Commentary

P16 Biomarker in Cervical Intraepithelial Lesions and Carcinoma: A Review

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Introduction

Cervical cancer is one of the leading cancers in women worldwide with 400,000 estimated new cases and 250,000 deaths occurring every year with highest incidence reported in Eastern Africa in 2012. It is one of the common cancers in females causing mortality and morbidity [1-3]. It is the third most common cancer accounting for 7.8% and fourth cancer related deaths among women worldwide. In developing countries, it constitutes about 6% of cancers in women and is the third most common cause of death among cancer deaths in females. In developed countries, it is the seventh common cause of cancer death. It is second common cancer in women in India after breast cancer in urban population. The average incidence is 25 per 100,000 females. Kalyani et al in their study at a tertiary health care in South India has observed a prevalence of 17% among all female cancers [4-6]. In Sub-Saharan Africa the prevalence is 24% which is highest globally [3].It gives rise to agonizing morbidity and mortality. The increasing incidence of Human Immunodeficiency Virus (HIV) infection associated with Human Papilloma Virus (HPV) infection accelerated the malignant transformation of pre-malignant lesion. However it is a preventable disease and early detection & treatment result in decreasing the mortality and morbidity [7-10]. It is the major killer disease in developing countries including India [8].

Cervical Intraepithelial Neoplasia (CIN) is the early changes in cervical epithelium that undergo malignant transformation. It is classified as CIN1, CIN2 and CIN3. Recent classification of World Health Organization (WHO) for CIN is low grade squamous intraepithelial lesion (LSIL) andhigh grade squamous intraepithelial lesion (HSIL). CIN1 (LSIL) is not a precancerous lesion and needs no treatment. CIN 2/3 (HSIL) is the premalignant lesion and the rate of progression is 10 – 40% [11]. As per the 2012 lower anogenital squamous terminology (LAST) project, the cervical dysplasia in biopsy has been classified into LSIL and HSIL [12]. CIN 1-3 causes alteration of cellular proteins at different levels that can be analyzed at every step of

progression of cancer. CIN2/3 indicates the HPV persistence and integration into cellular genomic DNA [8,13].

Cervical cancer has a long pre-cancerous period that provides an opportunity for screening and treatment that can be done before it progresses to frank cancer. Therefore improved screening tests help in early diagnosis [10]. The screening of cervical cancer by Pap test was started in the year 1940 and it took 30 long years to be implemented. The cervical cancer screening by Pap test has decreased incidence of cervical cancer by 70% especially in developed countries having programmed cervical cancer screening. However cervical cancer still persists in adequately screened population because of false negative rates which are due to variable sampling technique, inter and intra-observer variability and misinterpretation of abnormal squamous cells giving rise to false assurance to the patients. False positive results are due to misinterpretation of reserve cell hyperplasia, atypical immature metaplasia and inflammatory atypia giving rise to unnecessary investigation, over treatment or surgical intervention which will be an unnecessary burden and cost to the patients [2,5,7,13]. In Pap test, false negative rate is 20-30% and false positive rate is 5-70%. These leads to the requirement of an additional sensitive and specific biomarker to improve screening program and quality control in histlological diagnosis [2].

Certain biomarkers are used to decrease the false positive, false negative rates and increase Positive Predictive value (PPV) which has a role in cell cycle in signal transduction, DNA replication and cell proliferation. Altered expression of biomarkers is due to HPV E6 and E7 protein which causes deregulation of cell cycle. Significant higher agreement of Pathologists diagnosis of CIN2+ on cervical biopsy specimens was noted with H&E morphology used in conjunction with P16 IHC compared with H&E morphology alone [14-16].

P16 Biomarker and Human Papilloma Virus (HPV)

P16 is a tumour suppressor gene, member of INK4 class, cell cycle inhibitor/regulator, located on chromosome 9p21. P16 along with pRb normally inhibits cell cycle at G1-S phase. P16 and retinoblastoma protein (pRb) have reciprocal expression, where P16 inactivatescyclin dependent kinase 4/6 (CDK4/6), prevents formation of CyclinA- CDK4/6 complex and hence decreases the phosphorylation of pRb protein by which the hypophosphorylatedpRb in complex with E2F suppress cell proliferation [2,8,9,11,17,18]. pRb in hyperphosphorylated form causes detachment with E2F protein (transcription protein). P16 is a pRb regulator. pRb inhibits transcriptionof CDK. In normal cell, P16 is a negative regulator of proliferation, working through a negative feedback loop to down regulate CDK4 (Figure 1). P16 expression is not appreciated in normal cervical epithelium, inflammatory lesions and CIN1 associated with LR-HPV significantly. Its expression is controlled in normal cells. P16 is detected in low concentration in healthy cells. Expression of P16 protein increases in aging cells. P16 over expression causes cell death and apoptosis.P16 is positive in metaplastic and atrophic cells. Dysplastic and neoplastic cells express diffuse and strong expression of P16 in both smears and biopsy [2,13,19-21]. Therefore P16 is a surrogate marker, indicates transforming HPV infection and discriminates between integrated and non-integrated forms of HPV infection [2,8,9,11,17,18].

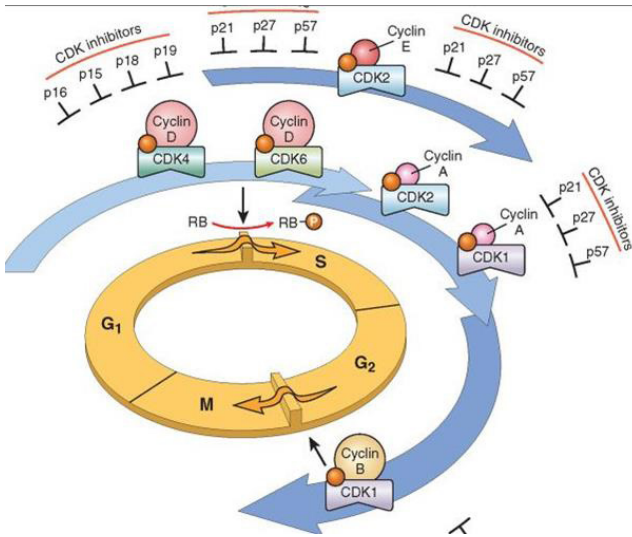

Figure 1: Shows the function of P16 biomarker in normal cell [19].

More than 200 subtypes of HPV are described having varied tropism towards epithelium of different organs. HPV are classified as low risk, intermediate risk and high risk types. Thirteen high risk HPV (HR-HPV) types are reported.Some of the high risk types like 16, 18, 31, 33, 39, 49, 51, 52, 56, 58, 59, 68, 73 and 82are proved to cause cervical cancer [9,18]. HPV 16 & 18 are positive in 99% of premalignant and malignant lesion of cervix [7,11].

HPV is proved to infect cervix and squamous epithelium which supports virion production. If the co-ordination control between viral gene expression and epithelial differentiation is broken then viral gene over expression results in cell clonal proliferation of undifferentiated cells with persistence of virus leading to colposcopy abnormality and malignant transformation. Hence the cell transformation depends on viral and epithelial interaction [15,22].

In HPV related tumours, the pathogenesis is integration of viral and host genome and synthesis of E6& E7 oncoprotein. The HR-HPV E6 degrades p53 protein resulting in decreased apoptosis. The high risk HPV (HR-HPV) E7 binds the pRb at the same site as E2F, inactivatespRbby which the E2F is released which being a transcription factor causes cell cycle activation at S phaseand cell proliferation (Figure 2). E2F also causes increased transcription of P16 suggesting HPV mediated carcinogenic process. Hence there will be increased P16 expression (nuclear and cytoplasmic) as a positive feedback loop which can bedetected by IHC especially with HR-HPV infection and hence P16 is a surrogate marker. Hence transcriptionally active HPV results in very high levels of expression of P16 [2,8,9,11,17,18,20,23]. HPV infection with P16 expression suggests release of E2F factor for proliferation of cells [1,15].

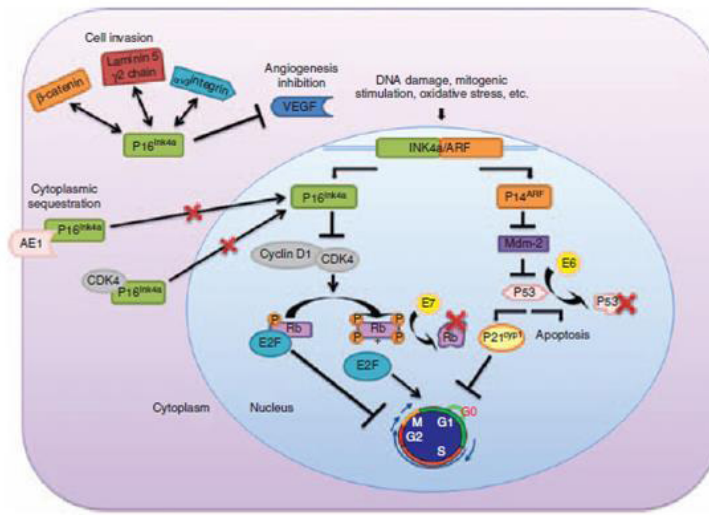

Figure 2: Shows the molecular alteration following HR-HPV infection in cervix [23].

Majority of HPV induced lesions disappear in 6-12 months after development. A few progress to HSIL and cervical cancer [24]. Cer-

vical cancer is caused by HR-HPV which causes transformation of normal epithelium into HSIL, a treatable premalignant lesion, which otherwise transforms into frank cancer [16]. P16 is expressed in cells transformed by HR-HPV. The E7 protein of LR-HPV differs from HR-HPV in biochemical and biological properties resulting in no P16 accumulation. LR-HPV presenting with CIN1 shows focal P16 positivity. Hence P16 is a sensitive marker for HR-HPV induced tumours.CAP and ASCCP have included P16 in new nomenclature for lower genital tract lesions [15,16,20].As per NTCC (New technology for cervical cancer screening), P16 over expression in HPV positive women increases cross sectional sensitivity and specificity for detection of HSIL [2,20].

CIN1 is associated with HPV infection and has low risk of malignancy. Less than 10% of CIN1 transform into HSIL or invasive carcinoma.CIN3 usually associated with HR-HPV and has risk of malignant transformation in 30% of cases. The CIN2 is also precancerous and is threshold for treatment [13].

In non-HPV related cervical cancer P16 is inactivated through mutation, deletion or hypermethylation of the gene.pRb can be directly inactivated at nucleic acid or protein level and cells proliferate even with high level of P16 (negative feedback control through pRb) [2]. Over expression of P16 correlates with high grade pre-malignant lesions, high grade tumours and senescence [24].

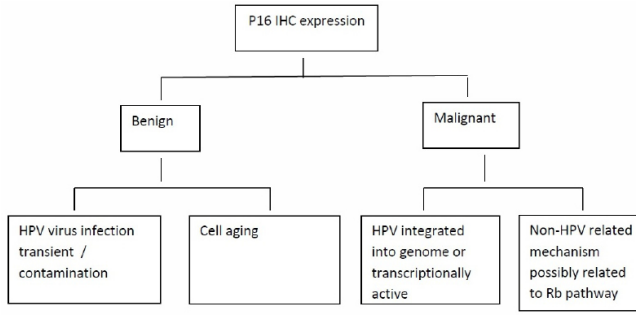

Figure 3: Mechanisms of p16 IHC in benign and malignant lesions [20].

P16 Biomarker in Cervical Biopsy and Cancer

The squamocolumnar junction (SCJ) of the cervix is the site of residual embryonic cells which is also the site of infection of HPV and initiation of neoplastic change or carcinogenesis [21]. HPV starts as initial transient infection, which when persists causes morphological and phenotypicchange as seen in HSIL and carcinoma [2,8,9,17,18,25]. Immunostaining of P16 was implemented in 2004, and until mid 2007 it was appraised using scoring method. Later positive and / or negative qualitative classification was considered [26].

Indications of P16 staining in cervical biopsy are; atrophy, metaplasia, atypical metaplasia, inflammation with reactive / reparable changes, small focus of abnormality, poor orientation, distinction between CIN1 & CIN2, discrepancy between the histologic preliminary diagnosis & a prior Pap test of a diagnosis of ASC-H, LSIL and HSIL. The frequency of use of P16 stain is inversely proportional to the experience of surgical pathologist and directly proportional to the super specialty as gynecologic pathology and cytopathology. The age of the patient is not a factor to decide or order P16 stain. Optimal percentage of cases to be subjected to P16 staining depends on the baseline HPV positivity and risk factors of the population. P16 is useful in approximately 10% of cervical biopsies routinely [27].

P16 expression only in nucleus or both in nucleus and cytoplasm is considered positive, only cytoplasmic staining is considered as negative [18]. In normal epithelium and in benign lesions, the P16 expression is basal because of spontaneous senescent cells[9]. Normal cervical squamous cells variably express P16 from negative to focal. Cervicitis and metaplastic squamous epithelium with absence of CIN does not express P16. The expression of P16 in LSIL varies and is confined to lower third of the epithelium associated with HR-HPV infection and then progress to HSIL than p16 negative cases. In HSIL, upper two thirds of squamous epithelium shows P16 expression. This helps to decrease the inter-observer reproducibility in histopathological diagnosis of CIN. It helps in diagnosis of focal area of HSIL and differentiates from HSIL mimics as atrophic squamous epithelium, immature squamous metaplasia and transitional metaplasia in which P16 is negative. P16 expression is also used in cauterized cervical resected margins in those of normal squamous epithelium from cauterized HSIL with MIB1 expression. In squamous cell carcinoma the P16 expression is diffuse [5,18]. In reserve cell hyperplasia, no staining or sporadic staining is observed. Diffuse staining was associated with HR-HPV infection[26].P16 helps in detecting CIN in follicular cervicitis[28]. P16 is focally or diffusely positive in non-HPV related mechanisms [2]. P16 is one of the molecular biomarkers designated as "Test of disease" in cervical biopsy [29].

In glandular component of cervix, normal endocervical glands are P16 negative or focally and weakly positive. In pre-neoplastic and neoplastic lesions, P16 is diffusely positive with those associated with HR-HPV infection. A few cervical adenocarcinomas are P16 negative which are not associated with HR-HPV. The intensity of P16 expression is less strong in cervical adenocarcinoma than in squamous cell carcinoma. Adenoid basal carcinomas, SMILE (stratified mucin-producing intraepithelial lesion; a form of reserve cell hyperplasia) with CIN and adenocarcinoma in-situ are P16 positive. Some cervical adenocarcinoma, adenoma malignum (usually non-HPV related),

minimal deviation adenocarcinoma, adenocarcinoma of mucinous type and mesonephric adenocarcinoma are P16 negative. Some HPV negative adenocarcinoma shows over expression suggesting non-HPV mechanism in pathogenesis of endocervical adenocarcinoma. The incidence of adenocarcinoma of cervix is increasing which may be true increase or due to greater recognition of the entity. The mimics of endocervical adenocarcinoma are some benign cervical glandular lesions such as; microglandular hyperplasia, tubuloemdometrial metaplasia, endometriosis in which P16 is negative or focally positive. The P16 is negative, focally positive and rarely diffusely positive in endometriod variant of endometrial adenocarcinoma unlike in endocevical adenocarcinoma where P16 is diffusely positive [12]. Cervical small cell carcinoma which is clinically aggressive is strongly P16 positive in majority and therefore associated with HR-HPV [18]. The rare subtype of cervical adenocarcinomas as; gastric, intestinal, mesonephric, clear cell, serous and hepatoid subtype express P16 with absence of HR-HPV. Here HPV RNA ISH can be used to prove the role of HPV. P16 can be used to differentiate AIS (block positive) from benign mimics as tubal and tuboendometrioid metaplasia (focal patchy positive) correlating with morphology [20].

With HR-HPV E7, P16 transcription can also be induced by the histone demethylase KDM6B. HR-HPV increased expression causes acute dependence on KDM6B expression for cervical cancer cell survival. Hence P16 is the critical KDM6B downstream transcriptional target and its expression is critical for cell survival [9].

P16 has to be used judiciously in the designated circumstances correlating with staining pattern as supplementary guidance. One has to classify the biomarker's utility and Lower Anogenital Squamous Terminology (LAST) recommendations have to be implemented in routine practice. The LAST project represents comprehensive evidence based clinical guidelines for the diagnosis and management of HPV related regions of lower genital tract [30].

In cytology, dysplastic cells show P16 expression without non-specific staining. However the mucus in Pap smears hampers the P16 staining of dysplastic cells because of prevention of sufficient diffusion of primary and secondary antibodies [2].

Percentage of Expression of P16 Marker in Different Grades of Cervical Intraepithelial Lesion

P16 is expressed in different grades of cervical intraepithelial lesion. The rate of expression is directly proportion to the grades of the lesion. However the percentage of expression in different studies varies.

In a study by Kleas et al, P16 expression was negative in normal inflammatory and metaplastic lesions. In CIN I, lower third epithelium showed strong and diffuse expression of P16 and upper 2/3rd weak, absent or focal staining. CIN III showed diffuse intense P16 expression throughout the epithelium and in CIN II lower 2/3rd showed the staining. In SCC all cases showed strong, diffuse and uniform staining. The expression of P16 in different lesions were; 27%, 38%, 60% and 100% in inflammatory, metaplastic (Sporadic or focal), CIN I and CINII/III and above respectively indicating transformed cells [2].

In a study by Zouheir et al, the expression of P16 was 100% in HSIL and 50% in LSIL. The diffuse staining observed was 18%, 64% and 87% in LSIL, HSIL and invasive cancer respectively[10].Wang and colleagues have stated that the PPV for diffuse staining of P16 in CIN2 is 39% and NPV 85%[31]. A study done by Lesnikova et al stated that P16 is not expressed in normal epithelium and stromal cells. The score was directly proportional to the degree of cervical dysplasia and invasion which has prognostic and predictive value [17]. A study by Gupta et al has reported that, P16 expression progressively increases with the grade of the CIN. It is predominantly negative in normal epithelium. It is a sensitive marker for CIN. The fibroblasts in the stroma are positive for P16 and hence can act as an internal positive control [8].

In a study by Hebbar et al, Low or absence of P16 expression in CIN1 is due to LR-HPV where E7 protein has lower affinity for Rb protein than that of HR-HPV. P16 is positive in HSIL and in a few LSIL which transforms into HSIL [7]. In a study by Xing et al the expression of P16 marker reported was, in CIN1 24.4% and in CIN2/3 87.5%. The sensitivity, specificity, PPV, NPV and accuracy of P16 marker for differentiating CIN1 and CIN2/3 was 87.5%, 75.6%, 76.1%, 87.2% and 81.2% respectively having high sensitivity and relatively less specificity. HR-HPV was detected in 72.4%, 81.4% and 88.1% in CIN1, CIN2 and CIN3 respectively [11].

In a study by Liu et al the sensitivity and specificity of only IHC P16 expression in diagnosis of HSIL is 96% and 92% respectively. The chance of HSIL was more in P16 block positive cases than negative cases [12]. In a study, P16 was positive in 20.7% of CIN1 and in 80.0% in CIN2&3 [22]. With P16 marker, the detection rate of CIN2+ was 9.5% to 24.1% and CIN1/CIN2 ratio was 0.7% to 4.5% [27].

P16 is at least focally expressed in 93% of invasive adenocarcinoma, 100% of AIS and 32% in negative samples. P16 is positive in 94% of glandular dysplasia cases. The score differences between neoplastic and non-neoplastic samples were highly significant ($p < 0.001$) [13].

There is difference in P16 expression in various studies because of different technical methods used, different clones of antibody used and difference in HPV types in CIN. A few CIN2 is also caused by low risk HPV (LR-HPV) and may also belong to the heterogenous group [4].The expression of P16 is not uniform as increased P16 expression is seen in majority of CIN and SCC; a few show decreased expression of P16. In a study by Klaes et al, the use of different clones / antibodies of P16 showed that the clone JC8 does not show non-specific staining of either in normal epithelial or mesenchymal cells or both [2].

Table 1: P16 positivity in cervical biopsy reported by various authors [7].

Authors and year	WNL	CIN1	CIN2+
Shi et al 2007	0%	77%	100%
Badr 2008	5%	35%	93%
Pinto 2008	37%%	ND	84%
Hallourch 2008	66%	92%	100%
Conesazamara 2009	14%	63%	87%

WNL – Within normal limits

Table 2: P16 expression in various squamous lesions of cervix by different authors.

Sl.No	Author & Year	Normal	CIN I	CIN II	CIN III	SCC
1	Soumpou et al [31].	2%	38%	68%	82%	-
2	Lesnikoua et al [17].	0%	72.3%	91.0%	98.3%	98.5%
3	Gupta et al [8].	10%	50%	60%	70%	95%
4	Hebbar et al [7].	-	50%	70%	90%	100%
5	Liao et al [32].	2.3%	50%	95.8%	95.8%	100%
6	Liao et al [1].	2.7%	42.7%	75.5%	79.6%	100%
7	Liao et al [1]. (Cytology)	14.4%	65%	78.8%	93.6%	100%

CIN: Cervical intraepithelial neoplasia; SCC: Squamous cell carcinoma.

This disparity in P16 results are due to technical variability which influence the test results (antibody kit and antigen retrieval method), interpretation variability as threshold for P16 positive is still imprecise both in distribution and intensity which needs to be standardized [27,30,32]. The variation in staining distribution and the intensity are challenges for interpretation [30]. P16 staining pattern is variable, i.e. nucleus and cytoplasmic or both, the importance is not fully understood. The comparison of biomarker performance in the detection of high grade cervical disease in different studies is difficult because of variability in specimens, study design antibodies and scoring al-

gorithms utilized [13]. P16 marker has to be interpreted correlating anatomic, clinical and technical aspects, pathologists expertise, morphological correlation and interpretative knowledge [20].

Regarding the technical aspect, there is lack of standard of P16 immunostain across institutions. Knowledge of immunohistochemistry is a requirement. Pre-analytical variables related to tissue processing and fixation also alters the P16 expression. Analytical variables related to protocol, reagent variability and technical expertise are to be taken care. Automated methods are of quality and reproducible. Post-analytical evaluation as control, interpretation, reporting of results and expertise of the pathologists has to be monitored. Inter-observer variability and various P16 clones used which hinder comparing protocols have to be kept in mind. E6H4 (MTM laboratories AG, Heidelberg, Germany) is the commonly used clone [19].

Table 3: Commercially available P16 specific monoclonal antibodies [20].

Sl.No	Clone	Manufacturer
1	E6H4	MTM Laboratories, Heidelberg, Germany
2	DCS-50.1/H4-NA29	Oncogene Research Products, Cambridge, MA
3	375P	BiogenexLaboratories, San Ramon, CA
4	ZJ11	Neomarkers, Freemont, CA
5	JC8	Neomarkers, Freemont, CA
6	16P04	Neomarkers, Freemont, CA
7	05-418	Upstate Biotechnology, Lake Placid, NY
8	G175-405	PharMingen, San Diego, CA

Regarding the interpretation of P16 expression, increased expression of P16 is due to increased P16 mRNA and P16 protein which causes strong and diffuse immunoreactions in the nucleus or nucleus & cytoplasm. In English literature different investigators have interpreted differently. Whether the different pattern of expression as nuclear or cytoplasmic depends on the clone used is not known. Positive and negative control had to be used [20].

Table 4: p16 interpretation in lower anogenital tract in different studies by various authors [20].

Study	Site/nature of the lesion	Antibody Clone	Criteria for positivity		
			Subcellular Localization	Intensity	Distribution
Galgano / 2010	Cervix / in situ squamous	E6H4	Nuclear & Cytoplasmic	Strong	Diffuse or patchy
Klaes / 2000	Cervix / in situ squamous	E6H4	Nuclear & Cytoplasmic	Strong	Sporadic, focal,diffuse[a]
Benevolo / 2006	Cervix / in situ squamous	E6H4	Nuclear &/ or Cytoplasmic	Not mentioned	Not mentioned
Bergeron / 2010	Cervix / in situ squamous	E6H4	Not specified	Strong	Diffuse[b]
Dijkstra / 2010	Cervix / in situ squamous	JC2	Nuclear &/ or Cytoplasmic	Strong	Diffuse[c]
Bernard / 2008	Anus / in situ squamous	JC8	Nuclear	Strong or week	Rare, patchy, contiguous[d]

Additional criteria applied in different studies shown in the table 4

a. <5% - sporadic, >5% to >20% - focal, >25% - diffuse

b. Focal staining – negative

c. <5% cells – negative, focal – scattered positive cells or clusters, basal staining – low intense diffuse in lower third of epithelium, diffuse – full thickness staining.

d. <10 positive nuclei per HPF – rare, >10 positive nuclei per HPF but cells not adjacent to each other – patchy, >10 positive nuclei per HPF within cells that are adjacent to each other – contiguous.

Table 5: Differential expression of P16 in LSIL and HSIL in lower anogenital tract biopsies [20].

Pattern of P16 Staining	Subcellular Localization	Biopsy	HPV Subtypes	Pathogenesis
Patchy / Focal	Nuclear / Cytoplasmic / Nuclear & Cytoplasmic	LSIL (-IN 1)	Low risk HPV or High risk HPV	Transient HPV infection
Strong, diffuse, block	Nuclear / Nuclear & Cytoplasmic	HSIL (-IN 2/3)	High risk HPV	Transcriptionally active HPV

LSIL: Low grade squamous intraepithelial lesion; HSIL: High grade squamous intraepithelial lesion; CIN: Cervical intraepithelial neoplasia; HPV: Human Papilloma Virus.

Role of P16 Biomarker in CIN3

P16 expression has significant positive correlation with severity of CIN and improves inter observer accuracy between pathologists [32]. Diagnosing HSIL / CIN3 by P16 marker has very high risk of malignant transformation. 30-50% of untreated HSIL has risk of malignant transformation [33].

Role of P16 Biomarker in CIN2

Morphologically CIN2 is a heterogenous group and is the least reproducible diagnosis with histological features suspicious but not definite for HSIL with uncertain biologic implications [12]. Therefore decreasing the frequency of diagnosis of CIN2 is advantageous. P16 marker is used in CIN2 cases as per LAST guidelines to differentiate HSIL from LSIL and increased P16 positive indicates HSIL diagnosis. Hence adding P16 to morphologic interpretation improves the agreement between Pathologists for CIN2+ diagnosis comparable to expert panel diagnosis. Therefore P16 is recommended to be used in equivocal CIN2 as per LAST standard project. It also increases positive predictive value of P16 positive cervical biopsy HSIL diagnosis

[26,34,35]. Regression rates are highest in the setting of P16 negative HSIL (CIN2) biopsies and spontaneous regression is 64% in 12 months follow up. Hence conservative management is considered safe in these cases. In P16 positive HSIL (CIN2) cases the risk of transformation to HSIL (CIN3) is high in 12 months follow-up. Therefore P16 is an important marker to distinguish the different behavior of HSIL (CIN2) lesions. Among P16 positive HSIL / CIN2 cases, 57% regressed spontaneously in a study by Miralpeix et al. However there was no association to predict the prognosis when P16 positivity was stratified (epithelial) and correlated with evolution of the disease. Therefore it was concluded that some other factors and biomarkers must be having role in regression or progression of the lesion along with HR-HPV. [14]. LAST group has supported the use of P16 marker as a complimentary to the histomorphology only in equivocal HSIL / CIN2 cases to guide the final opinion between P16 negative LSIL and P16 positive HSIL. In a study by Miralpex et al, total and partial regression of 53% and 47% respectively was reported in P16 negative HSIL / CIN2 cases. Among P16 positive cases, total regression, partial regression, persistence and progression was 21%, 36%, 33% and 10% respectively. Hence P16 marker can be used as a predictive marker to determine HSIL (CIN2) outcome. In a study by Guedes et al, the spontaneous total regression, partial regression, persistence and progression in HSIL (CIN2) cases were 42%, 20%, 11% and 22% respectively which had the disparity with previous reported study. The disparity between the two studies was probably due to the different definition used for P16 staining as week, moderate and strong staining which were considered positive in latter study. [14]. A study by Liao et al showed that, in CIN2+ cases, the sensitivity and specificity of HPV DNA test is 100% and 22.9% respectively against the sensitivity and specificity for P16 IHC of 85.7% and 60.3% respectively in CIN2 cases [1].

A study by Liu Yuxin et al showed that 23% of CIN2 cases showed P16 positivity of which, 8% showed strong / basal positivity (28% positive for HPV), 7% showed strong / focal (27% positive for

HPV) and 8% showed weak / diffuse positivity (35% showed HPV positivity). CIN2+ showed block positivity, negative and ambiguous staining of P16 was seen in 18%, 59% and 23% respectively. Among ambiguous cases, 70% were negative for LR-HPV and HR-HPV and 30% were positive for HR-HPV. All P16 block positive cases were HR-HPV positive and none of P16 negative cases showed HR-HPV. Hence P16 block positivity is associated strongly with HR-HPV with PPV of 97% and NPV of 86%. HSIL outcome later in 12 months follow-up was significantly higher in the P16 block positive group (35%), lower in the P16 negative group (1.5%) and intermediate in P16 ambiguous group (16%). Special guidelines for intermediate category were required to prevent diagnostic errors and help to implement P16 IHC in general practice. Morphological interpretation has to be integrated with biomarker and HPV genotypes [12].

A study by Carozzi et al stated that, CIN2+ was detected during follow-up more in P16 positive women (8.8%) than P16 negative women (3.7%) with relative risk of 2.61. The relative risk (RR) was more in women aged 35 – 60 years (3.37%) than between 25-34 years (2.15%). In addition CIN3 was detected in 4.4% P16 positive cases on follow-up and 1.3% in P16 negative cases with RR of 3.90, RR for women between 35-60 years was 6.05 and for 25-34 years was 2.69. The sensitivity of P16 test in detecting CIN3+ during follow-up of all ages was 77%. P16 expression is a marker of CIN2+ or suggests development of CIN3+ within three years especially with HPV positive cases in age group of 35-60 years. CIN2+ risk increases in P16 positive women than P16 negative women. Initial P16 test is a good predictor of CIN2+ in HPV positive women especially between 35-60 years of age [20].

Role of P16 Biomarker in CIN1 (LSIL)

CIN1 showed P16 positivity in 29.2% and 38% cases in two different studies. In a study by Cortecchia et al, P16 positive CIN1 were significantly seen in younger age group than the patients with P16 negative CIN1. Progression rate is 5.5 fold greater among P16 positive CIN1in first year of follow-up. In second and third year of follow-up,

the progression was limited and non-significant, however it was more in patients with 36 years of age or older. CIN1 P16 positive cases had a 50% decrease in the regression rate in the first year of follow-up. In another study, in HPV positive cases, free of CIN when associated with P16 positive expression, the progression was earlier, 2 fold and was more for patients older than 35 years. Interval between detection of P16 over expression CIN1 and related progression was less than one year i.e. shorter latency. P16 positivity indicates persistent HR-HPV infection which subsequently transform into HSIL. The first follow-up for P16 positive CIN1 has to be conducted with great care and screened for the prevalent lesions which may be missed. It is often followed up with median time of first appointment to be shorter. The rate of progression of P16 positive CIN1 to CIN2+ at first, second and third year was 12.3%, 9.9% and 13.0% respectively. The progression with P16 negative CIN1 to CIN2+ at first, second and third year was 2.2%, 7.5% and 11.4% respectively. Regression to normal with P16 positive CIN1 at first, second and third year was 26.3%, 49.4% and 47.8% unlike P16 negative CIN1 at first, second and third year which was 48.1%, 61.2% and 57.1% respectively. This study showed a mean of 5% to 8% progression in first two years of follow-up. Other studies reported the progression rate of 40% to 60% for P16 positive CIN1 and 15% to 30% for P16 negative CIN1. Hence in P16 negative CIN1, normal screening interval can be followed because of risk of progression is low. P16 has role in planning short term surveillance of patients with CIN1 [26].

P16 is a predictive marker of low grade lesion, i.e. LSIL / CIN1 with diffuse P16 staining has an increased tendency to progress to CIN2 and HSIL (CIN2+ or CIN3) in varying time periods than P16 negative cases. P16 positive CIN1 cases suggests worse follow-up than P16 negative CIN1 cases and also concurrent high grade lesion elsewhere [15,26,36]. Hence many studies have shown enough evidence that P16 IHC can be used in routine practice [36]. CIN1 (20-40%) with diffuse P16 staining usually have evidence of persistent HR-HPV infection resulting in increased risk of cervical pre-cancer and cancer

[32]. P16 expression will be significantly higher in the progression group than in the regression group. The sensitivity and specificity in predicting the CIN1 progression in a study was 86% and 60% respectively with NPV and PPV of 88.6% and 40.4% respectively [7].

P16 is positive in CIN1 cases with HR-HPV positive cases and the accuracy of diagnosis increases when associated with Ki67 (lower 1/3rd staining) [27]. 15% and 9,4% of P16 positive and negative LSIL progress to HSIL respectively [30]. A study by Liao et al showed that two year cumulative incidence of CIN2+ for P16 positive and P16 negative cases which progressed from CIN1 were 10.71% and 1.30% respectively. In CIN1, HPV positive and P16 positive is seen in 65.8% against HPV negative and P16 positive in 13% cases. 7.1% of CIN1 cases with HPV positive progressed and no CIN1 cases with HPV negative and P16 negative progressed. It means P16 positive women with CIN1 might require closer follow-up at every 6 months yearly compared to P16 negative women as many years are required before P16 becomes over expressed in dysplastic HPV induced cellular transformation. Hence P16 over expression is strongly associated with grade of CIN and risk of progression to high grade CIN in women with low grade lesions [1]. Hence P16 is useful in triage of LSIL associated with HR-HPV infection for careful follow-up[18].

P16 Negative Expression in Cervical Intraepithelial Neoplasia and Carcinoma

Non-HPV dependent P16 negative pathway may also exist in cervical carcinoma [17]. P16 can be inactivated due to mutation, deletion, loss of heterozygosity and hypermethylation (causes decreased or negative expression of P16) causing increased CDK4/6 activity, premature phosphorylation and inactivation of pRb [2,5]. The P16 promoter region hypermethylation and homozygous deletion constitutes about 6.5% and 8.7% of samples respectively as reported by Tripathy et al [8]. The epigenetic mutation of P16 gene as hypermethylation of promoter region of P16 gene presents as P16 negative in CIN2/3 and may not correspond to the HPV status or CIN grading [8].

In addition pRb can be inactivated by mutation, deletion, directly inactivated at nucleic acid or protein level and by HR-HPV E6 resulting in increased expression of P16 as negative feedback mechanism and increased cell proliferation [2].

Grading of P16 Biomarker Expression

Some authors consider 5% expression as positive and others 25% or 50% as positive staining. Different Pathologists use different criteria and threshold for diagnosis leading to bias. Therefore use of P16 biomarker objectively with repeated objective feedback and training is necessary [27,30].

The expression of P16 was classified into two groups in a study by Xing Y et al as those with nuclear and cytoplasmic positivity of less than 25% (grade 0 / negative, 0-5% and 1+, 6-25%) and more than 25% (grade 2+, 26-50% and 3+, >50%) [11].

There are many scoring systems for P16 marker of which the scoring system of Klaes and colleagues is widely used. Klaes and colleagues classified the P16 staining as a semi-quantitative score as; Negative: less than 1% of cells positive; sporadic: isolated cells positive but less than 5%; focal: small clusters of cells positive but less than 25% of cells are positive; diffuse: more than 25% of cells are positive. The positivity is considered only with diffuse staining with 25% and more cells positive, P16 with diffuse staining suggests increased tendency to progress to a high grade lesion than P16 negative cases [2,31]. Semi-quantitative P16 scoring methods of Klaes scoring systemare widely used [12].

Lee et al have interpreted P16 immunoreactivity under following parameters [25].

1. Intensity: a. Strong (dark brown as positive or similar to control) b. Weak (yellow colour)
2. Extent: a. Diffuse (>50% of the epithelium) b. Focal (<50% of the epithelium)

3. Continuity: a. Continuous (extent laterally to a significant distance) b. discontinuous (altered clusters of positive / negative stained cells).
4. Location: a. Lower 1/3rd b. Lower 2/3rd c. Full thickness
5. P16 immunoreactivity can be classified as; [25]
6. Block positive: Strong, diffuse, continuous and involve basal layer upwards >1/3rd.
7. Negative: Total absence or weak focal discontinuous staining.
8. Ambiguous: a. strong / basal / diffuse b. weak / diffuse / discontinuous up to 2/3rd thickness of the epithelium c. strong / focal / discontinuous at any level.

LAST defines "block positivity" P16 as the supporting evidence for the diagnosis of HSIL. The criteria are; 1. Demonstrates or expresses strong nuclear positivity with or without cytoplasmic signal. Cytoplasmic staining alone was considered negative. 2. Extends from basal layer upwards at least 1/3rd of the epithelium. 3. Extends laterally over a significant distance. P16 IHC has increased very much in most clinical practice since the publication of LAST recommendation [25].

The present author has scored the P16 biomarker expression by selecting a microscopic area having good expression and having minimum of 100 cells (hotspot area). If section does not show the P16 marker expression, scoring for that case is not done and considered as negative or zero. The scoring is done under five parameters only when the tissue section shows expression of marker as follows;

1. Nuclear and cytoplasmic positivity: 1 for cytoplasmic positivity, 2 for nuclear positivity and 3 for nuclear and cytoplasmic positivity.
2. Intensity: 1 for week, 2 intermediate and 3 for strong.
3. Extent: 1 forfocal <30%, 2 for 30-50% and 3 for diffuse >50%.

4. Continuity: 1 for discontinuous, 2 for extend laterally continuously less significantly and 3 for extend laterally continuously significantly.

5. Location: 1 for lower 1/3rd, 2 for lower 2/3rd and 3 for full thickness.

The author has graded the scores as follows; Grade 1: score 5 and 6, Grade 2: score 7 and 8, Grade 3: score 9 and 10, Grade 4: score 11 and 12 and Grade 5 score of 13 to 15. The author has used these criteria for validation and is working on cervical tissue biopsy which will be published.

P16 Biomarker & LAST Project Recommendation

As per the March 2012 the lower anogenital tract squamous terminology (LAST) standardized project for human Papilloma Virus (HPV) associated squamous lesions and new nomenclature of HPV related lower genital tract for cervix which was put forth in July 2012, it has been classified as two tiers which is superior to the three tiered intraepithelial neoplasia because it reflects the latest knowledge of HPV pathogenesis. The two tiered classification are, Low grade squamous intraepithelial lesion (LSIL / CIN1) & high grade squamous intraepithelial lesion (HSIL / CIN3) where –IN can be mentioned in parenthesis and CIN2 was eliminated. LSIL is HPV transient infection with low risk of progression to cancer, low grade lesions and requires continued surveillance versus HSIL which is a persistent infection with more stable precancerous lesions (Carcinoma in situ),having transformation high risk of progression to cancer and requires excision procedure. CIN2 is considered as an intermediate lesion. In United States where the maximum sensitivity prevails for the medical practice, CIN2 and CIN3 are considered as HSIL [14,30,35]. Two tiered system better reflects the biologic and morphologic equivalence of HPV associated squamous cells proliferation across the lower anogenital tract, decreases the variability and the management outcome is better. It gives unified histologic nomenclature supported by the

biomarker giving "--IN" terminology as HPV infection in cervix results in 1. Transient active HPV replication which is a self limited low grade lesion. 2. Persistent HPV infection associated with pre-cancer high grade lesion which may progress to invasive carcinoma. 3. Intermediate grade IN2, which does not correlate biologically, mix of low grade & pre-cancerous disease, cannot be reliably distinguished on H&E morphology, not a reproducible histologic category, has high interobserver variability , risk is also intermediate between IN1 & IN3 and good percentage represent CIN3 on follow-up. The CAP-ASCCP had consensus with LAST. However in two tiered system, the primary concern was for CIN2 and CIN3 in adolescent and young adults where conservative management was done for CIN2 and not for CIN3. HPV biology and biomarker does not support three tiered system [33].

Two tiered harmonizes lower anogenital tract terminology with other published systems. Observer variation is decreased in two tiered system with increased agreement among Pathologists, has consistent & reproducible diagnosis and has more valid clinical outcome data. However CIN2 is considered as intermediate or equivocal category and as per LAST consensus, CIN2 is retained because of conservative approach in young reproductive women and concern of over treatment if merged with CIN3 as HSIL. Hence the LAST recommendation is to consider two tiered system and further subcategorize (--IN) if required. The two tiered classification harmonizes the descriptive terminology for cytology and histopathology for biologically similar HPV associated squamous lesions of lower anogenital tract. Two tiered system is similar to The Bethesda System of cervical cytology [33].

The objectives of LAST were, [33].

1. Historical perspective of the origins of terminology and nomenclature that influence malignant transformation.
2. Terms for intraepithelial lesions and early invasive carcinoma.

3. Available biomarkers to support proposed terminology and improve diagnostic reliability and reproducibility to histopathology interpretation.
4. Dissemination, implementation of terminology into clinical practice and utilize for education, regularity and clinical processes.

The LAST also has the guidelines for the use of P16 biomarkers for cervical lesions. The recommendations are;

Recommendation 1, P16 stain to be used to differentiate between HSIL and its mimics as immature metaplasia, atrophy, repair and tangential sectioning.

Recommendation 2, P16 stain to be used to classify histomorphologically diagnosed intermediate equivocal lesion CIN2 as LSIL (P16 negative, weak or patchy) or HSIL (block positivity) as downgrading some CIN2 to LSIL decreases overtreatment or excision. Hence CIN2+ with P16 positive is considered as HSIL.

Recommendation 3, P16 stain to be used to resolve professional disagreement if Pathologists differ over a possible interpretation of HSIL. It increases interobserver agreement, consistency, reliability of cervical biopsy reports and decreases missed opportunities to treat potentially preinvasive lesions. However it may increase the expenses of test for P16 IHC along with follow-up treatment.

Recommendation 4a, P16 stain to be used in high risk colposcopy referral cases where biopsy with H&E stain is LSIL or lower and if the previous screening results indicate a more than 30% risk of HSIL as; ASC-H, AGC-NOS, ASC-US with HPV 16 positive in reflux or co-testing [13,20,22]. The rate of use of P16 biomarker in a study by Clinton et al has increased from 2.79% to 6.16%. The use of P16 has increased in category 2 and 4a, in category 2, it has increased from 0.16% to 1.42% and in category 4a from 0% to 1.9% [34,35].

The use of P16 marker is discouraged on unequivocal cases of CIN1/CIN3 lesions. The LAST project states that P16 IHC should not be used on morphologically unequivocal CIN I lesion or LSIL with features of prominent koilocytotic atypia with occasional mitosis at middle level of epithelium and tangential sectioned epithelium with expanded basal zone [31]. WG4 (working group 4) group of LAST project recommended that approximately 10% of the biopsy which are diagnosed as CIN2 and 10% of most problematic CIN1 cases (i.e. 4% based on 40% of CIN1 in total biopsy specimens), together of 14% of cases designated as "indeterminate lesions" to be considered for P16 staining with an average of 19% to less than 25% of cases overall. WG4 anticipated dramatic increase in use of P16 biomarker. The LAST WG4 recommendations expected increase in HSIL rate following the use of P16 stain compared to prior CIN2/CIN3 rate [34]. As per the LAST recommendation for diagnosis of HSIL (CIN3+ and P16 IHC positive CIN2) by pathologist the mean sensitivity and specificity were 90% and 87% respectively [32].

Some practical issues are also encountered in LAST project as; a few HSIL does not show P16 staining, LAST recommends to evaluate negative and equivocal P16 staining in conjunction with morphologic findings. Strong and diffuse expression in a very small focus of HSIL may be missed in routine H&E evaluation. Sometimes difficult to interpret the stain in minute biopsies, unoriented tissue, tangential sections and free floating single cell because of lack of morphologic correlation [35].

As per the LAST classification, if P16 is positive, it is HSIL and can be subjected for excisional procedure and if test is negative, it is LSIL and requires continuous surveillance. Therefore the LAST classification improves diagnostic accuracy especially with inter observer morphologically ambiguous and challenging cases. P16 biomarker block positivity is considered positive for the diagnosis of HSIL. Block pattern staining means, staining is strong, continuous, nuclear positivity with or without cytoplasmic staining, extending from basal layers upwards for at least 1/3rd thickness of the epithelium (basal &

parabasal layers) which can be further graded as 1/3rd, 2/3rd and more than 2/3rd and laterally over a significant distance (diffuse >25% of cells with staining) [15,22,26,30,34]. Galgano et al have stated 86.7% sensitivity and 82.8% specificity in identifying CIN2/3 lesions using the block pattern. However the specificity was 71.7% and 51.7% when patchy but strong staining (less than 50% area in question of middle and superficial cell layers are considered) and rare single dispersed positive cells (alternative positive and negative small cell clusters in mosaic like pattern) respectively are considered. The latter two types of expression had negative follow-up with low positive predictive value. Clinton et al reported that after adopting LAST, P16 positive HSIL increased from 1.4% to 2.3% and PPV significantly increased from 48% to 76%. LSIL or normal epithelium having non-block staining is considered negative. Non-block staining is a common cause for false positive diagnosis of HSIL. As per LAST guidelines, the patterns of P16 staining which are considered negative are; only cytoplasmic staining, wispy, blob like, single cells, puddle and scattered which are non-HPV related. Non- block staining includes patchy staining and minute pattern of staining. Patchy staining means, staining seen as alternating clusters of positive and negative stained cells where the positive cells reside either in the middle or in superficial cell layers and staining intensity is week or strong. Minute pattern of staining means, strong positive P16 staining of cells but limited in a very small focus or small detached epithelium having less than 10 cells in length and less than 5 cells layer in thickness [12,30,34]. Ambiguous staining is one where there will be strong nuclear and cytoplasmic positivity but limited to a part of epithelium [12]. "Negative" staining is total absence or weak or focal and discontinuous. Ambiguous staining is considered under following situations as; 1. Strong and basal (strong, diffuse, continuous, involves lower 1/3rd without upward extension) 2. Weak, diffuse and discontinuous staining, involving at least 2/3rd of the epithelium. 3. Strong, focal and discontinuous located at any level of the epithelium [12].

Implementation of P16 marker as recommended by LAST guidelines increases use of P16 marker (doubling the rate). The LAST guidelines predicted P16 use rate of approximately 20% which can decrease the discrepant cytohistological results, improve cytohistology correlation rates, decrease variability in frequencies of histologic diagnosis (especially when used in >10% of cervical biopsy) and decrease interobserver variability. In high risk Pap with cervical biopsy as LSIL or lower, the P16 staining has demonstrated small fragment of tissue with HSIL [26,35].

Utility of P16 Biomarker in Cervical Lesions

- Both quality and quantity of P16 immuno reactivity are considered in predicting high risk HPV and HSIL outcomes [12].
- P16 with HPV L1 capsid protein expression indicates association of HPV and correlates with severity and is a prognostic marker [13].
- In middle age, about 70-80% HPV prevalent infection clear in 2-3 years having high P16 negative women [22].
- P16 express strongly in high grade lesions, potential to identify severe lesions when associated with a positive hybrid capture 2 test for HR-HPV [24].
- P16 and Ki67 indicate the impact of HPV infection, integration and proliferation of immortalized cells [27].
- P16 and Ki67 helps to differentiate between persistent HR-HPV infection and dysplasia [11].
- The P16 "high users" diagnosed more biopsies with low cytohistologic discrepancy (12.62% Vs 19.92%), higher rate of CIN2+ diagnosis (19.9% Vs 16.4%, 1.3 times) especially with pap test showing ASC-US & above diagnosis and positive for HR-HPV test and less variability in frequency of CIN2+ diagnosis. Use of P16 for greater than 10% of cervical biopsies was associated with improved cytohistologic correlation rates and with lower variability in the frequency of histologic diagnosis [27,30].

- Use of P16 as biomarker serves as a surrogate marker for persistent infection with HR-HPV. It is a sensitive and specific marker to identify of dysplastic cervical cells in sections or cervical smears [2].
- P16 decreases the inter observer and intra observer variability, increase reliability of the histologic diagnosis of CIN, increases sensitivity of cytologic screening and increases accuracy of the histologic diagnosis of CIN2+[10,25]. It is used to standardize for quality control of histopathology diagnosis [2,7].
- Strong and diffuse positivity of P16 staining can be dysplasia at the cauterized margin as P16 staining is not affected by cautery artifact [20].
- P16 provides new opportunities for cost reduction, decreases insecurity for patients, avoidance of unnecessary diagnostic and therapeutic surgical procedures [2].
- P16 positivity along with HPV positive cases has to be subjected to immediate colposcopy unlike P16 negative with HPV positive cases where immediate colposcopy can be avoided and routine screening can be considered as HPV is detected in many transient spontaneously regressive infections and its specificity for HSIL is low [21].
- P16 staining sensitivity and specificity is 88% and 61% respectively in CIN2+ and HPV positive women [21].
- P16 marker is applied for both cytology and histopathology samples in relating to the degree of severity. Diffuse staining in histopathological specimens is predictor of disease progression and suggests requirement of intense follow-up [31].
- P16 is a convenient adjunctive tool that promptly offers straightforward answers especially for morphologically challenging cases [10].
- The nucleo-cytoplasmic expression of P16 in CIN1 indicates persistence of virus and tendency to progress, which needs appropri-

ate treatment and aggressive follow-up. Hence it is suggested to incorporate P16 in routine surgical Pathology practice [8].

- P16 is used to triage lesions especially of morphologic differential diagnosis of CIN2 into LSIL versus HSIL categories. Specific guidelines for this intermediate category should prevent diagnostic errors while planning to implement LAST guidelines in general practice [12].
- P16 is used to differentiate HSILs from their mimics as reactive / reparative epithelial changes, immature squamous metaplasia and atrophy which are problems in histopathological interpretation [7,12].
- P16 Solves disagreement between Pathologists [12,36].
- P16 will search for subtle HSIL in cases with high risk cytology [12].
- It helps in identifying regions of focal disease which is important because to take decision regarding the invasive procedure as LEEP and cone biopsy which may lead to pregnancy complication in reproductive females in future pregnancies and hence the procedure should be done only with true high grade cervical lesions [13].
- HSIL with strong P16 expression and only very focal Ki67 staining results in HSIL with low proliferative activity indicating early stages of HSIL regression [13].
- Grading of CIN with P16 is easier and faster than the histomorphology with Hematoxylin and Eosin stain [27].
- One has to differentiate between CIN1 and CIN2/3 by using P16 marker to prevent overtreatment of false positive cases or under treatment of false negative cases [1].
- Only histomorphology is challenging and subjective. Judicious use of P16 with proper interpretation improves the diagnostic accuracy of HSIL [12].

- P16 marker has increased diagnostic reproducibility and has prognostic factors in low grade lesions [15,33].
- The P16 biomarker in Pap smears helps in triage of mildly abnormal and intermediate cytology cases from high grade lesions [13].
- P16 highlight potential abnormal cells from normal / reactive cells drawing attention of slide screeners [13].
- P16 over expression increase with severity of cytological abnormality. 12%, 45% and 89% in normal, ASC-US / LSIL and HSIL show positivity with P16 respectively. P16 was 95% sensitive, 84% specific for ASC-US and 100% sensitive, 82% specific for LSIL for detection of biopsy proven HSIL. Sensitivity and specificity of P16 staining with CIN2+ as endpoint is 88% and 66% (HPV positive). For CIN3+ as endpoint 61% and 59% respectively (HPV positive) suggesting that P16 can be considered as a sensitive biomarker in cervical cancer screening [7,13,18].
- P16 can be used as reflux test for atypical Pap test, primary screen to increase the accuracy of the Pap test and as an alternative screening algorithm [13].
- HPV test and P16 test has relative high sensitivity than cytology screening [22].
- The baseline P16 specificity was lower in women more than 35 years than in those of less than 35 years of age i.e. HPV infection has already progressed to P16 over expression although not to HSIL. Therefore P16 testing decreases intervals between screenings [21].
- The cross sectional sensitivity of P16 is higher (91%), hence longer intervals can be considered for screening [21].
- It improves diagnostic accuracy for pre-cancerous lesions i.e. early diagnosis, improved screening approach of lesions progressing to cancer [7,10,12].

- P16 is a marker to assess risk of progression to cervical cancer. Positivity of P16 increases with progression of lesions [1]. P16 predicts the behavior of CIN2 cases. All P16 negative CIN2 regressed and 43% P16 positive CIN2 persisted or progressed to CIN3 at 12 months duration. P16 positive ambiguous CIN2 group posed an intermediate risk of persistence and progression [12,37]. P16 expression in CIN1 suggests tendency to progress to CIN2/3 and hence its importance [7]. The sensitivity, PPV and NPV is 96%, 39% and 96% respectively for diffuse staining of P16 in detecting those CIN1 biopsies which transforms to CIN2[31]. P16 test can be used as triage test to assess risk of CIN3 in next 3 years [21].
- P16 marker can be used by Pathologists to confirm their own diagnosis and thus overcome the conformational bias [27,36].
- P16 expression is useful in squamous and glandular dysplasia of cervix. P16 is the marker for all degree of dysplasia and carcinoma, surrogate marker of HPV infection and it is robust, objective specific and sensitive biomarker [17].
- P16 is connected with increased overall and disease free survival and thus marks a better prognosis. P16 is not associated with tumour TNM staging, the tumour grade, the tumour size or vascular invasion. However it is associated with lymph node metastasis [9]. Decreased expression of P16 results in poorer prognosis in cervical cancer than over expression. HR-HPV is the triggering factor which induces P16 mediated protection mechanism. Probably over expression of P16 is recognized by the immune system as a naturally low expressed protein which can initiate an antitumor response in cervical cancer patients [9,31].
- Strong P16 expression is the useful surrogate transcription marker for tumours with transcriptionally active HR-HPV, associated less with genetic alteration, has less complex tumours, respond better to treatment, has improved outcome, has favourable prognostic marker, causes suppression of cell division, sup-

presses lymph angiogenesis, has decreased lymphatic spread and increased susceptible to radio-chemotherapy. HPV negative cervical cancer has increased risk of relapse, increased risk of distance metastasis, increased mutations in genes coding for the cell cycle regulatory proteins, increased therapy resistance and less susceptible to radiotherapy. Hence P16 expression is significantly associated with better prognosis in terms of disease free survival and overall survival in cervical cancer [9].

- UK National External Quality Assessment service for Immunocytochemistry suggests high quality results with cell blocks sections followed by cytospins FFPE specimens, LBC and conventional smears [21].
- P16 and Ki67 were introduced in Europe in March 2010 to avoid the need for morphological criteria and increase the specificity and reproducibility [21].
- P16 is widely used in lower anogenital tract, oropharynx and also gynecologic tumours [20].

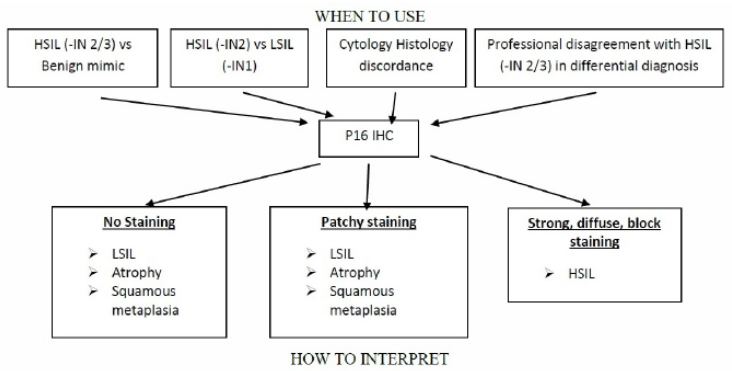

Figure 4: Utility and interpretation of p16 IHC in lower anogenital tract [21].

- P16 should not be performed on cases that can be morphologically categorized as LSIL (-IN 1)
- Occasionally LSIL and immature squamous metaplasia can show strong diffuse staining. Hence it is necessary to complete P16 expression with morphology

Role of P16 Biomarker in Clinical Management of CIN

Approximately 80% of LSIL/CIN1 regresses spontaneously and 10% progress to HSIL and cervical cancer. Hence LSIL/CIN1 cases are managed by regular follow-up without treatment. P16 positive low grade lesions are associated with higher rates of progression and negative cases associated with regression, hence predicts the outcome of low grade lesions [14]. For HSIL (CIN2 & CIN3) the standard treatment is excision by different methods as approximately 40% of HSIL spontaneously regress and 20% progress to frank carcinoma. However over-treatment results in obstetric complications in females of reproductive age group. Hence conservative management is recommended by ASCCP by follow-up for 24 months especially in adolescent and young adults with HSIL (CIN2) [14]. The treatment of HSIL is loop electrosurgical excision procedure (LEEP) or conization which has short term (infection, bleeding and pain) and long term (premature rupture of membranes, preterm labour and cervical os stenosis) complications. As approximately 85% of the patients are in reproductive age group, the diagnosis of P16 positive HSIL is of great clinical significance. P16 marker improves HSIL detection in cases that can be appropriately followed up with treatment [35]. Therefore P16 staining is required for clinical management, reliability of HSIL/CIN2 diagnosis and decide the closer follow-up[14]. P16 biomarker is important because it improves the clinical performance of cervical cancer screening to treat only high risk cases [13].

Disadvantages of P16 Marker

P16 is less specific as sometimes CIN 1 and 2/3 shows similar expression, in addition it also expresses in inflammatory lesions [11].

Wide variation of P16 positivity is observed in equivocal and low grade lesions where there is lack of reproducibility [31]. The warty variant of squamous cell carcinoma of cervix are P16 negative and is a diagnostic pitfall [38]. The other disadvantages of P16 expression in cervical tissue is P16 expression is positive in benign endocervical lesions as tubuloendometrial metaplasia, endometriosis and normal endometrium [18]. In endocervix, benign ciliated cells and immature squamous metaplasia show P16 positivity. The reporting Pathologist should be aware of these entities [20,27]. The P16 staining in non-dysplastic cells as atrophic or metaplastic cells needs additional criteria to discriminate from the dysplastic cells to increase the specificity [9]. However some studies have observed no non-specific staining in normal squamous cells [2].

In a study by Xing Yet alit was observed that this marker found no correlation between P16 and HPV status and hence P16 might be an unreliable surrogate marker for HPV status even though some studies have showed expression of P16 correlation with HPV status. The inconsistency is attributed to absence of uniformity or standardization in cutoff value of P16 expression and variation in HPV status among patients from different geographic origins [11].

The clinical application P16 marker is hampered by lack of reproducibilityand standardization of cut off points for positive expression and there is no general consensus for establishing threshold values above which a sample becomes P16 positive especially in P16 cytology interpretation. Features of P16 staining have not been analyzed systematically as; nucleus versus cytoplasmic staining and the intensity of staining. These parameters are considered not relevant by many authors. There are no clear guidelines used in routine practice. Consensus to be reached regarding evaluation of P16 staining and the biomarker needs to be evaluated in various clinical settings [9,31]. Diffuse positive P16 staining (i.e., >25% of cells with positive signal) is noted in 2% of normal epithelium, 38% of CIN1, 68% of CIN2 and 82% in CIN3. Therefore, solely relying on P16 results and theoretically classifying or under-classifying will results in under or

over treatment. Debate continues regarding the weightage of IHC P16 staining in cervical intraepithelial lesions. There are reports of negative follow-up of HSIL cases which may be due to small lesions which might have been removed completely, lesion regressed spontaneously, false positive HSIL result of preceding biopsy, over use of P16 IHC or upgraded questionable lesion to HSIL (non-block P16 staining) [30]. Approximately 30% of CIN1 shows block positivity with P16. Hence P16 is not specific for HSIL. If all P16 positive cases are considered as HSIL, there will be overcalls especially in young women [34]. P16 can result in over diagnosis of CIN2+, but only a few cases are reported and use of P16 along with Ki67 increases the specificity [27].

Nuclear scoring was introduced for P16 in LBC as; nuclear size, nuclear-cytoplasmic ratio and other nucleomorphologic features by which four tiered classification of nuclear abnormality was considered to increase the significance in specificity for detection of CIN2 positive instead of P16 positive slide. But morphological assessment in P16 stained smear is difficult and requires training and experience. Ideally biomarker should not rely on subjective criteria [31].

Discrepancies in the interpretation of P16 staining in both cytology and histology have decreased its reproducibility which also has hampered the interpretation of the data across different studies [31].

Expression of Other Biomarkers in Cervical Biopsy Correlating with P16 Expression

In a study by Lee H et al, The expression of P16 observed was weaker in SCC than CIN3. The expression of P16 was concordant with CK19 (marker for squamocolumnar junction) and HR-HPV in SCC and CIN3, i.e., expression was basal and less intense in normal, the expression increased in intensity from basal to superficial layer of stratified squamous epithelial layer (bottom up expression) with increasing grade of CIN. The expression of P16 was diffuse in CIN3 and SCC indicating HPV integration with host genome and stable production of HR-HPV E7 protein. P16 expression had inverse cor-

relation with expression of CK7, in CIN 1 predominantly strong in superficial squamous cells and extends downwards with progression towards CIN 2/3 (top downward expression) indicating downward clonal expansion of CK7 positive metaplastic and dysplastic cells in SCC however was not a consistent findings [25].

A study by Hebbar et al has stated that the P16 expression correlates with the prevalence of HR-HPV infection and the sensitivity, specificity, PPV and NPV between P16 expression and HPV diagnosis was 76.2%, 87.5%, 96.9% and 41% respectively. P16 was a better predictive marker than HPV-DNA for HSIL and the expression increased with the grade of the dysplasia and neoplasia.P16 marker is more specific for dysplasia and neoplasia than Ki67 and HR-HPV tests.P16 marker has better follow-up than Hybrid capture test which is specific for HR-HPV associated dysplasia [7].

As per the semi-quantitative P16 scoring methods ofKlaes scoring system where focal and diffuse is referred to <25% and >25% staining and association with HR-HPV is 27% and 76% respectively. The PPV and NPV of HR-HPV in P16 block positive cases was 97% and 86% respectively [18]. LR-HPV showed no or weak staining. HR-HPV showed diffuse and strong P16 positivity indicating genetic instability of the cells which replicate resulting in dysplastic cells [2]. The P16 expression and HR-HPV status correlated with histological grade. P16 expression has positive correlation with HR-HPV prevalence [10].

In a study, the prevalence of P16 and Ki67 positivity in CIN2 is 92.7% and 98.4%[40]. In United States, P16 biomarker is not used as a standalone test, instead used in conjunction with other tests as Ki67. The dual staining of p16 and Ki67 increases the reproducibility and specificity [5,39,40]. Studies have been done with dual markers P16 and mcm2. No significant difference in results between P16/Ki67 and P16/mcm2 was noted [41]. The dual markers P16 and P63 indicates squamous metaplasia and progression towards higher grades

of dysplasia [43].Hence some authors believe that dual biomarkers eliminates morphological criteria for diagnosis of dysplasia and malignancy in cervical biopsy [41].

Role of P16 Biomarker in Cancers of other Organs

P16 expression in human tissue varies with age. In infants, it is confined to thymus. In adults, it is seen in proliferative endometrium, breast ducts, gastric antral cells, esophageal squamous epithelial cells, salivary glands and some neuroendocrine cells [18].

P16 is expressed in gynecologic tumours related and unrelated to HPV (non-HPV related tumours), oropharangeal squamous cell carcinoma and carcinoma of breast, pancreas, colon, melanomas and head & neck region (related to smoking). The alteration of P16 here are probably deletion, mutation and epigenetic silencing resulting in P16 IHC negative [20]. P16 shows positive expression in some tumours as liposarcoma, gastric adenocarcinoma, Hodgkin & non-Hodgkin lymphomas, pulmonary adenocarcinoma, neuroendocrine carcinoma and subset of uterine carcinoma. The pathogenesis in these cases are HPV independent mechanisms probably gene deletion, point mutation, functional mutations or other mechanisms in pRb pathway as inactivation of pRb resulting in increased expression of P16. Therefore P16 is not 100% specific for proving HPV tumourigenesis [20].

The HPV is related to Squamous cell carcinoma and intraepithelial neoplasia of anal canal, perianal region, vulva, vaginaand penis. In vulva, P16 is negative in normal squamous cells and in squamous cell hyperplasia. Differentiated vulvar intraepithelial neoplasia commonly arises in a background of Lichen Sclerosis, which is unrelated to HPV and is P16 negative. Hence P16 can be used to rule out this entity [20]. The invasive squamous cell carcinomas with HR-HPV related are P16 positive and those with non-HPV related are either P16 positive or negative [18]. P16 positive expression of squamous cell carcinoma of

vulva are associated with less aggressiveness, less possibility of recurrences and less mortality [43].

Among the gynecologic tumours P16 is used to differentiate adenocarcinoma arising from the endocervix and endometrium on biopsy specimens. Endocervical tumour shows strong and diffuse P16 staining. Endometrial adenocarcinoma shows patchy pattern of P16 staining (10% in grade 1 / 2 to 90% in grade3). P16 shows strong and diffuse expression in uterine serous carcinoma, high grade serous carcinoma of mullerian origin, malignant mixed mulleriantumours and undifferentiated carcinoma [20]. In uterus, P16 is negative or focally positive in endometriod variant of endometrial carcinoma. Diffuse positive is seen in a few cases. However squamous elements are strongly positive.P16 is involved in pathogenesis of type II uterine carcinoma. Increased expression of P16 in leiomyomasarcoma is used to differentiate from leiomyoma and STUMP [18]. All tumours are HPV unrelated. The mechanism may be deregulation of Rb pathway like pRb point mutation, deletion or genetic alteration as CCNE1 amplification. Therefore P16 staining has to be correlated with morphological subtype [20].

In ovary, P16 is rarely expressed in benign and borderline ovarian tumours, low in malignant lesions. Increased expression is seen in increased stage. Serous carcinoma is P16 positive and ismoreintense in high grade lesions. Metastasis of endocervical adenocarcinoma to ovary shows P16 expression [18].

Table 6: Practical utility of p16 IHC expression in various gynecologic tumors [20].

P16 IHC expression	Gynecologic tumours
Strong diffuse positivity	Endocervical adenocarcinoma, usual type Endocervical adenocarcinoma in situ Uterine serous carcinoma High grade serous carcinoma of mullerian origin
Patchy (Positive in 10-90% of tumour cells)	Endometrial adenocarcinoma (The extent of staining varies with the grade of the tumour, a few tumour cells with negative staining will be present.

In oropharyngeal Squamous cell carcinoma, P16 is used to detect HPV related SCC of oropharynx as it has better prognosis and distinct from smoking induced SCC of oropharynx. The association of HPV and SCC is strong for those in oropharynx especially of lingual and palatine tonsil and sinonasal carcinomas. However the association is not clear in SCC of other locations of head and neck including nasopharynx and hypopharynx. Patchy false positive is seen in benign tonsillar, non-dysplastic squamous epithelium, tumour-stroma and benign papilloma. HPV RNA ISH is currently considered the gold standard for detecting transcriptionally active HPV. Other mechanisms apart from HPV with P16 expression are noted in SCC of nasopharynx and hypopharynx [6]. P16 is also positive in HPV related neoplasms as tonsillar and anal carcinoma [18].

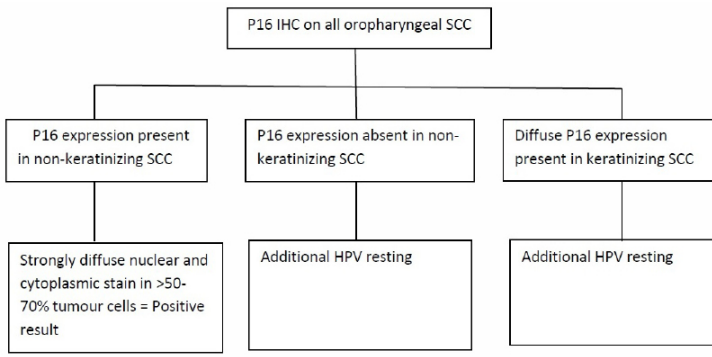

Figure 5: Algorithm for p16 IHC interpretation in oropharyngeal SCC [21].

P16 gene deletion and inactivation is seen in familial melanoma. Diffuse expression is seen in dermatofibrosarcomaprotruberans, gastric adenocarcinoma, Hodgkin's lymphoma, Non-Hodgkin lymphoma, pulmonary neuroendocrine carcinoma, carcinoids, large cell neuroendocrine carcinoma, small cell carcinoma, pulmonary squamous and adenocarcinoma [18].

P16 expressing high grade osteaosarcoma with neoadjuvant chemotherapy has good and pathological complete response compared to P16 negative tumour especially in white population. Hence

it is considered as potential predictor andnon-invasive marker having significant clinical application. However it has to be evaluated among Africans and Asians [44,45].

In metastatic lesions, p16 has limited use. P16 expression is seen in variety of tumours arising from various sites and because of various mechanisms. P16 is positive in cells of branchial cleft cyst and is a differential diagnosis for lymph node metastasis with SCC [20].

Hence sound knowledge of clinical scenarios, histologic findings, technical aspects and criteria for interpretation are necessary for proper utility of P16 IHC in daily practice [20].

IHC P16 Marker and Role of Non-Pathologists

In a study by Liao et al regarding alternative approach by using video trained non-Pathologist readers at Peking Union Medical College in Beijing, China, where P16 diffuse staining was considered as positive and focal / no staining was considered as negative, using pathological analysis as end point, the mean sensitivity and specificity for the non-Pathologist reader's rating of the P16 IHC were 91.7% and 94.1% respectively. Mean total agreement between the non-Pathologist reader (participants) and the study pathologists in reviewing the P16 IHC was 92.8% and kappa was 0.857. The mean sensitivity and specificity for CIN2+ of the non-Pathologist readers were 88% and 87% respectively with Youden index of 75% and positive likelihood ratio of 6.8. The mean sensitivity and specificity for CIN2+ of the study Pathologist scoring P16 IHC was 96% and 92% respectively with Youden index of 88% and positive likelihood ratio of 12. Hence it was concluded that didactic training preferably histotechnologists and educated non-pathologist to evaluate P16 IHC had excellent agreement with senior Pathologists and performance was in par with pathologist's diagnostic significance with H&E stained tissue which can be useful in low and middle income countries especially where Pathologists are insufficient to address the gap in pathologic services for cervical cancer screening [38].

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