stages[3]. Though cervical cancer is preventable, yet it is an important cause of disability and death of women in Bangladesh. Invasive cervical cancers are preceded by long phase of pre invasive disease, cervical intraepithelial neoplasia (CIN).CIN can be treated effectively to prevent progression to cervical cancer Most of the low grade CIN (CIN-1) lesions regress spontaneously and is recommended to a strict follow-up without any treatment[4]. CIN2 & CIN3 are usually treated actively as 5% of CIN II and 12% of CIN3 will progress to invasive cancer[5].

Cervical cancer is by far the most common HPV-related disease. Nearly all cases of cervical cancer can be attributable to HPV infection. Although most HPV infections clear up on their own and most pre-cancerous lesions resolve spontaneously, there is a risk for all women that HPV infection may become chronic and pre-cancerous lesions progress to invasive cervical cancer. It takes 15 to 20 years for cervical cancer to develop in women with normal immune systems. It can take only 5 to 10 years in women with weakened immune systems, such as those with untreated HIV infection[6].There are more than 100 types of HPV, of which at least 13 are cancer-causing also known as high risk type. Of these, HPV 16, 18 are implicated in 70% cases of high-grade CIN and cervical carcinoma[7].

The histologic diagnosis of cervical biopsies that is often considered as the gold standard for the diagnosis of different grades of cervical lesions can be significantly hampared by intra- and inter-observer variability[8,9]. By the histopathological diagnosis, it is not possible to give any clue about the risk of persistent infection or chance of progression or regression. The main interpretive categories include distinguishing from low-grade (CIN 1) lesions from high grade lesions[10].

Truly precancerous or high-grade lesions are sometimes misclassified as negative for dysplasia in the setting of reactive or metaplastic changes or when a biopsy is fragmented or poorly samples an underlying small lesion. Conversely, diagnostic errors resulting in the overcalling of negative as high-grade lesion also occur[11]. Errors in histologic diagnosis lead to either overtreatment of patients who will not benefit from intervention conversely, inadequate treatment of patients with clinically significant high grade lesions with false negative diagnosis[10].

Therefore, using a suitable marker to predict persistent hr-HPV infection would be of considerable clinical value. Supplementary methods using potential biomarkers are now being used in the developed countries to achieve more accurate diagnosis, as well as to identify those patients with high-grade CIN who are at risk for progression to cancer[12].

HPV is a double-stranded DNA virus. The E6 & E7 genes of high-risk HPV specifically bind to and inactivate P53 tumor suppressor protein and the retinoblastoma protein P^{RB}. It leads to disruption of cell cycle, increased cell proliferation and ultimately giving rise to carcinoma. Persistent infection with high risk HPV types is the most important factor in the development of CIN and invasive cancer[12]. The P16 protein is an INK4a cyclin dependant kinase (CDK) inhibitor that decelerates the cell cycle by inactivating CDK that phosphorylates retinoblastoma (Rb) protein. The status of Rb expression strongly affects P16 expression, and P16 over expression has been demonstrated in cervical cancers because of functional inactivation of Rb by HPV E7 oncoprotein. Immunohistochemical expression of P16 has been associated with dysplastic/neoplastic cells but not seen in normal cervical epithelium[13]. Thus P16 is a specific biomarker used for identification of dysplastic cervical epithelium with tendency to invasive cervical cancer[12].

Ki-67 is a proliferation marker. It is expressed in the parabasal cell layer of normal stratified squamous epithelium having proliferation capacity[10]. In CIN, dysplastic cells show increased cell cycling[5]. So Ki-67 is over- expressed in different extent in correlation with different grading of CIN.

The study was carried out to determine the clinical utility of these two biomarkers, p16 and Ki-67 in diagnosis and management of high grade cervical intraepithelial neoplasia. This study will play an

important role in the accurate diagnostic interpretation of cervical biopsy specimen to identify high-grade cervical intraepithelial lesion to avoid overtreatment of false-positive cases and under treatment of false-negative cases.

Materials and methods:

As per granted research proposal dated 14/03/2017,this retrospective cross-sectional study has been carried out among thirty two biopsy confirmed cases of CIN2 and CIN3 in which P16 and Ki-67 immunostain was performed. The place of the study was Colposcopy clinic, Bangabandhu Sheikh Mujib Medical University (BSMMU) & Department of Pathology, BSMMU. All cases of CIN 2, CIN 3 and CIS diagnosed histopathlogically were included in the study by purposive sampling from 14-03-17 to 31 - 05-2018. Histopathologically proven case of benign lesions, CIN 1, and cancer cervix were excluded from the study.

General objective of the study was to study clinical utility of biomarkers in diagnosis and management of high grade cervical intraepithelial neoplasia. Specific objectives were to determine positivity of P16 immunostain in the biopsy confirmed histopathology slides of CIN2 and CIN3 and to determine positivity of Ki-67 immunostain in the biopsy confirmed histopathology slides of CIN2 and CIN3.

Regarding the study procedure an approval letter from the Institutional Review Board (IRB) of BSMMU was obtained at the outset. The information was collected in a pre-designed data collection sheet (Research instrument). Primary selection of the cases were made from the slides which were histologically diagnosed as CIN 2, CIN 3/CIS on H& E stain from the Department of Pathology,BSMMU . Then these slides were selected for p16 and Ki-67 immunohistochemical staining according to the standard protocol followed at the Department of Pathology, BSMMU. All the cases of CIN 2 and CIN3 with immunostain positive and immunostain negative were reviewed by consensus meeting.

The research instrument (Data Collection Sheet) was prepared keeping in mind the research questions, objectives and variables of the study. Pertinent socio-demographic data, and diagnosis were recorded in the data collection sheet. Modification of collected data was performed as per need. Data were also collected from documents review like hospital record from the Department of Pathology and from Colposcopy clinic, BSMMU.

Results:

A total of 32 formalin-fixed paraffin-embeded-samples of high grade CIN (CIN 2 and CIN 3/CIS) were collected from the Department of Pathology, BSMMU. Samples those were biopsied from colposcopically diagnosed high-grade lesions at colposcopy clinic of BSMMU from 14-03-17 to 31 - 05-2018 were selected for the study.

Among the 32 cases, 25 were diagnosed as CIN 2 (78.13 %) and 7 cases as CIN 3 (21.87 %). All of the cases were subjected to immunohistochemistry for P16 and Ki 67 [Table I]. Among the CIN 2 cases, P16 immunostain was found positive in 8 (32%) cases and negative in 17 (68%) cases whereas among the CIN 3 cases, P16 immunostain was found positive in all 7 (100%) cases [Table II]. Regarding the status of Ki67 imunostaining, among the CIN 2 cases, it was found positive in 9 (36 %) cases and negative in 16 (64%) cases but similarly among the CIN 3 cases, Ki 67 immunostain was found positive in all 7 (100%) cases [Table III].

Table-I

Histopathological diagnosis of the study cases (n=32)

Histopathological diagnosis	No. of cases	Percentage
CIN 2	25	78.13%
CIN 3	7	21.87%

Table-II

Status of P16 immunostain in the study cases (n=32)

Histopathological	P16 immunostain	
diagnosis	Positive	Negative
CIN 2 (25)	8(32%)	17(68%)
CIN 3 (7)	7(100%)	0(0%)

Table-III

Status of Ki67 immunostain in the study cases (n=32)

Histopathological	Ki 67 immunostain	
diagnosis	Positive	Negative
CIN 2 (25)	9(36%)	16(64%)
CIN 3 (7)	7(100%)	0(0%)

Table-IV

Confirmation of diagnosis of HSIL after immunostain with P16 and Ki 67

Histopathological diagnosis	Confirmation of HSIL after immunostain with P16 and Ki 67	Percentage
CIN 2 (25)	8	32
CIN 3 (7)	7	100

Table-V

Reviewed diagnosis by consensus opinion of CIN 2 cases after immunostaining.

Immunostain	No of cases (%)	Reviewed diagnosis by consensus opinion
Both P16 and Ki 67 immunostain positive	8(32%)	CIN 2
P16 negative but Ki 67 positive	1(4%)	Immature squamous metaplasia
Both P16 and Ki 67	13(52%)	CIN 1
negative	3(12%)	Immature squamous metaplasia

When HSIL (High grade squamous intraepithelial lesion) was confirmed by immunostain, only 1/3rd (32%) cases of CIN 2 were confirmed as HSIL. On the other hand, all cases of CIN 3 7(100%) was confirmed as HSIL with the immunostain [Table IV].

All the cases of CIN 2 (25) were reviewed by consensus opinion. Only 8 (32%) cases of CIN 2 were both P16 and Ki 67 immunostain positive and reviewed diagnosis was also CIN 2 in those 1/3rd cases. One case (4%) of CIN 2 was P16 negative but Ki 67 positive that revealed as immature squamous metaplasia in consensus opinion. Sixteen cases of CIN 2 were negative for P16 and Ki 67. Of these sixteen negative cases, in 13 (52%) cases reviewed histopathological diagnosis revealed CIN 1 and in 3 (12%) cases as immature squamous metaplasia [Table V]

Among the initial histopathologically diagnosed (with H & E stain) CIN 2 cases, only 1/3 rd cases were positive for immunohistochemistry. In the remaining 2/3rd cases the confirmatory diagnosis was CIN I in 50% cases and immature squamous metaplasia in 12% cases both of which are not premalignant condition. Therefore, an HSIL diagnosed lesion could be false positive in a significant number of cases in histopathological examination using H & E staining. However, the confirmed diagnosis can be obtained by immunohistochemistry for P16 and Ki-67.

Discussion:

Until now several biomarker has been evaluated for the diagnosis of cervical precancer and cervical cancer. In this retrospective observational study, we assessed the clinical utility of biomarkers in the diagnosis of high grade squamous intraepithelial neoplasia in biopsy samples.

In the present study, the age of the study subjects ranged from 20 to 70 years with the mean age 38.93 ± 10.62 years. A study by Tebeu et al found the mean age of cervical precancerous lesions 41.59 years, the results of which correlated with the present study[14].

In this study, among the initial histopathologically diagnosed CIN 2 cases, only 1/3 rd cases were found positive when stained with P16 and Ki-67 immunostain. In the remaining 2/3rd cases both the immunostains were negative.

Recently, a systematic review and meta-analysis concluded that interobserver agreement of the diagnosis of CIN 2+ improve with the conjunctive use of p16 immunostaining compared to H&E morphology alone[15].

The College of American Pathologists and the American Society for Colposcopy and Cervical Pathology recently included p16 immuno-histochemistry in their revised nomenclature for lower genital tract lesions[16]. Infection with hr-HPV results in integration of hr-HPV into the host genome. The early gene E7 is the urgent oncogene of hr-HPV. E7 binds to the tumor suppressor retinoblastoma protein (p^{RB}) and thus in activates p^{Rb}, resulting in G1-S transition of the cell cycle. The HPV-E7 determines the inactivation of p^{Rb} with a consequent increase of free E2F in the cell, leading to aberrant proliferation

(marked by increased levels of Ki-67 expression)[17,18].

P16, a p^{Rb} regulator, underlies a negative feedback controlled by the p^{RB} whose inactivation results in overexpression of p16. As to the relation between hr-HPV and p16 as well as Ki-67 expression, most researchers believe that p16 is a surrogate marker for CIN induced by hr-HPV[17,18].

A study by Srivastava et al showed 100% P16 positivity in invasive carcinoma of cervix, and increased P16 positivity with increasing severity of CINs. Murphy et al. also observed 100% P16 positivity in invasive squamous cell carcinoma and significant linear relation ($p<0.0001$) between P16 staining and increasing grades of squamous dysplasia[19,20]. They concluded that P16 has a clinical utility as a biomarker because it is a measure of HPV gene expression and activity, rather than solely a detector of viral presence[21].

Ki-67 is an antigen expressed in proliferating cells that can be detected in formalin–fixed tissue using the MIB-1 antibody[22,23]. MIB-1/Ki-67 is an important immunohistochemical marker used to assess the proliferation activity and has been suggested as a sensitive biological indicator of progression in CIN lesions. Van Hoven et al. in 1997 formulated a "stratification index" (SI), which indicates, how high Ki-67 positive nuclei are located in the epithelium; the higher the SI, the higher the CIN grade[24]. Srivastava et al in their study also found that Ki-67 positivity increased with the severity of CIN to carcinoma group[19].

Garzetti et al. analyzed the performance of Ki-67 immunostaining as an index of cellular proliferation in CIN and micro-invasive carcinoma with the aim to identify a relationship with the degree of dysplastic lesion and the risk of neoplastic progression. Their results showed that positive Ki-67 immunostaining had increased progressively from squamous metaplasia to CIN and micro-invasive carcinoma, ($P<0.001$). Ki-67 index showed a significant increase with respect to CIN grades ($P<0.0001$)[25].

Similarly, Godoy et al and Carrilho et al found Ki-67 expression restricted to the basal layer in normal epithelium, and also Ki-67 expression crossed the basal layer and reached different level of epithelial thickness in relation to lesion grade[26,27].

Although presence of Ki-67 positive nuclei in the upper two-thirds of epithelial thickness is outstanding criteria for Ki-67 positivity, there are few false positive interpretations of the staining, such as in cases of cervicitis, immature squamous metaplasia and areas of repair[28,29]

In this present study, regarding confirmation of HSIL(High grade squamous intraepithelial lesion) with immunostain, both P16 & Ki-67confirmed only one third cases of CIN 2 as HSIL. On the other hand, all cases of CIN 3, was confirmed as HSIL with the immunostain for both P16 & Ki-67.

Galgano et al. conducted a community-based population based evaluation in 1500 cervical biopsies in order to evaluate the utility of HPV L1, p16, and Ki 67 immunohistochemical staining for improving diagnostic accuracy. This well designed study showed that the addition of p16 and Ki-67 to H&E increase sensitivity for the diagnosis of high grade cervical lesions[11].

In the present study, all the cases of CIN 2 (25) were again reviewed by consensus opinion. Only one third cases of CIN 2 were both P16 and Ki 67 immunostain positive, the reviewed report was also CIN 2. But in the remaining cases, the consensus opinion revealed CIN 1 in more than fifty percent cases and immature squamous metaplasia in 12% cases

There are certain benign conditions that mimic CIN like, immature squamous metaplasia/reactive epithelial changes/ reparative atypia/ atrophy/ tangential sectioning etc. that may lead to an overdiagnosis of CIN 2[30].

A study by Ankita et al. showed that immunostaining for p16 showed negative staining in cases of immature squamous metaplasia except one case which was also positive for Ki-67[31], the result also correspond to this present study.

A study by Agoff et al included 597 specimens of cervical biopsy/LEEP and compared the p16 staining with Ki-67 and they found p16 to be more specific for neoplasia and dysplasia than Ki-67[32].

In the LAST project (Lower Anogenital Squamous Terminology Standardization) Project for HPV-associated Lesions, a consensus process was sponsored by the College of American Pathologists(CAP) and the American Society for Colposcopy and Cervical Pathology (ASSCP) regarding comprehensive reevaluation of the

terminology of HPV –associated lesions of lower anogenital tract.LAST project recommendation is to use of P16 immunostain in a few specific situations specially in cases with histological diagnosis of CIN 2 which are actually biologically equivocal cases between CIN 1 and CIN 3. P16 immunostain should be used as an adjudication tool for cases in which there is a professional disagreement in histologic specimen interpretation with the differential diagnosis of a precancerous lesion (CIN 2/CIN 3).But it should not be used as a routine adjunct to histologic assessment of biopsy specimens with a benign disease, CIN 1 or CIN 3[30].

The results of the present study also support the recommendations of LAST project. All CIN 3 cases were positive with P16 and Ki-67 as expected. On the other hand, only 1/3rd cases of CIN 2 were confirmed as high grade squamous intraepithelial lesions with both the P16 and Ki-67 immunostains. Treatment for high grade cervical intraepithelial lesion involves cryotherapy, thermocoagulation, loop electrosurgical excision procedure or cervical conization that are associated with risk of haemorrage, infection, cervical stenosis, cervical incompetency and subfertility in young women. So instead of treating all the cases of histologically diagnosed CIN II, we can manage them in two ways; simply by observation or to subject them to P16 and Ki67 immunostaining. In this way we can spare the patients from developing such complications, unnecessary financial burden and psychological breakdown. However further large study is warranted to demonstrate it.

Conclusion:

Discordance on histopathological diagnosis of high grade squamous intraepithelial lesions has been documented in several literatures, suggesting a need to identify biological marker that could help the pathologist to make a correct diagnosis in equivocal lesion. The immunostain is not essential for CIN 3 lesion but it may help in the confirmatory diagnosis of CIN 2 lesion. Thus testing with P16 and Ki immunostain appears to be a gold addition to more accurately diagnose the HSIL. The clinical utility of these two biomarker (P16 and Ki 67 immunostain) is important to ensure accurate and proper management of the patients.

Recommendation:

Thus all cases of CIN 2 should be carefully dealt with either review of the slides by consensus opinion if possible or by doing immunohistochemistry where facility is available.

Acknowledgment:

This study was granted by University Grants Commission (UGC).

References:

1. Kitchener HC, Castle PE, Cox JT.C hapter 7: Achievements and limitations of cervical cytology screening. Vaccine 2006;24(suppl 3):S63-70.
2. Ferdous J,Begum SA, FeedousNE,Nahar Q, Khatun SF ,Khatun S.Presentation of Invasive Cervical Cancer in Bangladesh.BSMMU J 2013; 6 (1) :29-32
3. Nessa A, Hussain MA, Rahman JN, Rashid MH, Muwonge R, Sankaranarayanan R. Screening for cervical neoplasia in Bangladesh using visual inspection with acetic acid. International Journal of Gynecology & Obstetrics. 2010;111(2):115-118.]
4. Rouzier R.Management of CIN. J Gynecol Obstet Biol Reprod (Paris) 2008;37(Suppl1): S114-20.
5. Grigore M, Teleman S, Ungureanu D, Mares A. Molecular markers in cervical screening- a promise for the future. Revista Romana de Medicina de Loborator. 2013;21: 231-237.
6. Human papillomavirus (HPV) and cervical cancer [https://www.who.int/news-room/fact-sheets/detail/human-papillomavirus-(hpv)-and-cervical-cancer]accessed on 2-8-2020
7. Zur-Hausen H. 1991. Human papillomaviruses in the pathogenesis of anogenital cancer. Virology. 1991; 184: 9-13.
8. Kavatkar AN, Nagwanshi CA, Dabak SM. Study of a manual method of liquid-based cervical cytology.Indian J Pathol Microbiol.2008;51:190-4.
9. Esquivias L0pez-Cuervo J, Montalban Beltran E, Cuadros Lopez JL, Alonso Castillo A, Nieto Sanchez T. Preeliminary study of a new, fully automated system for liquid-based cytology: The NovaPrep processor system. Acta Cytol.2011;55:281-6]

10. Hwang SJ, Shroyer KR. Biomarkers of cervical dysplasia and carcinoma.J Oncol.2012: 1-9.

11. Galgano MT, Castle PE, Atkins KA, Brix WK, Nassau SR et al. 2010. Using Biomarkers as objective Standards in the Diagnosis of Cervical Biopsies. Am J Surj Pathol.2010; 34: 1077-1087.

12. Aslani FS, Safaei A, Pourjabali M, Momtahan M. 2013. Evaluation of Ki-67, p16 and CK17 Markers in Differentiating Cervical Intraepithelial Neoplasia and Benign Lesions. Iran J Med Sci.2013; 38: 15-21.

13. Patel T. Role of P16 & Ki67 immunohistochemistry in the evaluation of cervical dysplasia. News Path. 2014; 10: 1-3.

14. Tebeu PM, Sando Z, Ndoumba A, Sandjong I, Mawech-Fauceglia,P, Anderson SD. Prevalence and Geographical Distribution of Precancerous Lesions of the Uterine Cervix in Cameroon. J Cytol Histol.2013; 4: 183.

15. Reuschenbach M, Wentzensen N, Dijkstra MG, von Knebel Doeberitz M, Arbyn M. p16INK4a immunohistochemistry in cervical biopsy specimens: a systematic review and meta-analysis of the interobserver agreement. Am J Clin Pathol 2014;142:767-72.

16. Darragh TM, Colgan TJ, Thomas Cox J, Heller DS, Henry MR, Luff RD, et al; Members of the LAST Project Work Groups. The Lower Anogenital Squamous Terminology Standardization project for HPV-associated lesions: background and consensus recommendations from the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology. Int J Gynecol Pathol 2013;32:76-115.

17. Khleif SN, DeGregori J, Yee CL, Otterson GA, Kaye FJ, Nevins JR, Howley PM. Inhibition of cyclin D-CDK4/CDK6 activity is associated with an E2F-mediated induction of cyclin kinase inhibitor activity. Proc Natl Acad Sci U S A. 1996;93:4350–4.

18. Reuschenbach M, Waterboer T, Wallin KL, Einenkel J, Dillner J, Hamsikova E, Eschenbach D, Zimmer H, Heilig B, Kopitz J, Pawlita M, Doeberitz Mv, Wentzensen N. Characterization of humoral immune responses against p16, p53, HPV16 E6 and HPV16 E7 in patients with HPV-associated cancers. Int J Cancer. 2008; 123:2626–31.

19. Srivastava S. P16INK4A and MIB-1 Expression in Preneoplasia and Neoplasia of Cervix, Intraepithelial Neoplasia, Dr. Supriya Srivastava (Ed.),2012 ISBN: 978-953-307-987-5, InTech, Available from: http://www.intechopen.com/books/intraepithelial-neoplasia/p16ink4a-and-mib-1-expression-in-preneoplasiaand-neoplasia-of-cervix.

20. Murphy N, Ring M, Heffron CC, King B, Killalea AG, Hughes C, et al. $p16^{INK4a}$, CDC6, and MCM5: Predictive biomarkers in cervical preinvasive neoplasia and cervical cancer. J Clin Pathol. 2005; 58: 525–534.

21. Stanley MA. Prognostic factors and new therapeutic approaches to cervical cancer. Virus Res.2002; 89: 241-248.

22. Brown CA, Bogers J, Sahebali S, Depuydt C.E, De Prins F, Malinowsk DP. Role of Protein Biomarkers in the Detection of High-Grade Disease in Cervical Cancer Screening Programs. Hindawi Publishing Corporation Journal of Oncology. 2012: 11.

23. Cattoretti G, Becker MH, Key G. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB-1 and MIB-3) detects proliferating cells in microwave processed formalin fixed paraffin sections. J Pathol.1992; 168: 357-363.

24. Baak JPA, Kruse AJ, Robboy SJ, Janssen EAM, van Diermen B, Skaland I. Dynamic behavioural interpretation of cervical intraepithelial neoplasia with molecular markers. J Clin Pathol.2006; 59: 1017–1028.

25. Garzetti GG, Ciavattini A, De Nictolis M, Lucarini G, Goteri G, Romanini C, Biagini G. MIB 1 immunostaining in cervical intraepithelial neoplasia: prognostic significance in mild and moderate lesions. Gynecol Obstet Invest.1996; 42: 261-6.

26. Godoy AEG, Bazzo KO, De Moura LB, Serafini ED. Expression Analysis of p53, Ki-67 and bcl-2 in Pre-Malignant Lesions of the Cervix. Open Journal of Obstetrics and Gynecology.2014; 4: 462-469.

27. Carrilho C, Gouveia P, Cantel M, Alberto M, Buane L, David L. 2003. Characterization of Human Papillomavirus Infection, p53 and Ki-67 Expression in Cervix Cancer of Mozambican Women. Pathology—Research and Practice.2003; 199: 303-311.

28. Keating JT, Cviko A, Riethdorf S, Riethdorf L, Quade BJ, Sun D. 2001. Ki-67, cyclin E, and p16INK4 are complimentary surrogate biomarkers for human papilloma virus-related cervical neoplasia. Am J Surg Pathol.2001; 25: 884-91.

29. Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy D, Petry U, et al. Overexpression of p16ink4a as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int. J. Cancer.2001; 92: 276–284

30. Waxman AG, Chelmow D, Darragh TM, Lawson H, Moscicki AB. Revised Terminology for Cervical Histopathology and Its Implications for Management of High-Grade Squamous Intraepithelial Lesions of the Cervix. Obstet Gynecol;2012; 120: 1465-1471.

31. Ankitha H and Venkataramappa SM. Role of p16/INK4a and Ki-67 as specific biomarkers for cervical intraepithelial neoplasia: An institutional study.J Lab Physicians. 2017 Apr-Jun; 9(2): 104–110.

32. Agoff SN, Lin P, Morihara J, Mao C, Kiviat NB, Koutsky LA. p16(INK4a) expression correlates with degree of cervical neoplasia: A comparison with Ki-67 expression and detection of high-risk HPV types. Mod Pathol. 2003;16:665–73.