Chapter 2

Markers in Cervical Cancer Screening and Diagnosis

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Abstract

Cervical cancer is the common cancer in females worldwide and is common cause of death due to cancer. Screening of cervical cancer by Pap test has reduced the incidence especially in developing countries. However some of the markers are used to detect dysplastic cells in early phase of the disease as a screening method and others suggests the spread of the disease. Many markers are studied and published in English literature right from the Human Papilloma Virus as marker to various proteins which are altered in cell cycle and metastasis. However only a few are used in clinical practice as p16 and Ki-67 markers. Here a list of markers is discussed with their role in cervical cancer etiopathogenesis and its use in screening and diagnosis.

Introduction

Cervical cancer is one of the commonest cancers in females especially in developing countries. The incidence of the disease has decreased in developed countries which are due to organized screening methods. More than 80% of cases are diagnosed at an advanced stage in developing countries in young, working and child rising period which lead to social problems. Pap test is used for cervical cancer screening since 1950 by which the incidence and prevalence has decreased remarkably. However the sensitivity of the test is 60-80%, specificity is 70-95%, false negative rate is 15-50% and false positive is 30%. Many of the tests like HPV test, colposcopy and other markers are
Recent Advances in Cervical Cancer

used to increase the sensitivity of the test [1-3].

Some markers are used to detect HPV virus infection, which is a risk factor. Some markers detect byproducts of HPV virus as E6 / E7 or L1 / L2 and others detect alteration in various proteins which monitor cell cycle in dysplastic / carcinoma cells. The E6 and E7 proteins bind and degrade certain tumour suppressor proteins as pRb, p53, etc which monitor cell cycle. The affinity to bind varies with the oncogenic potential of HPV, with HR-HPV (high risk Human Papilloma Virus) having higher affinity to bind compared to LR-HPV (low risk Human Papilloma Virus). Hence these alterations in different proteins are used to detect the dysplastic cells which help in screening, better diagnosis and reduce diagnostic variability of cervical cancer. Some of the commonly used or studied markers are discussed here [4].

The markers can be broadly classified depending on the site of action of protein in the cell:

Single Marker

- Proved etiological factor: HPV test
- Products of HPV virus: E6 / E7 and L1 proteins
- Growth factor receptor: EGFR, C-erb-2
- Signal transduction protein: RAS, β-catenin, E-cadherin (adhesion molecule), Cytokeratins, PIK-3CA
- Nuclear Regulatory proteins: C-myc

- Cell cycle regulators: p14, p16, p53, pRb, p63, cyclins / cyclin inhibitors, Ki-67, MN antigen
- Regulators of metastasis: Metalloproteinase, Cathepsin, nm23-HI

Combined (Cocktail) Markers

- P16 + Ki-67
- ProExC

HPV Test

Human Papilloma virus (HPV) is a small double stranded DNA virus which infect epithelia of the skin and mucosa. There are 120 HPV types and HPV 16 is the most common high risk type. HPV 16 and 18 is responsible for 60 – 99% cases of cervical cancer. However cervical cancer develops only in a fraction of females with HPV infection with many years following initial infection. Hence HPV is required but insufficient to develop cervical cancer. 11.4% of general population harbor HPV infection in cervix globally. The prevalence of combined HPV 16 and 18 infections are shown in the table 1. Prevalence of HPV 16 and 18 infections separately are shown in table 2. In India, HPV 16 is detected in 4.7% and HPV 18 in 1.3% in NILM. In reactive cases, 22.6% shows HPV 16 and 18 infections [5,6].
Table 1: Shows prevalence of combined HPV 16 and 18 infections.

<table>
<thead>
<tr>
<th></th>
<th>NILM (%)</th>
<th>LSIL (%)</th>
<th>HSIL (%)</th>
<th>Cervical cancer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Worldwide</td>
<td>3.8</td>
<td>24.3</td>
<td>51.1</td>
<td>70.9</td>
</tr>
<tr>
<td>India</td>
<td>6.0</td>
<td>29.4</td>
<td>56.0</td>
<td>82.5</td>
</tr>
</tbody>
</table>

NILM – Negative for intra-epithelial lesion or malignancy

Table 2: Shows prevalence of HPV 16 and 18 infections separately.

<table>
<thead>
<tr>
<th></th>
<th>NILM (%)</th>
<th>LSIL (%)</th>
<th>HSIL (%)</th>
<th>SCC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV 16</td>
<td>13.9</td>
<td>36</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>HPV 18</td>
<td>6.9</td>
<td>8</td>
<td>16.7</td>
<td>-</td>
</tr>
</tbody>
</table>

SCC – Squamous Cell Carcinoma

HPV virus may be transient infection or persists in host cells as episomal form or get integrated into host genome. HPV infection causes risk of cervical cancer once HPV DNA get integrated to host genome and cause expression of viral mRNA protein i.e., E1 to E8, of which E6 and E7 are important to cause inhibition of function of P53 and Rb proteins respectively which is important for cell transformation into a dysplastic cell. Expression of HPV 16-E7 oncogene in cervical epithelial cells is sufficient to initiate oncogenesis [6,7].

The HPV test can be done by various methods as, PCR (polymerase chain reaction), IHC/ICC (immunohistochemistry / immunoytochemistry), Hybrid Capture (HC) 1 and 2. The immunostaining results are in agreement with those of the PCR analysis. HC2 has a sensitivity of 90% and specificity of 62-90%. The HC2 along with Pap test increases the sensitivity. FDA has given approval for detection of HR-HPV (high risk HPV); 16, 18, 31, 33, 3, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 which are mucosotropic HPV genotypes and are carcinogenic. A single HPV test at the age of 30 years in females reduces the occurrence and mortality of cervical cancer in a significant way. In ASCUS-LSIL (atypical squamous cells of undetermined significance – low grade squamous intraepithelial lesion) triage study, HC2 has higher sensitivity and specificity in detecting subsequent HSIL (high grade squamous intraepithelial lesion) in ASCUS than in only Pap test [5-8]. HPV test is superior for stratifying the long term risk for cervical cancer.

However the disadvantages of HPV marker are, in young females HPV infection is transient and only indicates infection which may or may not persist for cell transformation. It does not confirm the cell has undergone immortalization.

L1 Protein

It is a major capsid protein of HPV, expressed in early productive phase of viral life cycle. Once viral genome gets integrated into host genome the L1 protein disappears. It is progressively lost during cervical carcinogenesis and
E6 & E7 Protein

These are early proteins synthesized and released by virus in the host cell following integration of viral genome into host genome. E6 and E7 causes immortalization of HPV infected cells by inhibitory effects on p53 and pRb protein respectively, causes alteration of cell cycle and chromosomal instability.

E6 and E7 proteins can be demonstrated by IHC. Increased expression of these markers indicates integration of HR-HPV DNA into host cell genome and probably immortalization of the host cells. However these markers does not confirm dysplastic change of the host cells, their proliferation and hence carcinogenesis. In addition the immortalized cells can be spontaneously cleared without further progress [4].

Epidermal Growth Factor Receptor (EGFR)

It is a 170 KDa transmembrane glycoprotein receptor encoded by Her-1-proto-oncogene located on chromosome 7 which regulates intracellular signal transmission required for cell growth. Its function is to monitor cell growth, differentiation and development.

Deregulation of EGFR in cervical tissue causes malignant transformation. However exact mechanism is not completely understood. EGFR mutations are uncommon in high grade cervical lesions and invasive cancer. Hence HPV protein probably affects EGFR at the protein level than at the genome level. One explanation is HPV E5 protein binds to a subunit of the protein pump ATPase causing decreased degradation of EGFR, increased EGFR recycling and over expression of EGFR. In addition HPV E6/E7 causes decreased stability of EGFR at post-transcriptional phase giving rise to altered growth rate of cervical cancer cells and oncogenesis [9].

It is normally expressed in the membrane of basal cells of ectocervical epithelium. As cell differentiate the expression shifts from membranous to cytoplasmic. Many studies of EGFR in normal and dysplastic epithelium have shown contradictory results. The intensity of expression varies from mild to strong. The intensity of cytoplasmic expression increases with increasing grades of CIN.
(cervical intraepithelial neoplasia) and is associated with HPV infection but cannot be correlated with HPV types. However increased expression of HR-HPV E6 is associated with an increase in EGFR levels. EGFR expression is higher in tissues of cervical cancer tissues from recurrent / metastatic sites than those obtained from primary sites. Hence EGFR over expression is associated with poor survival / disease outcome and this marker can be exploited for targeted therapy [3].

**C-erb B-2**

C-erb B-2 proto-oncogene codes for a transmembrane tyrosinase kinase 185 kD oncoprotein which is related to the EGFR. It has been suggested that higher levels of C-erb B-2 expression are seen in the late stage of cervical cancer. In early stage of the disease the expression can be low or higher [9,10].

**RAS Gene**

It is a family of oncogenes which code for transducing proteins example p21. The mutant RAS gene converts HPV immortalized keratinocytes into tumorigenesis state. The expression is higher in HSIL and carcinoma compared to normal and LSIL [9].

**β-catenin & E-cadherin**

E-cadherin and β-catenin are adhesion molecules and signal transduction proteins. E-cadherin is 120 kD transmembrane adhesion molecule encoded in chromosome 16. These two molecules act in co-ordination with α-catenin and gama-catenin (cytoskeletal network) responsible for intercellular adhesion, integrity of cells, epithelial polarization, differentiation and stratification. β-catenin in association with APC protein act along the Wnt signaling pathway and monitors the cell proliferation, differentiation and oncogenesis. Hence alteration / mutation in these genes and the protein molecules give rise to disruption in intercellular adhesion and abnormal proliferation of the cells which is property of a cancer cell and it happens in cervical cancer [11-13].

Normally β-catenin is seen in very small amount in cytoplasm as most is seen in complex with E-cadherin, cytoskeletal protein and APC protein. Following signals for proliferation at cell membrane receptor level, the β-catenin tyrosine phosphorylates, dissociate from the E-cadherin complex, redistributes from membrane to non-membrane cytoplasmic and nuclear distribution giving rise to loss of adhesion, interact with other proteins and cause transcriptional activation of variety of critical genes which are features of malignancy [12,13].

HPV infection causes only immortalization of cells. Activation of Wnt pathway in HPV infected cells at multiple levels leads to malignant transformation along with cytoplasmic and nuclear expression of β-catenin. Thus β-catenin accelerate cervical cancer progression [8,11].
These molecules can be demonstrated by IHC where normal or preserved expression gives bright membranous staining. In impaired or abnormal expression it is negative, no staining, cytoplasmic or nuclear staining. Cytoplasmic localization of the markers is seen in LSIL, HSIL and Invasive carcinoma. The marker can be demonstrated in ecocervical as well as endocervical cells. However stromal cells do not take this stain. Loss of expression of these molecules are seen in the early stage of cervical cancer cells i.e. the amount of stain is inversely proportional to the histologic grade in squamous intraepithelial lesions of the cervix where expression of both markers are marked in low grade lesions and is lost in high grade lesions and is observed in all histopathological subtypes (Table 3 & 4). Loss of expression indicates deregulation of cell cycle in early stage of cervical carcinoma. Adenocarcinoma has shown the highest percent of impaired / absent expression of β-catenin and it is significantly associated with advanced pathological stage and disease free survival. Increase expression is noted in HPV 16/18 infection. Hence these markers have role in the pathogenesis of cervical cancer. However some studies have reported that staining has no relation with grade of the lesion. In addition there are studies which states that the staining does not correlate with clinicopathologic parameters of prognostic significance [11-14].

The abnormal expression of these proteins, correlate with invasiveness of cervical carcinoma i.e. metastasis. As E-cadherin in one study has showed expression of 46.2% and 5.9% in superficial and deep muscular invasion respectively [13]. Also abnormal expression of E-cadherin has been associated with clinical stage, lymph node involvement and histological grades [11].

**Table 3:** Shows the relative expression of the two markers in different grades of lesions [13].

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>CIN I</th>
<th>CIN II</th>
<th>CIN III</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td>100%</td>
<td>80%</td>
<td>65%</td>
<td>45%</td>
<td>23%</td>
</tr>
<tr>
<td>β-catenin</td>
<td>95%</td>
<td>70%</td>
<td>30%</td>
<td>20%</td>
<td>13%</td>
</tr>
</tbody>
</table>

**Table 4:** Shows the relative expression of these two markers in different histological types [12].

<table>
<thead>
<tr>
<th></th>
<th>Adenocarcinoma</th>
<th>Squamous cell carcinoma</th>
<th>Adenosquamous carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td>94.3%</td>
<td>86.5%</td>
<td>100%</td>
</tr>
<tr>
<td>β-catenin</td>
<td>85.7%</td>
<td>66.7%</td>
<td>58.3%</td>
</tr>
</tbody>
</table>

**Cytokeratin (CK)**

Cytokeratins are fundamental markers of epithelial differentiation which forms cytoskeleton in all eukaryotic cells. It is required for stability and intracellular signaling pathways and monitors apoptosis, cell growth, tissue polarity, wound response, tissue remodeling and malignant transformation. Cytokeratins persists even in metastatic tumours where all other identifying features are lost. The normal distribution of cytokeratins are; for ectocervical squamous epithelium and mature squamous metaplastic
epithelium - 4,5,13,14; basal layer of stratified squamous epithelium - 5,14,19 and endocervical columnar cells - 16,18; subcolumnar reserve cells - 15,16,17,6 [15-17].

The studies of various cytokeratins in cervical dysplasia and cancer have given a diversified and inconclusive result. The type of cytokeratins positive in SCC differs depending on the histogenesis (arising from endocervical subcolumnar reserve cells or basal cells of ectocervix) and the differentiation. SCC can be positive for cytokeratins 5,14,19,17 (basal layer keratins), 6, 16 (hyperproliferative keratinocyte), 1, 10 (highly differentiated with pearl formation) and 8, 18, 19, 4, 13 (co-expression). Endocervical adenocarcinoma are positive for cytokeratins 7, 8, 14, 17, 18, 19 and adenosquamous are positive for 5, 6, 14, 17, 18, 19. CK 10 indicates suprabasal differentiated keratins although no morphological signs of keratinization is detected. Changes in cervical mucosa following application of acetic acid are due to CK 10. Circulating CK 19 expressing cancer cells are detected in blood samples of patients of untreated early stage cervical cancer suggesting CK 19 responsible for dissemination of cancer cells and hence it can be used as marker for early check point in the multi-step process for developing metastasis in cervical cancer patients. CK 14 is reported to stain predominantly the tumour cells at margin of the cancer lesion suggesting it as a marker for local invasion. Some of the studies have suggested CK 17 as marker of progression from CIN to carcinoma. CK 7 positive and CK 20 negative suggests cervical epithelial cancer and helps to differentiates it from colorectal and anorectal epithelial malignancy [16-22].

**PIK3CA**

This marker is important in lipid signaling pathway. Increased expression of this marker is seen because of 3q gain which occurs in cervical cancer. It can be used as a marker for SIL and can be used as an adjunct tool to HPV-DNA. It is best to detect CIN3 compared to P16 and MIB1 [9].

**C-myc**

These oncogenes function as nuclear DNA transcription factors associated with cell cycle progression, immortalization and differentiation. It also has capacity for transcriptional regression and an additional role in apoptosis. It is expressed in both CIN and invasive carcinoma with varying levels of expression according to the extent of the disease [9].

**P14ARF**

It is a cell cycle inhibitor which blocks minichromosome maintenance protein 2 (MDM2) induced p53 degradation resulting in an increase in p53 levels that leads to cell cycle arrest. p14 over expression is attributable to a negative feedback result in functional inactivation of the p53 protein by E6 HPV oncoprotein during the early immortalization step by HPV in cervical carcinogenesis and
not during late malignant transformation.

This marker gives nuclear positivity by IHC. Intense and high immuno reactivity of p14 in basal and parabasal cells in addition to superficial cells suggest a more advanced lesion. P14 expression is also found among cervical cancer cells negative for HPV. No non-specific staining of normal squamous cells was seen in normal smears with p14. Increased p14 expression is associated with disease progression and time taken for progression was shorter among those stained positive for p14. Hence p14 expression is valuable markers for prediction of cervical disease. The sensitivity and specificity of expression of p14 in HSIL is 74% and 83% respectively [23,24].

P16INK4a

It is a cell cycle regulatory protein having tumor suppression function in cells with intact cell cycle through Rb protein. It is a cell cycle dependent kinase inhibitor acting at S phase in cell cycle. P16 over expression is associated with E7 oncoprotein which inactivates Rb (retinoblastoma) protein. Inactivated Rb protein causes elevated levels of P16 in HR-HPV infected cells. P16 is over expressed as a negative feedback control of functional Rb protein. Hence P16 is a biomarker for detection of HR-HPV infection and a surrogate marker of the E7 mediated inactivation of the tumour suppressor function of Rb protein and indicates dysplastic cell. Hence P16 suggest the transformation of normal epithelium into immortalized cells [25].

P16 can be detected by IHC/ICC and it gives a nuclear and cytoplasmic stain. The staining is diffuse / weak and basal pattern in normal / metaplastic squamous epithelium of cervical biopsy. It is usually nuclear in CINI and cytoplasmic in CIN II, CIN III, invasive cancer and glandular intraepithelial neoplasia. All HPV positive cases express p16 whereas all p16 positive cases are not HPV positive. P16 gives strong and diffuse staining in all preneoplastic lesions (LSIL & HSIL), squamous cell carcinoma and in high / intermediate risk HPV infection. Staining intensity is focal and weak in HPV negative cases and cases with LR-HPV. P16 positivity suggests disease progression and cell takes shorter time to progress to malignancy. However p16 immunoreaction are occasionally positive in; tubal metaplastic cells, squamous metaplastic cells, endocervical cells, giant cells, malignant/benign endometrial cells, Trichomonas, atypical glandular cells, koiocytes and atrophic cells with minimal nuclear aberration. Hence scoring of P16 expression by combining morphologic criteria and p16 staining was put forth by Wentzenson et al which give good correlation between P16 score and Pap smear results with excellent inter-observer concordance. The p16 scoring is as follows; score 1 – No staining in any squamous epithelial cells, score 2 – staining in cells having increased N:C ratio / altered chromatin / altered nuclear shape or anisonucleosis, score 3 – staining in cells having increased N:C ratio + altered chromatin / altered nuclear shape / anisonucleosis, score 4 - staining in cells having...
increased N:C ratio + more altered chromatin and / or altered nuclear shape and / or anisonucleosis. Score 3 and 4 has a more favorable P value in follow-up group predicting HSIL more in association with HC2 positivity for HR-HPV [2,5].

P16 expression by IHC and HSIL detection by Pap smear has got good correlation. P16 expression by IHC is superior to assay showing HR-HPV viral load as P16 assess gene expression / alteration and not merely the presence of virus.

**P16 marker has following advantages:**
- Efficient triage to colposcopy in women with ASCUS/LSIL and finding / predicting the underlying HSIL.
- Has similar or slightly low sensitivity but has high specificity compared to HPV test.
- P16 is over expressed 100% in HSIL, SCC, adenocarcinoma and 70-100% in LSIL.
- Helps in differentiating cervical dysplasia (HSIL) and reactive lesions (immature squamous metaplasia).
- Sensitivity and specificity of p16 positivity in HSIL is 79% and 83% respectively.
- Utilized to detect HR-HPV than HC2.
- Avoids unjustified anxiety.
- Gives better correlation with subsequent tissue diagnosis as increase risk of subsequent HSIL in LSIL.
- Hence p16 is used as surrogative marker [2,5,22,25,26]

**P16 marker has following disadvantages** [2,5,22,26,27]:
- Lack of standard criteria in cytological evaluation regarding staining intensity, nuclear versus cytoplasmic staining, absence of consensus for threshold value of positivity, and use of p16 marker plus morphological interpretation to be used for better results
- Less access to the test especially in developing countries
- Cost of the test
- Inappropriate in low research setting without patient follow-up
- Cost to be balanced against the potential increased specificity which should be acceptable to risk sensitive potentials and health care professionals

Hence the clinical utility of p16 is yet to be determined.
P53

It is a tumour suppressor gene, has control on cell cycle through p21. The gene is located on chromosome 17 and wild type monitors the integrity of the cell. Loss of p53 function is believed to play an important role in the pathogenesis of cervical cancer.

P53 gene may be inactivated by three ways as; somatic point mutation, loss of heterozygocity, and by HPV oncoprotein E6 which binds wild type p53 and causes degradation by cellular ubiquitin pathway. The point mutation occurs at 72 codon where arginine is substituted by proline. The two variants of p53, p53arg and p53pro, behave differently. The p53arg are more efficiently inactivated by HR-HPV E6 protein than p53pro variant and women with p53arg are at higher risk of HPV associated cervical cancer than p53pro. Mutation causes functional inactivation of p53 with/without association of E6. Degradation and inactivation of p53 by HR-HPV E6/E6AP (associated protein) complex by binding to central region of p53 is important in cervical carcinogenesis. Other cellular proteins are implicated in the inactivation of HPV negative carcinoma. Hence inactivation p53 causes increased proliferation and decreased apoptosis [27-29].

The expression can be demonstrated by immunohistochemistry technique. The expression progressively increased from LSIL to HSIL, squamous cell carcinoma and a few cases of adenocarcinoma compared with normal cervix [28]. Some authors on contrary have found relative increased expression of p53 in CIN I compared to CIN II/III which may be due to p53 degradation by HPV. However p53 does not give prognostic information and not correlate with tumour recurrence [30].

p63/p73

These are homologue of tumour suppressor gene p53. Normally the expression is seen in basal cells and immature squamous cells of atrophic epithelium. The expression of these molecules is increased in CIN and expression increases in higher grade of CIN. It can be used as a marker for grading CIN. p63 and p73 are detected in HSIL and cervical cancer. Expression of p63 in ASCUS and p73 in LSIL correlated with increased rate of progression to HSIL. Morphological assessment in addition decrease false positives [22].

pRb

It is a product of tumour suppressor gene Rb (retinoblastoma) gene. It regulates cell cycle at the entry of cell into S phase. The E7 protein (nuclear resident) of HR-HPV interacts with / degrades by ubiquitin pathway the dephosphorylated form of pRb and release the growth promoting factors such as E2F-1, cyclins, Cdk5 and Myc. Mutation of Rb gene causes loss of normal Rb protein function [31].
The expression of Rb protein is detected by IHC. Rb gene alteration can be appreciated by Southern blot technique. The positive immunostaining is quite heterogeneous, some cells show nuclear staining, some cytoplasmic and others both nuclear and cytoplasmic. Nuclear reactivity also seen in normal endocervical glands and infiltrated lymphocytes which act as internal control. Expression of Rb protein is high in premalignant and malignant lesions compared to normal cervical squamous epithelium. The expression is higher in well differentiated tumours than in poorly differentiated tumours and complete loss of expression in undifferentiated tumours. In advanced / invasive carcinoma the expression decreases compared to squamous intraepithelial lesion. Expression of Rb protein has an inverse correlation with E7 expression especially well detected in HSIL / carcinoma where increased expression of E7 causes decreased / complete disappearance of Rb protein expression. However no relationship between the Rb protein expression and clinical stage is proved [28,31]. One study has showed that the nuclear pRb expression is maximum in intraepithelial neoplasia compared to normal and malignant lesion where as cytoplasmic expression of pRb progressively increased from intraepithelial lesion to frank malignancy [28].

Cyclins

Normally cyclins are weakly expressed in parabasal and basal cells (8%). It is synthesized in late G phase of cell cycle and required for moving into S phase. Expression is diminished rapidly as the cell enters into phase S. In premalignant and malignant cells, increased expression indicates prior detection of deregulation of the cell cycle at molecular level induced by HR-HPV [10,27].

Cyclin A activation and increased expression occurs due to degradation of p53 by E6. LSIL with HR-HPV show 100% positive for cyclin A. Cyclin E activation and increased expression is due E7 coupling with E2F causes prolongation of phase S. (cyclin E/E/cdk complex). HPV 16 with mixed and integrated forms shows expression of cyclin A (72%) and E (76%) respectively. Expression will increases as lesion grade increases. Over expression in early lesions indicates bad prognosis and poor outcome in cervical carcinomas associated with HR-HPV. Cyclin D1 aberration is detected in late events (CIN III & Invasive SCC). It forms complex with cyclin-dependent kinase 4 or 6 to carry out the phosphorylation of pRb and has a role in cell cycle. Over expression of cyclin D1 has been found to be an independent factor associated with poor prognosis in cervical cancer [10,27].

Ki-67

It is a proliferative marker and expressed in cells in all phases except in G0 phase of cell cycle with maximum intensity during mitosis. It is an antigen marker for G1, S, G2/M phase. It is a gold standard proliferative index. MIB 1 is a corresponding monoclonal antibody marker.
HPV infected cells following immortalization give rise to increase cell cycle progression with increase cell cycle kinetics results in increase Ki-67 / MIB 1 staining and index [22,28].

The marker can be demonstrated by IHC and it takes nuclear stain. In normal cervical epithelium, the expression is seen only in basal and parabasal epithelium. Increased expression is seen in cervical dysplasia and carcinoma. In CIN cases the expression is seen in throughout the different epithelial layers, increases with the grade of the dysplasia independent of histology, thus has potential prognostic value and valuable follow up. It is an additional prognostic marker and correlates with the histological grade of cervical carcinoma. The higher expression is seen in tumours with a higher grade / higher stage at diagnosis associated with poor outcome and tumours with HR-HPV 16/18 infection compared with LR-HPV. Ki-67 does not correlate with behavior of the cervical cancer [10,14,27,28].

**MN Antigen**

The membrane associated MN antigen has homology with carbonic anhydrase. Expression of this protein has only recently been detected in cervical neoplasia and correlate with loss of a tumour suppressor gene on chromosome 11, possibly at an early stage of oncogenesis. Low expression is noted with poor differentiation, the adenosquamous histologic type, deep stromal invasion, regional lymph node metastasis and HPV negativity. The role of this marker protein as a prognostic marker in cervical cancer is yet to be determined [9].

**Metalloproteinase (MP)**

The metalloproteinase cause degradation of basement membrane type IV collagen assisting early phases of tumour invasion and thereby metastasis. Increased expression is demonstrated by IHC in microinvasive squamous cell carcinoma compared with CIN lesions. A significant relationship is found between tumour MP index and presence of nodal metastasis, number of positive nodes and recurrence risk. Hence one can predict the prognosis and this marker can be used for targeted chemotherapy [9].

**Cathepsin**

This is a acid proteinase which degrades extra-cellular matrix and activates other proteinases. Various studies about this molecule with respect to cervical dysplasia and carcinoma have shown conflicting results. Some studies has shown that positive staining in SCC correlates with lymph node metastasis and lower relapse free survival having independent prognostic significance especially in early invasion. Hence its detection / expression in dysplasia and microinvasive stage I help in predicting metastasis [9].
nm23-HI

The nm23-HI gene is a metastasis suppressor gene; the protein product of this gene modulates intracellular signal transduction by phosphorylation of GTP binding proteins. It plays vital role in cell attachment and detachment to extracellular matrix. Increased expression is seen with high incidence of lymph node involvement and poor prognosis especially in the adenocarcinoma variant of cervical cancer [9].

P16 & Ki-67 Dual Staining

In normal cells, simultaneous expression of P16 and Ki-67 in same cell mutually exclude each other. However simultaneous expression of cell cycle dependent kinase inhibitor (tumour suppressor protein) p16 and expression of the proliferation marker Ki-67 within the same cervical epithelial cell indicate viral E7 mediated inactivation of tumour suppressor protein Rb protein, deregulation of the cell cycle, formation of immortalized cells with genetic instability and proliferation of the immortalized cells giving rise to the malignant transformation. In cervix it is seen in CIN especially in higher grades. Usually negative in CIN I. Hence this dual stain is utilized in cervical cytology to detect HSIL (CIN II /III) in pap cytology which is independent of the morphology based interpretation with P16 staining [22,25].

P16 – Ki-67 stain is available as CIN tec plus kit which is done in two steps (ICC) staining procedure where the primary antibody cocktail consists of mouse monoclonal antibody of P16 and rabbit monoclonal antibody of Ki-67. The antibody of P16 follows horse raddish peroxides method with secondary goat anti-mouse antibody with DAB (diamimo benzidine) and positivity is shown as brown colour. The Ki-67 antibody follows alkaline phosphatase method with secondary antibody goat anti-rabbit antibody with fast red and the positivity is shown as red colour. Presence of one or more than one cervical epithelial cells showing within the same cell a brown cytoplasm and red nuclear staining suggest P16 and Ki-67 expression respectively and the result is positive.

Advantages of P16 / Ki-67 stain:

- pCan achieve maximum test sensitivity
- Simplicity in interpretation
- Morphology independent
- Helps to develop computer assisted slide reading approaches in future giving higher level of automation in cell based cervical cancer screening
- Has comparable sensitivity but significant higher specificity compared to HPV test and P16 single stain cytology
- Can be used as triage in ASCUS / LSIL study.
- Used to correctly identifying dysplastic cells with-
out biopsy confirmation
- Identify HSIL (CIN II) with high sensitivity and specificity
- Strong in predicting immediate outcome
- Used to improve the management of women with equivocal/low grade abnormal cytology results and reduce unnecessary follow-up diagnostic procedure [4,14,22,23].

**BD-ProExC**

ProEx C has two monoclonal antibodies. One is MCM2 and the other is DNA topoisomerase II alpha (TOP2A). MCM2 and other MCM proteins has important role in early S phase of DNA replication and hence useful in detection of dysplasia and cancer. However this marker occasionally stains normal glandular cells, tubal metaplasia in cytology, parabasal cells in atrophic epithelium, benign endometrial cells and endometrial stromal cells. DNA topoisomerase II alpha has a role in cell cycle by modulating the topological structure of DNA during early S phase of replication.

ProExC is expressed when viral DNA integrates with host genome indicating increased levels of E6 and E7 with aberration of S phase induction. These markers take nuclear stain. Hence ProExC is a sensitive biomarker to detect HSIL, small cell carcinoma and SCC and used to detect HSIL in cytology since 2006. The sensitivity and specificity to detect HSIL are 92% and 84% respectively.

BD-ProExC has advantage over P16/MIB 1 as in staining by ProExC the background is clear, easy to interrupt, more selective and information for progression of LSIL better than MIB 1 [27].

**p16 & ProExC**

It is a cocktail of P16 and ProExC which indicates immortalization of host cells by HPV infection followed by alteration of DNA synthesis at early S phase of cell cycle. Hence suggest progression and proliferation of immortalized cells to malignancy. It detects HSIL in 100% and LSIL in 70-100% cases [2].

**P16 and HPV L1**

In cocktail marker, HPV L1 indicates the early phase of HPV infection and p16 indicates disruption of cell cycle and immortalization in HPV infected cells. Thus this marker predicts the progression of cervical lesion in HPV infected cells [14,22].

**Conclusion**

There are many markers to detect cervical lesion from phase of HPV infection to immortalization of cells by involvement of various proteins involved in cell cycle which are used to detect dysplastic cells. Later the proliferation of this immortalised cells finally progressing to carcinoma. The markers can be used as single or in combina-
tion knowing the advantages and disadvantages of each. Among single markers p16 is commonly used as surroga-
tive marker. Combined / cocktail markers are more useful and informative.

Markers can be used to detect dysplastic and malignant cells with High sensitivity and specificity. It gives bet-
ter understanding of genetics and biology of cervical car-
cinogenesis. It also helps in developing chemopreventive/ therapeutic strategies and targeted therapy.

However further studies is needed in depth using large sample size to prove the best marker for screening, early diagnosis and therapy. The availability and accessibility of these markers is difficult in developing countries which should be made up by organizations taking up cervical cancer screening programs.

References


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