www.jmscr.igmpublication.org
Impact Factor 5.244
Index Copernicus Value: 83.27
ISSN (e)-2347-176x ISSN (p) 2455-0450
crossref DOI: https://dx.doi.org/10.18535/jmscr/v5i1.37

IGM Publication
Journal Of Medical Science And Clinical Research
An Official Publication Of IGM Publication

Immuno-Histochemical Study of P16INK4A in Cervical Intraepithelial Neoplasia and Cervical Cancer

Authors
Dr Sharath Kumar H.K[1], Dr Kokila. S[2]
[1]Associate Professor, Dept of Pathology, Mysore Medical College & Research Institute Mysore, INDIA
Email: *sharath_hk@hotmail.com, 09743813949*
[2]Assistant Professor, Department Of Pathology, Bangalore Medical College & Research Institute, Bangalore INDIA
Email: *kokila.krishsan@gmail.com, 09902101000*

ABSTRACT
Background: *Cervical cancer is caused by human papilloma virus (HPV) strains 16 and 18. One of the consequences of viral genomic integration into the host cell is up regulation of p16INK4A, a cyclin dependent kinase inhibitor. This implies that detection of p16INK4A may be used as a surrogate marker of integration of high risk HPV into cervical epithelium and transition to malignant transformation.*
Aim: *The aim of this study is to assess utility of p16INK4A immunopositivity and evaluate the usefulness of this biomarker in the diagnosis of CIN and cervical cancer.*
Materials and Methods*: 65 cervical biopsy/ hysterectomy specimens of LSIL, HSIL and SCC were studied for H&E and compared with IHC staining. Subsequently, the immunohistochemically stained slides were graded on a 0-3 grading scale.*
Results*:*
- *65 evaluated cases on histopathological examination showed that 23 (35.4%) cases were LSIL, 8 (12.3%) cases were HSIL and 34 (52.3%) cases were SCC of uterine cervix*
- *23 cases of LSIL were studied for p16INK4A over expression in which 20 (87%) cases showed grade 1 staining and 3 (13%) cases showed grade 2 staining*
- *8 cases of HSIL were studied for p16INK4A over expression in which 1 (12.5%) case showed grade 2 staining and 7 (87.5%) cases showed grade 3 staining*
- *34 cases of SCC were studied for p16INK4A over expression in which 3 (8.8%) cases showed grade 2 staining and 31 (91.2%) cases showed grade 3 staining*

Conclusion: *The present study concludes that p16INK4A is a specific marker for dysplastic and neoplastic alteration in the cervical epithelium and thereby significantly improves cervical pre-cancer and cancer detection.*
Keywords*: Dysplasia, Kinase inhibitor, Cervical neoplasia, Biomarker.*

Introduction

Cervical cancer is the third most common cancer among women worldwide. It is especially common in developing countries like India.[1,2] Cytological screening for cervical cancer using the Pap smear is based on subjective diagnostic

parameters and is affected by a high rate of false-positive and false-negative test results. Similarly, histopathological assessment, considered the gold standard for diagnosis, is also hampered by intra and inter-observer variability and discrepancy.[1-3]

Cervical cancer is caused by the highly pathogenic human papillomavirus (HPV) strains 16 and 18.[1] One of the consequences of viral genomic integration into the host cell is the upregulation of p16INK4A, a cyclin dependent kinase inhibitor.[1] Therefore, over expression of p16INK4A indicates interference of viral oncoprotein with cellular proteins involved in cell cycle regulation, thus having a role in pathogenesis of cervical intraepithelial neoplasia and cervical cancer. This implies that the detection of p16INK4A may be used as a surrogate marker of integration of high risk HPV into cervical epithelium and transition to malignant transformation.[1-4]

The aim of this study is to assess utility of p16INK4A immunopositivity and evaluate the usefulness of this biomarker in the diagnosis of cervical intraepithelial neoplasia and cervical cancer.

Materials and Methods

Cervical specimens, either hysterectomy or diagnostic biopsy of uterine were received for histopathological examination at the Department of Pathology, Mysore Medical College and Research Institute.

On arrival to the surgical pathology wing of the department, the specimens were adequately fixed in 10% neutral buffer formalin. Based on the routine protocol for hysterectomy specimen/ cervical biopsies, the specimens were grossed.

The biopsy specimens were subjected to routine processing of paraffin embedding procedure. 4-5 micron thick sections were cut from paraffin embedded blocks and stained with haematoxylin and eosin (H&E) stain.

Sections stained with H&E were reviewed and slides showing features of dysplasia and/or neoplasia were classified according to the criteria of the World Health Organisation (WHO) into Low Grade Squamous Intraepithelial Lesion (LSIL), High Grade Squamous Intraepithelial Lesion (HSIL) and Invasive Squamous Cell Carcinoma (SCC).

According to the manufacturer's instructions, 65 slides diagnosed as LSIL, HSIL and SCC, were immunohistochemically stained for p16INK4A using the Biogenex Life systems Histology Kit.

Also, since it is established that normal cervix will have negative p16INK4A expression, 20 random normal cervical biopsies were also immunohistochemically stained for p16INK4A and studied to confirm the negative association.

Slides with the following diagnoses were considered as normal cervix:

a) Squamous metaplasia
b) Cellular evidence suggestive of HPV changes
c) c)Inflammatory/chronic cervicitides
d) Reserve cell hyperplasia
e) Micro glandular hyperplasia

Interpretation of P16INK4A Staining

All sections that showed either strong nuclear and/or cytoplasmic staining were considered positive and graded qualitatively according to the following arbitrary scale:

- 0 (no positive staining)
- 1 (<10% positive staining)
- 2 (>10% but <50% positive staining)
- 3 (>50% positive staining) (refer Table 1)

Statistical Methods Applied

The collected data was entered in excel sheets and analysed using the following statistical methods:

1. Descriptive statistics
2. Chi-Square test
3. Cross tabs (contingency coefficient test)

The *p* value of <0.05 was considered statistically significant.

All the statistical calculations were done using SPSS software for Windows version 16.0.

Inclusion Criteria

All the biopsies of uterine cervix forwarded by the Gynaecology unit of Cheluvamba Hospital to the Department of Pathology, Mysore Medical College & Research Institute, were subjected to standard grossing and H&E staining procedures.

Only slides diagnosed as LSIL, HSIL or SCC of uterine cervix on histopathological examination were taken up for p16INK4A immuno-histochemical study.

Exclusion Criteria

Sections that showed the following were excluded from the immuno-histochemistry study

a) Negative for LSIL, HSIL or SCC
b) Showed extensive tissue necrosis and/or
c) Contained inadequate viable tissue on histopathological examination.

Results

65 cases on histopathological examination showed that 23 (35.4%) cases were LSIL, 8 (12.3%) were HSIL and 34 (52.3%) cases were SCC of uterine cervix. 20 random normal cervical biopsies were also immunohistochemically stained for p16INK4A and studied to confirm the negative association.(Fig 1,2).

Among the 23 cases of LSIL, 20 (87%) cases showed grade 1 staining and 3 (13%) cases showed grade 2 staining.(Fig 3,4) 8 cases of HSIL were studied among which 1 (12.5%) case showed grade 2 staining and 7 (87.5%) cases showed grade 3 staining.(Fig 5,6) 34 cases of SCC were studied in which 3 (8.8%) cases showed grade 2 staining and 31 (91.2%) cases showed grade 3 staining.(Fig 7,8). Overall, grade 1 staining was observed in 30.8% of cases, grade 2 staining in 10.8% and grade 3 staining in 58.5% of cases. Analysis of values in the table showed that an association is observed between H&E and IHC grading where contingency coefficient of 0.685 was found to be highly significant (p = 0.000). This implies that LSIL was significantly associated with IHC grade 1, whereas HSIL and SCC were significantly associated with IHC grade 3. All 20 (100%) cases of chronic cervicitis showed grade 0 staining on immunohisto-chemistry. (Table 1,2)

Table 1. Grading system for p16INK4A staining

STAINING	GRADE
No positive staining	0
Less than 10% positive staining	1
10-50% positive staining	2
More than 50% positive staining	3

Table 2. Comparison of histopathological diagnosis with grade of immunostaining.

	0	1	2	3
NORMAL (n=20)	20(100%)	-	-	-
LSIL (n=23)	-	20(86.9%)	3(13.1%)	-
HSIL (n=8)	-	-	1(12.5%)	7(87.5%)
SCC (n=34)	-	-	3(8.9%)	31(91.1%)

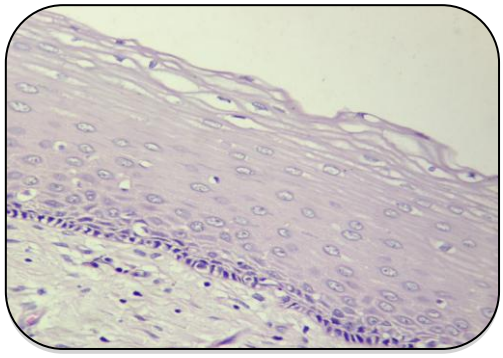

Figure 1: Normal cervix, H&E,400X

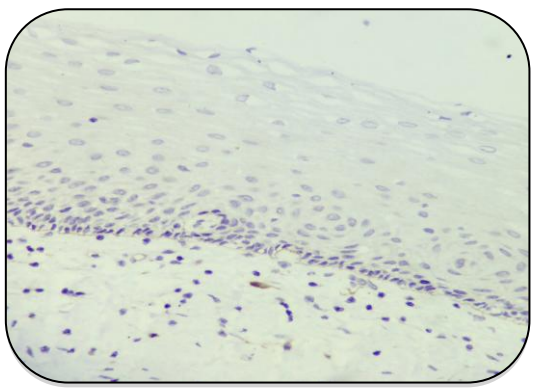

Figure 2: Normal cervix, p16INK4A Immunostaining grade 0, 400X

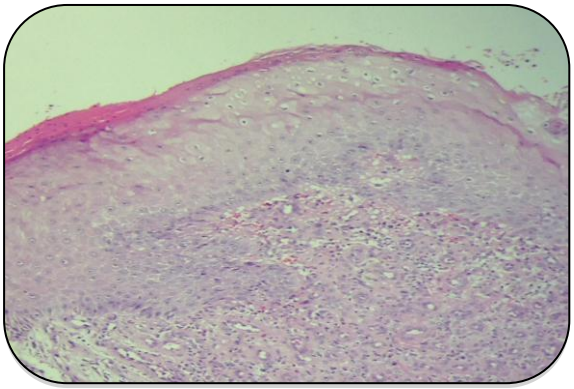

Figure 3: LSIL, H&E, 100X

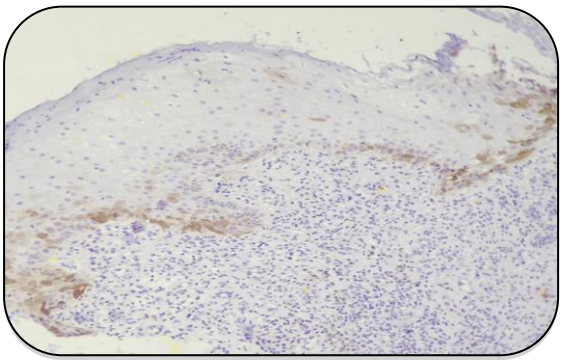

Figure 4: LSIL, p16INK4A Immunostaining grade 1, 100X

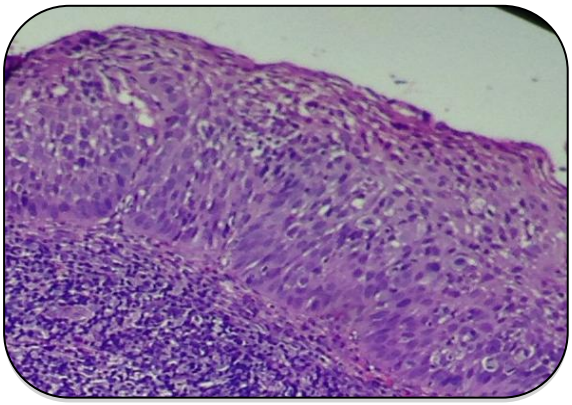

Figure 5: HSIL, H&E, 400X

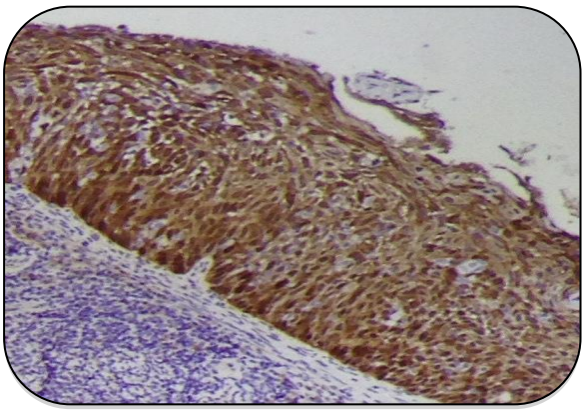

Figure 6: HSIL, p16INK4A Immunostaining grade 3,400X

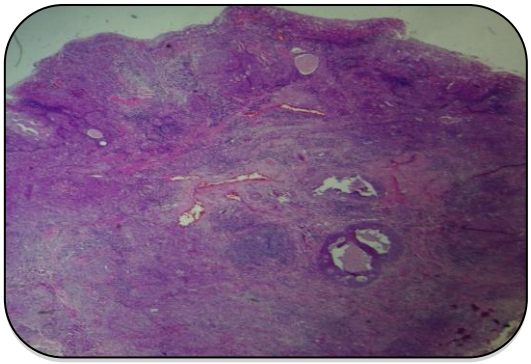

Figure 7: SCC, H&E, 40X

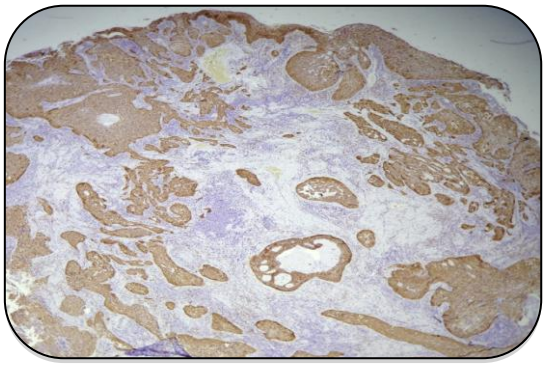

Figure 8: SCC, p16INK4A Immunostaining grade 3, 40X

Discussion

The present study was undertaken to study the over expression of p16INK4A in cervical intraepithelial neoplasia and cervical cancer. This was done to establish whether p16INK4A over expression could be used as a marker of dysplastic/neoplastic alteration in the cervical epithelium.

p16INK4A is a cellular correlate of the increased expression of oncogenic E6/E7 mRNA. Several properties of p16INK4A make this protein a promising biomarker for HPV-related cancers. The expression is directly linked to the HPV oncogene action because continuous expression of E7 is necessary to maintain a malignant phenotype in HPV associated cancer. The expression of p16INK4A seems to be independent of the HPV type causing the oncogenic infection, obviating the need to detect different HPV types in DNA and RNA assays. Also, in contrast to many classic tumour markers such as ki-67 or MYC, p16INK4A is not associated with proliferation, but rather with senescence and cell cycle arrest. It

is not expressed in normal basal cells or in other cells with proliferative capacity.[5]

Several studies have shown that normal cervical epithelium does not express p16INK4A. However, there is upregulation of p16INK4A in SILs and cervical cancers. In the present study, p16INK4A immunopositivity was evaluated in 65 cervical biopsy/hysterectomy specimens. Additionally, 20 random normal cervical biopsy/ hysterectomy specimens were studied to test the negative association that has been established in theory. p16INK4A immunostaining was done using Mouse Monoclonal Antibody (Biogenex Lifesystems Histology Kit) in all the study groups. Immunopositivity was considered when there was either nuclear and/or cytoplasmic staining of tumour cells. Immunohistochemistry should show nuclear staining because p16INK4A is basically a nuclear protein. However, in severe dysplasia and neoplasia, both nuclear and cytoplasmic staining with p16INK4A were observed possibly because of post transcription modification or overproduction of p16INK4A protein, forcing its transfer into the cytoplasm.[6-7]

Normal Cervix

In the present study, all 20 (100%) cases diagnosed as chronic cervicitis, showed p16INK4A grade 0 immunostaining. Results of the present study is consistent with Klaes et al (2001)[2], Murphy et al (2003)[3], Redman et al (2008)[8], Srivatsava (2010)[7] and Han et al (2011)[9], all of whose studies showed 0% p16INK4A immunopositivity in normal cervix. However, Liu Wang et al (2004)[10] showed 1/8 (12.5%), Bolana et al[11] (2007) showed 1/13 (7.6%), Lee et al (2012)[12] showed 16/60 (26%) and Kumari (2013)[1] showed 4/16 (25%) immunopositivity among the non-neoplastic cases.

In the study done by Lee et al, 16/60 (26%) cases were p16INK4A immunopositive. In their study, 14 cases showed p16INK4A grade 1 immunopositivity and 2 cases showed grade 2 immunopositivity. Following the reassessment of the histologic sections in combination with the Ki-67 labeling. However, the above cases were interpreted as an immature squamous metaplasia or a borderline condyloma-like lesion.[12]

In the study done by Kumari, 4 cases of chronic cervicitis showed p16INK4A immunopositivity. They re-reviewed the H&E stained slides and these 4 cases were reclassified to be either LSIL or HSIL. In these cases, the histopathological findings were so subtle that they were only picked up by p16INK4A immunostaining. This highlights the importance of employing the p16INK4A marker for identifying dysplasia in cases of chronic cervicitis.[1]

Low Grade Squamous Intraepithelial Neoplasia

In the present study 23/23 (100%), LSIL cases were p16INK4A immunopositive. These results are consistent with that of Klaes et al[2] (2001) who observed 40/40 (100%) and Murphy et al[3] (2003) whose study yielded 38/38 (100%) rates of immunopositivity. On the contrary, study by Hu et al[13] (2005) showed 40/45 (88%), Zhang[14] (2006) et al showed 81/157 (51%), Nam et al[15] (2008) showed 2/12 (16.6%), Ordi et al[16] (2010) showed 56/86 (65.8%), Balan[17] (2011) et al showed 81/157 (51%), Mood et al[18] (2012) showed 2/12 (16.6%) and Son et al[19] (2013) showed 56/86 (65.8%) rates of immunopositivity.

Study of Hu et al showed 40/45 (88%) rate of immunopositivity. He explained that HPV infection among p16INK4A negative LSIL cases was transient and that viral DNA was not integrated into the host cells. Hence, p16INK4A immunopositivity was absent in these cases.[13]

In the study done by Nam et al, 2/12 (16.6%) cases were p16INK4A immunopositive. He states that a possible reason for some significant lower rates of p16INK4A immunopositivity in low-grade lesions may be because a certain percentage

are thought to be caused by low-risk HPV types. There would not be over expression of P16INK4A if the affinity of the E7 protein of low-risk HPV for Rb was much lower than that of high-risk HPV types.[15]

High Grade Squamous Intraepithelial Neoplasia

Present study showed 8/8 (100%) immunopositivity in HSIL cases. Results of the present study is consistent with that of Klaes et al[2] (2001) who observed 92/92 (100%) and Bolana et al[11] (2003) whose study yielded 35/35 (100%) rates of immunopositivity. On the contrary, Murphy et al[3] (2003) showed 78/79 (98.7%), Agoff et al[20] (2003) showed 52/56 (92.8%), Guimaraes et al[21] (2005) showed 13/18 (86.6%), Zhang et al[14] (2006) showed 101/135 (74.8%), Cheah[22] (2012) et al showed 24/27 (88.9%), Mood et al[18] (2012) showed 10/11 (90.9%) and Wei et al[23] (2013) showed 26/36 (72.7%) rates of immunopositivity.[12]

In the study by Guimaraes et al, 13/18 (86.6%) cases were p16INK4A immunopositive. He explained that many studies have also reported a significant association between p16INK4A overexpression and HSIL related to high-risk HPV types. It is possible that not all HPV types classified as high-risk possess the same potential for the cell cycle disruption or altered gene expression that leads to p16INK4A upregulation. Thus, these results highlight the possible potential of p16INK4A as a marker for type-specific HPV-related HSIL and cervical cancer progression.[21]

Squamous Cell Carcinoma

In the present study, 23/23 (100%) cases of SCC were p16INK4A immunopositive. These results are consistent with that of Srivatsava[7] (2010) who observed 15/15 (100%), Kumari et al[1] (2013) whose study yielded 16/16 (100%) and Wei et al[23], who showed 25/25 (100%) rates of immunopositivity. On the contrary, Klaes et al[2] (2001) showed 52/53 (98.1%), Volgareva[24] (2004) et al showed 20/21 (95.2%), Lesnikova et al[25] (2009) showed 131/133 (98.4%), Tan et al[26] (2010) showed 70/71 (98.5%), Cheah[22] (2012) et al showed 46/53 (86.8%) and Mood et al (2012) showed 18/20 (90%) rates of immunopositivity.

Volgareva et al, stated that p16INK4A-negative SCC had been detected. Substantial variability of the data may be due to small numbers of samples analysed to utilisation of different types of monoclonal antibodies and to different criteria used by different research groups for the results interpretation.[24]

Tan et al[26], observed that nearly all (98.6%) SCC cases showed p16INK4A over- expression. However, a few patients with cervical cancer had p16INK4A negativity. Other studies too have showed that a proportion of their cervical cancer cases had neither HPV infection nor P16INK4A expression. The possible explanation for the absence of p16INK4A expression in these lesions could be methylation of the p16INK4A promoter resulting in silencing of the gene.

The present study has adapted the immune-histochemical grading system which was used by Murphy et al[3]. Results of p16INK4A over expression in both studies were mostly similar.

In the study by Murphy et al[3], p16INK4A overexpression in all 21(100%) cases of normal cervical tissue showed grade 0 immunostaining. In 38 cases of LSIL, 3 (7.8%) cases showed grade 1 staining, 11 (28.9%) cases showed grade 2 staining and 24 (63.1%) cases showed grade 3 staining. 79 cases of HSIL were studied in which 1 (1.2%) case showed grade 0 staining, 12 (15.1%) cases showed grade 1 staining, 23 (29.1%) showed grade 2 staining and 43 (54.4%) cases showed grade 3 staining. 10 cases of SCC were studied in which 10 (100%) cases showed grade 3 staining.

In normal cervical tissues examined, all normal epithelia, metaplastic, endocervical, reactive and inflammatory regions were not stained with the monoclonal anti-p16INK4A antibody. In addition, all normal regions adjacent to SIL lesions showed no detectable p16INK4A expression. Murphy et al, could not ascertain as to why 1 case of HSIL did not show p16INK4A immunostaining. The grades of p16INK4A immunopositivity progressively increased from LSIL to HSIL and later to SCC in both studies. All SCC cases examined exhibited strong over expression of the p16INK4A protein. Although a small number of LSIL cases showed exclusive nuclear staining, interestingly, the remaining LSIL, HSIL and invasive cancer cases showed a combination of nuclear and cytoplasmic staining. The presence of p16INK4A in the cytoplasm may result from a type of post-transcriptional modification or, more simply, overproduction of the protein may force its transfer into the cytoplasm.

The present study establishes that p16INK4A over expression was restricted to LSIL, HSIL and SCC of cervix. No detectable p16INK4A over expression was observed in normal cervical epithelium. The rates of immunopositivity increased from normal cervical epithelia to dysplasia of varying severity and to carcinoma. These findings clearly support previous studies in confirming that p16INK4A is indeed over expressed in dysplastic and neoplastic cells of the uterine cervix.[3]

References

1. Kumari K, Arcot AV. p16INK4A expression in cervical intraepithelial neoplasia and cervical cancer. Brunei Int Med J. 2013;9(3):165-171.
2. Klaes R, Friedrich T, Spitkovsky D, et al. Overexpression of p16INK4A as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int J of Cancer. 2001;92:276–284.
3. Murphy N, Ring M, Killalea AG, et al. p16INK4A as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and thin preparation of smears. J Cln Pathol. 2003;56:56-63.
4. Cheah P, Looi L, Teoh K, Mun K, Nazarina AR. p16INK4A is a useful marker of Human Papillomavirus integration allowing risk stratification for cervical malignancies. APJCP. 2012;13:469-472.
5. Kate Cuschieri, Nicolas Wentzensen. Human Papillomavirus mRNA and p16 Detection as biomarkers for the improved diagnosis of cervical neoplasia. Cancer Epidemiol Biomarkers Prev. 2008;17:2536-2545.
6. Raluca Balan. Immunohistochemical assessment of p16, COX-2 and EGFR in HPV-positive cervical squamous intraepithelial lesions. Rom J Morphol Embryol. 2011;52(4):1187–1194.
7. Supriya Srivastava. P16INK4a and MIB-1: An immunohistochemical expression in preneoplasia and neoplasia of the cervix. Indian Journal of Pathology and Microbiology 2010 Jul-Sep;53(3):518-24.
8. Rachel Redman. The Utility of P16INK4a in Discriminating Between Cervical Intraepithelial Neoplasia 1 and Nonneoplastic Equivocal Lesions of the Cervix. Arch Pathol Lab Med 2008 May;132.
9. Han. Decreased D2-40 and increased P16INK4a immunoreactivities correlate with higher grade of cervical intraepithelial neoplasia. Diagnostic Pathology 2011; 6:59.
10. Jian-Liu Wang, Bi-Ying Zheng, Xi-Dan Li. Predictive Significance of the Alterations of P16INK4a, P14ARF, p53, and Proliferating Cell Nuclear Antigen Expression in the Progression of Cervical Cancer. Clin Cancer Res 2004; 10:2407-14.
11. Ines Krivak Bolana, Sreko Ciglar. Short communication Evaluation of P16INK4a in Cervical Lesion of Premenopausal and

Postmenopausal Women. Coll Antropol 2007;31(2):107–11.
12. Sangho Lee, Hyunchul Kim, Hyesun Kim, Chulhwan Kim, Insun Kim. The utility of P16INK4a and Ki-67 as a conjunctive tool in uterine cervical lesions. Korean J Pathol. 2012;46:253-260.
13. Lulin Hu. Human papillomavirus genotyping and P16INK4a expression in cervical intraepithelial neoplasia of adolescents. Modern Pathology 2005; 18:267-73.
14. Qi Zhang. Impact of utilizing P16INK4a immunohistochemistry on estimated performance of three cervical cancer screening tests. Int J Cancer 2006; 120:351–6.
15. Eun Ji Nam. Expression of the P16INK4a and Ki-67 in relation to the grade of cervical intraepithelial neoplasia and high-risk human papillomavirus infection. J Gynecol Oncol 2008 Sep;19(3):162-8.
16. Jaume Ordi. International Society of Gynecological Pathologists, P16INK4a Immunostaining Identifies Occult CIN Lesions in HPV-positive Women. International Journal of Gynecological Pathology 2008;28:90–7.
17. Raluca Balan. Immunohistochemical assessment of p16, COX-2 and EGFR in HPV-positive cervical squamous intraepithelial lesions. Rom J Morphol Embryol 2011;52(4):1187–94.
18. Izadi-Mood N, Asadi K, Shojaei H, Sarmadi S, Ahmadi SA, Sanii S, et al. Potential diagnostic value of P16 expression in premalignant and malignant cervical lesions. J Res Med Sci 2012;17(5):428-33.
19. Seung-Myoung Son. Evaluation of P16INK4a, pRb, p53 and Ki-67 expression in cervical squamous neoplasia. P16INK4a Expression Correlates with Degree of Cervical Neoplasia: A Comparison with Ki-67 Expression and Detection of High-Risk HPV Types. J Biomed Res 2012;13(3):209-17.
20. Nicholas Agoff S. P16INK4a expression correlates with degree of cervical neoplasia: a comparison with Ki-67 expression and detection of high-risk HPV types. Mod Pathol. 2003;16 (7):665–673.
21. Márcia CM Guimarães. Immunohistoc-hemical expression of P16INK4a and bcl-2 according to HPV type and to the progression of cervical squamous intraepithelial lesions. J Histochem Cytochem. 2005;53(4):509–516.
22. Cheah P, Looi L, Teoh K, Mun K, Nazarina AR. P16INK4a is a useful marker of Human Papillomavirus integration allowing Risk Stratification for cervical malignancies. Asian Pacific Journal of Cancer 2012;13,469-72.
23. Qingzhu Wei. Combined detection of P16INK4a and IMP3 increase the concordance rate between cervical cytologic and histologic diagnosis. Int J Clin Exp Pathol 2013;6(8):1549-57.
24. Volgareva G, Zavalishina L, Andreeva Y. p16 as a marker of dysplastic and neoplastic alteration in cervical epithelial cells. BMC Cancer. 2004;4:58.
25. Iana Lesnikova, Marianne Lidang, Stephen Hamilton-Dutoit, Jørn Koch. p16 as a diagnostic marker of cervical neoplasia: a tissue microarray study of 796 archival specimens. Diagnostic Pathology 2009;4:22.
26. Tan. Immunohistochemical study of P16INK4a and surviving expressions in cervical squamous neoplasm. Indian J Pathol Microbiol. 2010 ;53(1).