Improving Early Diagnosis Of Cervical Cancer Lesions Using p16INK4a Biomarkers On Cellblocks From Cervical Smears

*Martha W. M[1], Kyama M.C[2], Kibet P.S[2]

1. *Jomo Kenyatta University of Agriculture and Technology, Department of Medical Laboratory Sciences.*
2. *University of Nairobi Department of Pathology*
3. *Kenyatta National Hospital*

Corresponding Author: Martha W. Maina, P.O Box 55623-00200 Nairobi, Kenya: E-Mail: mainamartha79@gmail.com

Summary

INTRODUCTION

Globally, cervical cancer is the second most regular malignant growth in females after breast cancer. Timely recognition of *premalignant* lesions is an essential segment in the decrease of related morbidity and mortality. Though Pap -Smear test has been a helpful screening device despite hampered by high intra and interobserver changeability, false negatives at 20– 30% and false positives at 5– 70%.

OBJECTIVE

The study was to determine the role of p16INK4a biomarker in the identification of low grade *Squamous Intraepithelial* lesions in cellblocks prepared from Pap smears and subsequently compared with previous Pap smear results with *Colposcopy* and Cellblock results.

METHODOLOGY

This was a laboratory-based, prospective study with a parallel comparative arm at Kenyatta National Hospital Reproductive health clinic (66). All patients who had abnormal Pap smear reports and referred for Colposcopy, and consented for the study were enrolled. A smear was taken just before taking a Colposcopy biopsy. The cytobrush was immediately put in Acid alcohol fixative centrifuged and deposits wrapped in a filter paper and processed histologically to form a cellblock. Colposcopy biopsies were then retrieved from KNH histology Lab and both samples subjected to Routine histological stain and eventually with biomarker p16. Total of 85 samples was collected.

RESULTS

There was a significant level of agreement between Pap smears and cellblock findings on the routine Histological stain. Of the 58 cases analyzed Colposcopy had (39%) 27 negatives and (45%) 31 positives while cellblock had (48%) 33 negative and (36%) 25 positive for pre and malignancy with a confidence interval of 0.016 as the margin of error. Biomarker Colposcopy had (43%) 30 negativity and (41%) 28 positivity while cellblock had negativity of (46%) 32 and positivity of (38 %) 26

CONCLUSION

Poor inter-rater agreement resulting to mortality and morbidity associated with false positives and false negatives, cellblock prepared from residues of cytobrush stained with *Haematoxyline* and *Eosin* and biomarker is likely to circumvent all the above, together with minimizing loss to follow up as patients only visit health facility once and they acquire all the results without resampling hence drastically reducing the cost of colposcopy, which require highly specialized equipment and experienced personnel who are very few and difficult to find.

[***Afr. J.*** Health Sci. 2019; 32(2): 39 - 48]

Introduction

Worldwide, cervical *malignancy* is the second most regular disease in ladies after breast cancer. Opportune location of *premalignant* sores is a critical part to decrease the related morbidity and mortality [2].

Pap test has been a helpful "screening tool however hampered by high inter and intra-observer fluctuation". This has brought about high false negative and false positive rates that extend between 20– 30% and 5– 70% respectively [3, 4].

Specificity and sensitivity in Pap smear for HSIL using LBC have not been shown to improve though. Colposcopic cervical biopsies are "considered as the gold standard, though hampered by intra- and inter-observer variability" [5, 6].

HPV testing is productively incorporated into the essential screening, either as an assistant to cytology or as a sole essential test. In any case, it fails in the triage of low-grade lesions, and regardless of whether actualized as an essential screening test, it is "important to have a more disease explicit triage marker to distinguish women that would need to undergo colposcopy"[7].

Moreover, a solitary HPV DNA test can affirm infection by the virus, present in 99.7% of every cervical malignancy; it doesn't segregate among transient and unending disease [8].

Performing IHC on cervical smears could evade this impediment [9], circumvent invasive interventions and avert possibly increased morbidities by using target choices at the negligibly intrusive cervical cytology stage.

The epidemiology of cervical cancer, just as experimental examinations, demonstrates that the development of cervical cancer predetermined by various etiological factors but the infection with the human papillomavirus (HPV) is the utmost factor towards the development of the disease [10]

HPV DNA is available in about 95% of *Squamous Cell Carcinomas* while in *Adenocarcinoma* of the cervix, has been recognized in just about 70% to 90% [10]. Because of population-based Pap smear screening, the frequency of Squamous Cell Carcinoma has reduced remarkably in most developed and some developing nations, while amid a similar period, there has been a relative increment in the rate of adenocarcinoma among women. A few investigations have detailed this escalation, particularly in young women [7].

The development of *adenocarcinomas* cannot be effectively prevented by cytological screening alone. The various grades of *dysplasia* can be used to distinguish the precancerous phases of invasive cervical carcinoma and characterize the stages of development. It is proposed that around "12% of all carcinomas in-situ, when left untreated, advanced into obtrusive malignancy after 13 years" [11, 12].

The "history of cervical intraepithelial neoplasia (CIN) is extremely variable" and unpredictable when left untreated, CIN may revert to normal, either persist or eventually advance to intrusive cervical malignant growth. The investigation of those that will progress or relapse has critical clinical value[13]

p16INK4a, a tumor silencer gene, is a component of the p16INK4a/cyclinD - CDK4/6/Rb pathway[14]. It can initiate G1 cell cycle capture by repressing the phosphorylation of pRb by CDK4 and CDK6[12]

Recent investigations have demonstrated that immunohistochemical staining techniques with p16INK4a is a promising marker for dysplastic and cancerous cervical epithelia [15].

Different examinations have likewise shown that overexpression of p16INK4a aids the recognition of high-hazard HPV-related cervical squamous lesion [16].

It was suggested that p16INK4a is valuable for recognizing immature *Squamous Metaplasia* which forms a high-grade *Squamous Intraepithelial* lesion, where the previous could act like a morphologic differential diagnosis [17].

p16INK4a immunostain is used as a marker for identifying dysplastic lesions caused by HR HPV and in distinguishing non-HPV-related changes, such as *squamous metaplasia,* inflammatory conditions or reactive conditions that either mask or mimic dysplasia in the cervical biopsy specimen, from HPV-related changes like CIN. p16INK4a has recently been described as a surrogate marker for HR-HPV.

Associated squamous and glandular intraepithelial lesions of the cervix. It has been also demonstrated that the immunohistochemical staining pattern of p16INK4a is different in high-grade intraepithelial neoplasia of the uterus (CIN2 and 3), where it diffuse, from sporadic or focal staining patterns in CIN1 [18, 19].

p16INK4a immunostaining allow precise identification of even small C1N or cervical cancer lesions in biopsy sections and helped to reduce interobserver variation in the *histopathologic* interpretation of cervical biopsy specimens [22].

Thus, p16 IHC can reduce false-negative and false-positive biopsy interpretation and thereby significantly improve cervical (pre)-cancer diagnosis, and also p16INK4a negative CIN1 may benefit from a less intensive follow-up as they rarely progress to high grade.

One of the significant disadvantages of the current existing screening tests is their inability to discriminate between lesions that will progress and those that do not [20].

To surmount the current tests limitations, a test is required that indicates that an oncogenic HPV virus has already enhanced genetic instability and rendered infected cells susceptible to transformation, that leads to the development of cancer, which is, a test for HR HPV that indicates the virus will exert its oncogenic potential in that particular woman [12]

A number of studies have focused on the utility of p16INK4a immuno-staining as a marker of identifying dysplastic lesions caused by HR HPV and also in distinguishing non-HPV-related changes, such as *squamous metaplasia,* inflammatory conditions or reactive conditions that either mask or mimic dysplasia in cervical biopsy specimen; from HPV-related changes like CIN1 [21].

p16INK4a has recently been described as a surrogate marker for HR-HPV associated squamous and glandular intraepithelial lesions of the cervix. It has been also demonstrated that the immunohistochemical staining pattern of p16INK4a is different in high-grade intraepithelial neoplasia of the cervix (CIN2 and 3), where it is diffuse, from sporadic or focal staining patterns in CIN1 [22]

According to Klaes et al., there was a significantly better agreement in the interpretation of p16INK4a expression which was restricted to CIN2 / CIN3, CIN1 associated with high-risk *H*uman *Papillomavirus,* or cervical cancer. p16INK4a immunostaining allowed precise identification of even small CIN or cervical cancer lesions in biopsy sections and helped to reduce interobserver variation in the *histopathologic* interpretation of cervical biopsy specimens [36].

Thus, p16IHC can reduce false-negative and false-positive biopsy interpretation and thereby significantly improve cervical (pre)-cancer diagnosis and also p16INK4a negative CIN1may benefit from a less intensive following up as they rarely progress to high grade [19].

AIM

To determine the role of p16INK4a in early diagnosis of cervical cancer from cervical lesions using cellblocks in patients referred for colposcopic biopsy

Methodology

Study Site

The study was carried out at KNH 's reproductive health unit clinic (clinic 66). This clinic deals with specialized gynaecological related complications

Study Design

This was a laboratory-based, prospective study with a parallel comparative arm.

Data Collection Methodology

Patients whose Pap smear reports had been recommended for colposcopy were recruited for the study. Those who consented had smear samples taken using a cytobrush. The cytobrush was put in a labelled centrifuge tube containing AA fixative and cocked ready for transportation to the laboratory.

They were then centrifuged, supernatant discarded and the brush discarded. 10ml of the fixative was added to each of the deposits centrifuged and the supernatant discarded. This procedure was repeated 2 times. 10% formalin was added and left to stand for 4 hrs. The deposits were picked using forceps wrapped in a filter paper and processed in a tissue processor, embedded and cellblocks prepared.

The corresponding colposcopic biopsies were retrieved from Histopathology laboratory at Kenyatta National Hospital and both the cellblocks and Colposcopy biopsies were subjected to the routine histological tests (H & E) and the P16 biomarker tests. The results were interpreted by the PI together with the pathologist and results recorded.

Results

Samples were collected from a study population (N) of 69 for analysis out of which a sample population (n) of 58 met the inclusion criteria. The sampling procedure which was based on the previous Pap smear test results were included in this study. The Pap smear specimens had either HSIL, LSIL, ASCUS AGUS

Table 1: *The Overall Distribution Of The Tests*

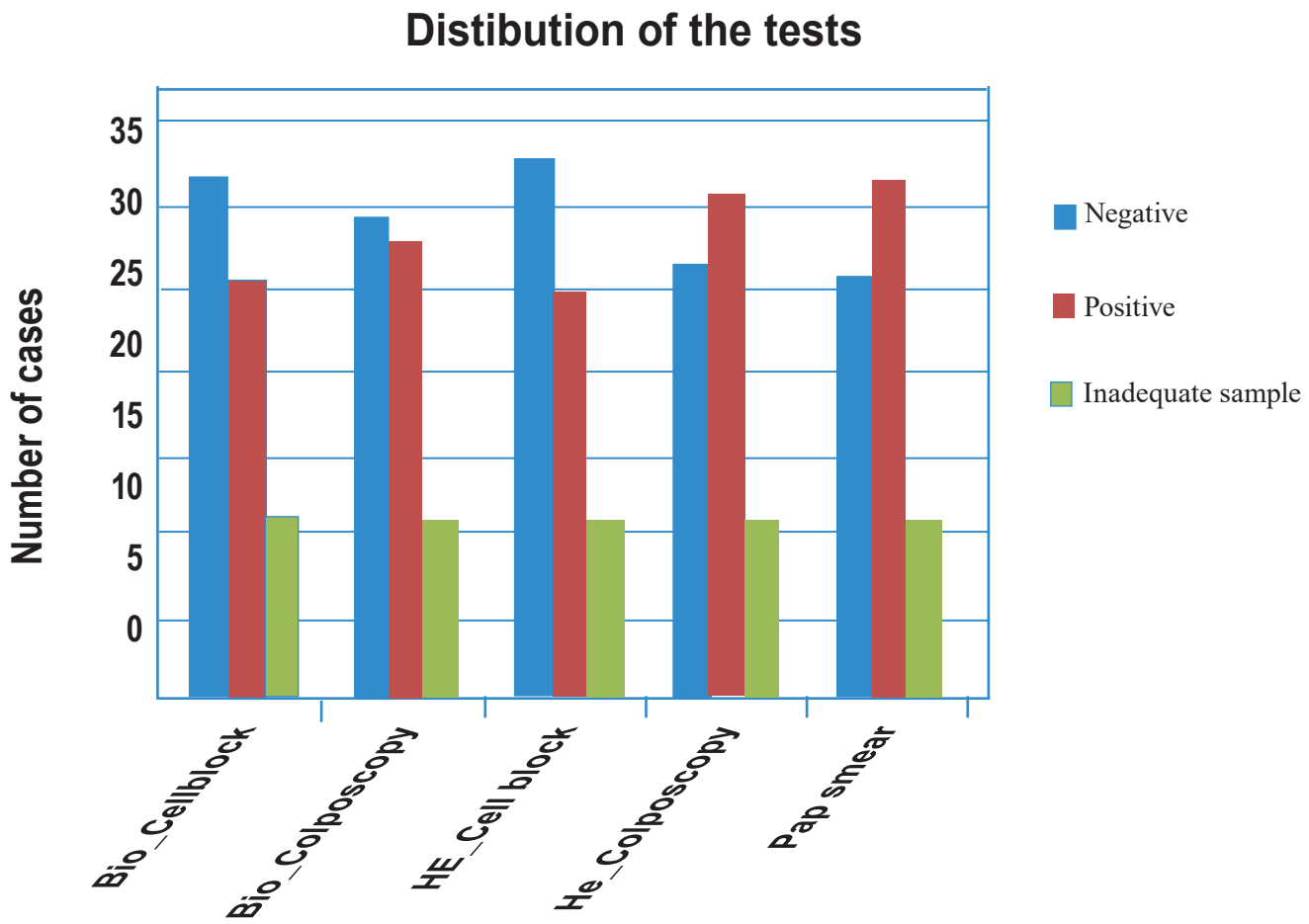
	Negative	Positive
Biomarker cellblock	32	26
Biomarker colposcopy	30	28
HE_cellblock	33	25
HE_colposcopy	27	31
Pap smear	26	32

From ***Table 1****:* 58 of 69 cases analyzed Biomarker Colposcopy had (43%) 30 negativity and (41%) 28 positivity while Cellblock had negativity of (46%) 32 and positivity of (38 %) 26 Colposcopy had (39%) 27 negatives and (45%) 31 positives while Cellblock had (48%) 33 negative and (36%) 25 positive for pre and *malignancy.*

Table 2:

Cellblock		**Colposcopy**	
Positive		***Positive***	
Lsil	11	**CIN1**	16
Hsil	9	**CIN2**	10
Ascus	5	**CIN3**	5
Total	25	**TOTAL**	31

Out of the 58 cases on the routine histological stain (*Haematoxyline* and *Eosin*) carried out, LSIL were 11(18%) of all cases HSIL 9 (15%) ASCUS 5 (8%) on Cellblock and on Colposcopy CIN1 were 16 (28%) cases CIN2 10 (17%), CIN3 5 (8%)



The table for P values

Table 1: *Biomarker Cellblock , Colposcopy and Pap Smear*

	Biomarker Cellblock				
		Negative	**Positive**	**Total**	**P-Values**
Biomarker Colposcopy	Negative	21	9	30	0.018
	Positive	11	17	28	
	Total	32	26	58	
Pap Smear	Negative	18	8	26	0.046
	Positive	14	18	32	
	Total	32	26	58	

Table 2: HE_Cellblock Colposcopy & Pap smear

Haematoxyline & Eosin Cellblock					
		Negative	***Positive***	***Total***	***P-values***
Haematoxyline & Eosin Colposcopy	Negative	19	8	27	0.047
	Positive	14	17	31	
	Total	33	25	58	
Pap Smear	Negative	21	5	26	0.001
	Positive	12	20	32	
	Total	33	25	58	

Specificity and Sensitivity of The Tests

Table 3: Biomarker Cellblock

Biomarker Cellblock		
	Sensitivity	***Specificity***
Biomarker colposcopy	56.7%	60.7%
Pap smear	72%	69.7%

Table 4: HE_Cellblock

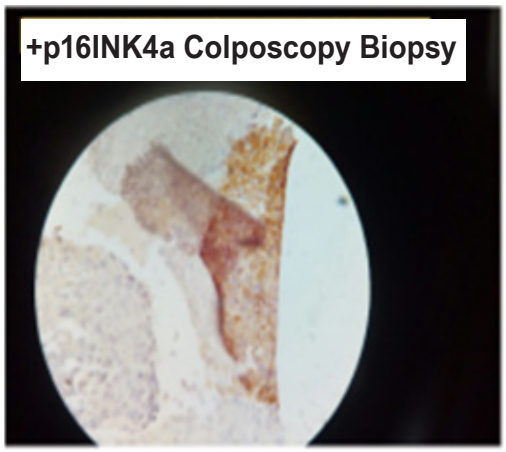
HE cellblock		
	Sensitivity	***Specificity***
HE Colposcopy	51.4%	65.2%
Pap smear	60%	66.7%

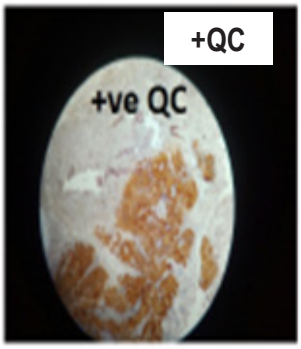
From ***Table 2***: it was observed that there was significance in the Biomarker tests carried on the Cellblock and Colposcopic biopsies having a significance of 0.018 which is below 0.05 indicating a level of agreement and hence a cellblock could be used in place of colposcopic biopsy.

Similarly for the routine histological stain, there was significance in both the cell block and colposcopic biopsy , Cell block and Pap smear with P values of 0.047 and 0.001 respectively indicating that cell block remains a useful screening and diagnostic tool for early cervical cancer detection.

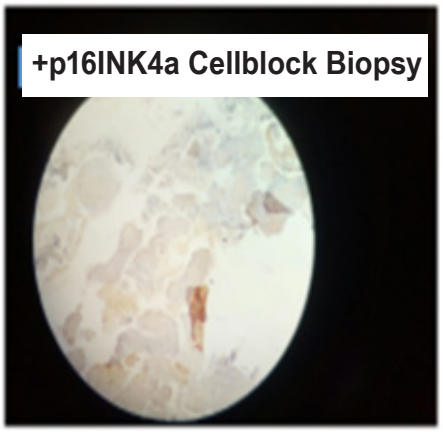
Expression of p16INK4a on Cell Blocks & Colposcopy Biopsies

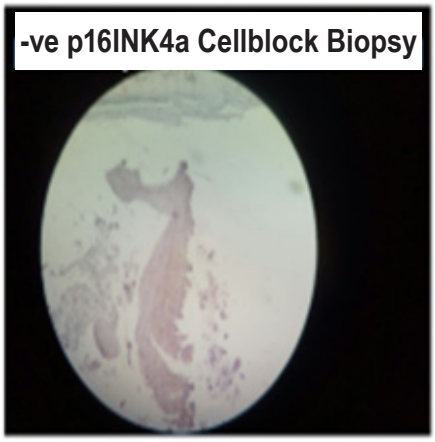
Colposcopy Tissue Biopsy





Cell Block Preparations





Discussion, Conclusion & Recommendation

1.1 Discussion

p16 is a widely used surrogate marker for HPV related cervical dysplasia. It is believed to have a high diagnostic efficacy in the determination of a transient and chronic HPV infection.

The study enrolled 69 participants whose samples were collected out of which 58 met the inclusion criteria, and 11 samples were inadequate to proceed for investigation. The age range of the participants was 21-70 years with a mean age of 43.2029 and the standard deviation of 9.771778.

There was a significant level of agreement between Pap smears and *Cellblock* findings on the routine histological stain.

Of the 58 cases analyzed, Colposcopy had (39%) 27 negatives and (45%) 31 positives while *Cellblock* had (48%) 33 negative and (36%) 25 positive.

Biomarker Colposcopy had (43%) 30 negativity and (41%) 28 positivity while *Cellblock* had negativity of (46%) 32 and positivity of (38 %) 26.

Pap smear compared to biomarker cellblock had a p-value of 0.046 showing a significance agreement and routine histology stain for Cellblock.

Pap smear had a p-value of 0.001. This indicated that a Cellblock can be useful for screening and diagnosis as there was a significant level of agreement between the two tests.

Hence, there will be a minimal loss to follow up and avoid the need for highly specialized and significantly cut the cost of screening equipment currently in use for the gold standard.

The difference in variation of Cellblock on H&E and Biomarker could be as a result of Cellblock preparations sampling techniques, processing of Colposcopic biopsies, degradation of the tissue block and the existing inter and intraobserver variability.

The sensitivity of Colposcopy is at 88.88% as compared to the low sensitivity of Pap test estimated to be 44.44%, the primary reason why colposcopy is regarded as gold test/confirmatory test for cervical cancers [40].

The sensitivity of Colposcopy is known to range from 87% to 99% as compared to the sensitivity of Cellblock ranges from 90% to 94.5%, but the sensitivity can be increased for cyto diagnosis at analytical stages.

Cervical screening, p16 is still an excellent surrogate marker highly useful for detection of *dysplastic* lesions. The cell cycle regulator overexpressed in *Squamous Dysplasia* and Cellblock together with Colposcopy can be used to further diagnostic accuracy of *dysplastic* lesions.

Mitigate inter and intra-observer variation, loss to a follow up, late presentation of patients, unnecessary invasive procedures and misdiagnosis results to mis-management.

The gold standard test for cervical *malignancy,* stained with H&E gives a false impression of a static process that lacks the ability to demonstrate the progression from pre-to *malignancy.*

On contrast cellblock with H&E and BM has the potential to distinguish CIN from other lesions and distinguish chronic from the transient.

Cellblock if well prepared and well sampled could serve as a tool for screening, diagnosing and a confirmatory for pre and *malignant* cervical lesions as it provides multiple sections paving way for additional markers that may be used for further testing and diagnostic markers that would improve on patient's further management.

The p16 expression has been shown to be associated with *Neoplastic Nquamous* and *Glandular Intraepithelial* lesions due to the complex molecular mechanisms, where high-risk HPV transforming proteins interact with cell cycle proteins to generate a futile feedback loop, resulting in p16 overexpression [39].

Diffuse p16 expression in *immunoperoxidase* study can thus serve as a surrogate marker of transformed high-risk HPV-related cervical lesions and represent a significant means for the pathologist to separate lesions requiring colposcopy from those that do not [40].

The single most common cause of an inadequate biopsy specimen is the failure to provide abnormal tissue of sufficient amount and depth[41].

Without the underlying *stroma,* an invasive *neoplasm* is likely to be interpreted as an in situ lesion. On the prognostic significance of p16 especially in LSIL prediction of regression or progression to malignancy.

1.2 Conclusion

The two histological testing techniques; Colposcopy and Cellblock, agree on the variables examined. The methods highly agree when *Hematoxylin*

& Eosin is used as a staining procedure for both colposcopy and cellblock. There was a considerable variation on the number of negative and positive results between the histological assessment using the Pap test as compared to both colposcopy and cellblock techniques.

For cervical cancer screening, p16INK4a is still an excellent surrogate marker useful for the detection of lesion likely to progress to *malignancy.*

The cell cycle regulator p16 INK4a is shown to be overexpressed in *Squamous Dysplasia* and cellblock together with colposcopy techniques can be used to improve further the diagnostic accuracy of ASC-H *Papanicolaou* smears and to reduce unnecessary procedures and over treatment cases.

From the study, it can be concluded that cellblock with *Haematoxylin* and *Eosin* staining and biomarkers p16INK4a is a singnificant tool for the confirmatory test of the cervical *cytological* specimen when examining *pre-malignant* lesions.

Also, the disparity arising from cellblock can be due to deviation from the standardized procedures of cutting surfaces towards achieving excellent cellularity.

Through the cellblock techniques, this study proves to be a confirmatory test for the diagnosis of ASC-H Pap tests. Multiple unstained slides producing adequate cellularity can be obtained from each cell block paving way for additional markers that can increase the diagnostic specificity and sensitivity to be used.

From this study, Colposcopy Technique. Cell block techniques ties with the gold test of colposcopy for cervical screening with some areas to be improved on the cell block.

Based on the study findings, sensitivity ranges for colposcopy and cell block methods for cyto-diagnosis is visible.

In consideration of other aspects such as affordability of the services, loss of follow-ups, equipment required and capacity building, it can be concluded that cell block seems to be a better choice for the Kenyan population.

With colposcopy, personnel needs to go through specialized training. Currently, the test is done by the obstetrician/ gynecologists as compared to Cellblock that can be done by a nurse or medical officer in the facility.

Although the sensitivity, specificity and diagnostic accuracy of cell block vary, through this study, is preferred as compared to colposcopy, Colposcopy is at crossroads due to issues with efficiency, cost and comfort consideration.

Most often, Colposcopy should not be recommended to be done as a random biopsy on patients with normal - appearing cervices.

This means, primary test such as Pap smear must be done first because Coloscopy makes it more unaffordable and inaccessible to many patients. The sensitivity of Colposcopy procedure can only be increased by taking two or more biopsies instead of one as the case of cell block where samples are obtained once.

References

1. **D. M. Parkin,** "Global cancer statistics in the year 2000," *Lancet Oncology. 2001.*

2. **N. P. et al.**, "Efficacy of HPV DNA testing with cytology triage and/or repeat HPV DNA testing in primary cervical cancer screening," *J. Natl. Cancer Inst., 2009.*

3) **L. Pantanowitz, M. Hornish, and R. Goulart,** "The impact of digital imaging in the field of cytopathology," *Cytojournal, 2009.*

4. **C. B. Visioli** *et al.,* "hr-HPV testing in the follow-up of women with cytological abnormalities and negative colposcopy," *Br. J. Cancer, 2013.*

5. **M. Safaeian, D. Solomon**, and **P. E. Castle,** "Cervical Cancer Prevention-Cervical Screening: Science in Evolution," Obstetrics and Gynecology Clinics of North America. 2007.

6. **P. Cristiani** *et al.,* "Rationale and development of an on-line quality assurance programme for colposcopy in a population-based cervical screening setting in Italy," *BMC Health Serv. Res., 2013.*

7. **J. Li, M. J. Poi,** and **M. D. Tsai,** "Regulatory mechanisms of tumor suppressor p16INK4a and their relevance to cancer," *Biochemistry, 2011.*

8. **S. K. Ghosh *et al.,*** "Human papillomavirus testing for suspected cervical cancer patients from Southern Assam by fast-PCR.," Asian Pac. *J. Cancer Prev., 2011.*

9. **V. B. Shidham, R. Mehrotra, G. Varsegi, K. D'Amore, B. Hunt,** and **R. Narayan,** "p16INK4a immunocytochemistry on cellblocks as an adjunct to cervical cytology: Potential reflex testing on specially prepared cellblocks from residual liquid-based cytology specimens," *Cytojournal, 2011.*

10. **E. Burd**, "Human papillomavirus and cervical cancer Burd E (2003) Human papillomavirus and cervical cancer. *Clin. Microbiol Rev* 16: 1–17 Available at: http: // www.sciencedirect.com / science / article / pii / S0140673607614160," *Clin. Microbiol Rev, 2003.*

11. **A. Castanon, R. Landy,** and **P. D. Sasieni,** "Is cervical screening preventing adenocarcinoma and adenosquamous *carcinoma* of the cervix," *Int. J. Cancer, 2016.*

12. **J. L. Wang** *et al.,* "p16INK4a and laminin-5γ2 chain expression during the progression of *cervical neoplasia*," *Acta Oncol. (Madr)., 2006.*

13. **U. K.-E.** *et al.*, "Prediction of spontaneous regression of cervical *intraepithelial neoplasia* lesions grades 2 and 3 by proteomic analysis," *Int. J. Proteomics, 2014.*

14. **S. Shrivastava, D. Barmon, P. Deka**, and **A. Kataki,** "Vaginal vault *carcinoma* as second primary in a treated case of ovarian cancer," *J. Midlife. Health, 2012.*

15. **S. Nishio** *et al.,* "p16INK4a immunohisto-chemistry is a promising biomarker to predict the outcome of low grade cervical *intraepithelial neoplasia:* Comparison study with HPV genotyping," *J. Gynecol. Oncol., 2013.*

16. **R. Klaes et al.,** "p16INK4a immuno-histochemistry improves inter-observer agreement in the diagnosis of cervical intraepithelial neoplasia," *Am. J. Surg. Pathol*., 2002.

17. **H. G. Kaspar and C. P. Crum,** "The utility of immunohistochemistry in the differential diagnosis of gynecologic disorders," *Arch. Pathol. Lab. Med*., 2015.

18. **D. Q. Liu et al.,** "Increased RIPK4 expression is associated with progression and poor prognosis in cervical squamous cell carcinoma patients," Sci. Rep., 2015.

19. **F. S. Aslani, A. Safaei, M. Pourjabali,** and **M. Momtahan,** "Evaluation of Ki67, p16 and CK17 markers in differentiating cervical intraepithelial Neoplasia and benign lesions," *Iranian Journal of Medical Sciences. 2013.*

20. **E. R. Nijhuis** *et al.,* "An overview of innovative techniques to improve cervical cancer screening," *Anal. Cell. Pathol., 2006.*

21. **I. Rufforny, E. J. Wilkinson, R. Redman, N. A. Massoll,** and **C. Liu,** "p16ink4a Is Helpful in Discriminating Between Cervical *Intraepithelial Neoplasia 1* and Equivocal Lesions of the Cervix," *J. Low. Genit. Tract Dis., 2006.*

22. **I. Tsoumpou** *et al.,* "p16INK4 aimmuno-staining in *cytological* and histological specimens from the uterine cervix: A systematic review and meta-analysis," *Cancer Treatment Reviews. 2009.*