"STUDY AND ANALYSIS OF p53, pRb AND Ki-67 EXPRESSION IN DYSPLASTIC AND MALIGNANT CERVICAL LESIONS"

By

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Dissertation Submitted to the
SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION AND RESEARCH
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In partial fulfillment
Of the requirements for the degree of

DOCTOR OF MEDICINE

IN

PATHOLOGY

Under the Guidance of
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APRIL - 2013

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LIST OF ABBREVIATIONS USED

AC	»	Adenocarcinoma
AC-VG	»	Adenocarcinoma- Villoglandular type
AC-EN	»	Adenocarcinoma- Endodcervical type
AS	»	Adenosquamous carcinoma
B-SA	»	Biotin Streptavidin
CIN	»	Cervical Intraepithelial Neoplasia
CK	»	Cytokeratin
HPV	»	Human Papilloma Virus
DAB	»	Di-amino Benzidine
DNA	»	De-oxyribonucleic acid
EGFR	»	Epidermal Growth Factor Receptor
FFPE	»	Formalin- Fixed Paraffin Embedded Tissue
HIV	»	Human Immunodeficiency Virus
HPE	»	Histopathological Examination
HSIL	»	High grade Squamous Intraepithelial Lesion
IHC	»	Immunohistochemistry
ISCC	»	Invasive Squamous Cell Carcinoma
L-NKSCC	»	Large cell Non-Keratinising Squamous Cell Carcinoma
LSIL	»	Low grade Squamous Intraepithelial Lesion
MDSCC	»	Moderately Differentiated Squamous Cell Carcinoma
MI	»	Micro-invasion
PAP	»	Peroxidase Anti-peroxidase

PDSCC	»	Poorly Differentiated Squamous Cell Carcinoma
PFPE	»	Perfluoropolyether
pRb(c)	»	pRb(cytoplasmic)
pRb(n)	»	pRb(nuclear)
Rb	»	Retinoblastoma
SCC	»	Squamous Cell Carcinoma
SIL	»	Squamous Intraepithelial Lesion
S-NKSCC	»	Small cell Non-Keratinising Squamous Cell Carcinoma
WDPV	»	White Discharge Per Vagina
WDSCC	»	Well Differentiated Squamous Cell Carcinoma

ABSTRACT

BACKGROUND

Cervical carcinoma is one of the most frequent malignancies in women worldwide. Human Papillomavirus (HPV) 16 and 18 are most important risk factors for cervical carcinogenesis. p53 and Rb are the two important tumour suppressor genes which interact with HPV oncoproteins and cause cervical cancer. Ki-67, a proliferative marker helps to know the tumour status.

OBJECTIVE

1. To grade the cervical dysplasia and malignancies based on histomorphological findings.
2. To study the expression of p53, pRb and Ki-67 markers in various grades of dysplasia and malignant lesions of cervix.

MATERIALS AND METHODS

A total of 120 cervical tissue samples were included. These represented normal, dysplasia and malignancy. Immunohistochemical staining was performed for p53, Ki-67 and pRb in formalin-fixed, paraffin-embedded tissue sections of the uterine cervix using horse-peroxidase method.

RESULTS

There was significant association of p53 expression between normal cervical epithelium and LSIL, SCC and AC ($p= 0.023$, $p<0.001$ and $p=0.004$ respectively), but no significant association with HSIL ($p=0.09$). In case of pRb(n) expression significant association was found when normal was compared with LSIL and HSIL ($p<0.001$, $p=0.001$), no association was found with SCC and AC. pRb(c) expression showed significant association when normal was compared with HSIL, SCC and AC

but no association was found with LSIL. Whereas Ki-67 expression showed highly significant association with LSIL, HSIL, SCC and AC.

CONCLUSION

Squamous cell carcinoma is the commonest histological type of carcinoma cervix which is commonly seen in 4^{th} decade of life in women. p53 expression progressively increases from normal cervical epithelium to intraepithelial lesion to malignant lesion. However pRb (n) expression was maximum in intraepithelial lesion compared to normal cervical epithelium and malignant lesion. pRb(c) expression as p53 protein progressively increases from intraepithelial lesion to frank malignancy. Ki-67 expression is directly proportional to the degree of dysplasia to malignant lesion. However further studies is required to exactly analyze the cytogenetic aberration in p53 and pRb genes. The present study can be used as a base line study of the expression of p53 and pRb protein in cervical neoplasia. p53 and pRb(c) can be used as marker for early detection of cervical intraepithelial neoplasia.

KEY WORDS

Cervical carcinoma, Human papilloma virus, Low grade squamous intraepithelial lesion, High grade squamous intraepithelial lesion, Squamous cell carcinoma, Adenocarcinoma, p53, Ki-67, pRb.

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INTRODUCTION

Cancer is the most dreaded disease in the world.[1] Among all the malignancies occurring in females carcinoma cervix is one of the most frequent malignancy in women worldwide, with an incidence of approximately 4,56,000 cases per year leading to 2,00,000 deaths per year.[2] In India it is estimated that approximately 1,00,000 women develop cervical cancer each year and it is posing a major public health problem.[3] Both the incidence and mortality rate have been dramatically reduced in the United States and other developed countries during the last several decades. This is due to the introduction of organised cervical cytological screening programme.[4,5]

Carcinoma cervix emerges from a defined series of preneoplastic lesions with increasing cellular dysplasia referred to as cervical intraepithelial neoplasia(CIN) grade I, II and III.[6] Many epidemiological studies conducted over the last 20 years have established a strong association between the high risk human papilloma virus (HPV type 16 and 18) and cervical cancer in upto 95% of cases . For the discovery of HPV as a cause of cervical cancer, Harald zur Hausen was awarded the Nobel Prize in 2008. HPVs are DNA viruses that are typed based on their DNA sequence and subgrouped into high and low oncogenic risk. High oncogenic risk HPVs are currently considered to be the single most important factor in cervical oncogenesis.[7] However viral infection alone is not sufficient to initiate malignant transformation. Additional genetic changes must occur in order for malignant transformation to take place.[4,5,6]

No form of cancer better documents the remarkable effects of screening, early diagnosis, and curative therapy on the mortality rate than does cancer of the cervix. In sharp contrast to this reduced mortality, the detection frequency of early cancers and precancerous lesions is high. Much credit for these dramatic gains belongs to the effectiveness of the Pap test in detecting cervical precancers and to the accessibility of the cervix to colposcopy (visual examination of the cervix with a magnifying glass) and

biopsy. While there are an estimated 11,000 new cases of invasive cervical cancer in the United States annually, there are nearly 1 million precancerous lesions of varying grade that are discovered yearly by cytologic examinations. Thus, it is evident that Pap smear screening not only has increased the detection of potentially curable, low-stage cancers but has also allowed the detection and eradication of preinvasive lesions, some of which would have progressed to cancer if not discovered and treated.[7]

The main interpretative categories include distinguishing normal from dysplasia (CIN) of any grade and low-grade (CIN1) lesions from high-grade (CIN2/3) lesions. The HPV life cycle and molecular events leading to cellular transformation have not been completely elucidated. These events have provided insight into potential biomarkers that can be used as adjunctive tests to improve diagnostic accuracy of cervical lesions. In addition they serve to identify those patients at risk for progression to cancer.[8]

Recently developed biomarkers include c-myc, Ras, c-reb B-2, PCNA, Ki-67, nm23-H1, MN protein and metalloproteins. These biomarkers may be useful for evaluating the biological potential of early CIN lesions.[9] Emphasis is given at a practical level to markers which can preferentially be applied to tissue sections rather than involving other modalities of investigation which may require specialised equipment and technology. No single marker of those previously listed was found to have outstanding prognostic significance. Although some have shown promise in initial studies, subsequent investigations have not provided corroborating evidence or in some situations, have also led to conflicting results. Difficulties inherent in establishing the prognostic value of individual markers also include the multifactorial complexity of cervical carcinogenesis itself. The future awaits a greater amount of data to be accrued across all stages of disease, with improved standardization of results.[1]

Most prominent among the regulators disrupted in cancer cells are two tumor suppressor genes, the retinoblastoma protein (Rb) and the p53 transcription factor. In an attempt to explain their potentially universal involvement in the etiology of cancer, the interconnecting signaling pathways controlled by Rb

and p53 are studied in detail and will be discussed ahead. The Ki-67 protein plays an important role in cell proliferation. Its antigen is expressed during the cell cycle with the exception of the G0 phase, and has been used as a marker for proliferation in various tumors, including cervical carcinoma.[12]

AIM OF THE STUDY

1. To grade the cervical dysplasia and malignancies based on histomorphological findings.
2. To study the expression of p53, pRb and Ki-67 markers in various grades of dysplasia and malignant lesions of cervix.

REVIEW OF LITERATURE:

History and background:

Carcinoma of the uterine cervix is the most frequent malignancy in women[2]. The majority of cervical cancers are squamous cell carcinomas. These lesions arise from the squamocolumnar junction and may be keratinizing or nonkeratinizing. Adenocarcinoma of the uterine cervix arises from the endocervical columnar cells and account for about 14% of cervical carcinomas. The percentage of adenocarcinoma has increased because, they are more difficult to detect at a preinvasive stage. Although most clinical studies on cervical neoplasia have involved patients with squamous-cell carcinomas, patients with adenocarcinoma are generally treated similarly. It was reported that adenocarcinoma has a worse prognosis than squamous cell carcinoma. However several recent investigations showed that long-term survival rates for these two histological types of disease are not significantly different.

Immunohistochemistry (IHC)

It is a method for localizing specific antigens in tissues based on antigen-antibody recognition. It seeks to exploit the specificity provided by the binding of an antibody with its antigen at a light microscopic level. IHC has a long history, extending more than half a century from 1940, when Coons developed an immunofluorescence technique to detect corresponding antigens in frozen tissue sections. However, only since the early 1990s has the method found general application in surgical pathology. A series of technical developments led eventually to the wide range of IHC applications in use today. The enzymatic label (horseradish peroxidase), developed by Avrarneasf and by Nakane and colleagues allowed visualization of the labeled antibody by light microscopy in the presence of a suitable colorogenic substrate system.

In Oxford, Taylor and Burns developed the first successful demonstration of antigens in routinely processed formalin-fixed paraffin-embedded (FFPE) tissues. A critical issue in the early development of immunoperoxidase techniques was related to the need to achieve greater sensitivity. Greater sensitivity would facilitate staining of FFPE tissues from a simple one-step direct conjugate method to multiple-step detection techniques such as the peroxidase antiperoxidase (PAP), avidin-biotin conjugate (ABC), and biotin- streptavidin (B-SA) methods, and would eventually lead to amplification methods (such as tyramide) and highly sensitive "polymer-based" labeling systems.

Use of IHC in diagnostic pathology has expanded such that the use of one or more IHC "stains" is routine in surgical pathology, especially in tumor diagnosis and classification. Furthermore, IHC has been adapted to the identification and demonstration of both prognostic and predictive markers, with corresponding requirements for semi-quantitative reporting of results.

Only when the IHC technique became applicable to routine PFPE (Perfluoropolyether) tissue sections did it usher in the “brown revolution”. The critical significance of rendering the IHC technique suitable for routine paraffin sections was illustrated in 1974 by Taylor and Burns, who showed that it was possible to demonstrate at least some antigens in routinely processed tissue. These initial studies led to serious attempts by pathologists to improve the ability to perform IHC staining on FFPE sections.[13]

Anatomy of the cervix[14]:

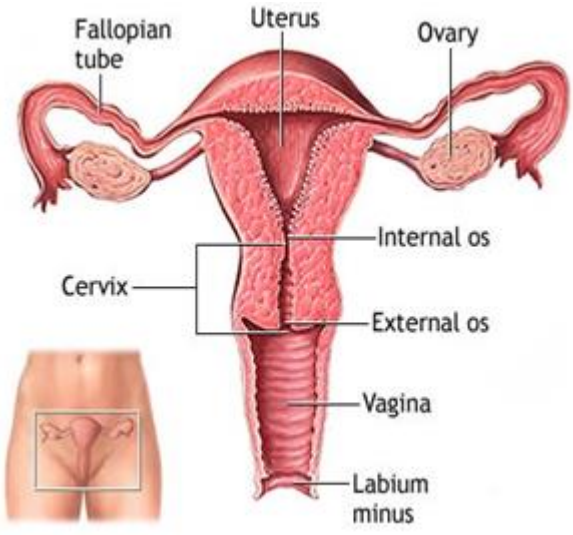


Fig 1:- Anatomy of uterus and cervix

Normal Histology

Cervical squamocolumnar junction showing mature, glycogenized *(pale)* squamous epithelium, immature *(dark pink)* squamous metaplastic cells, and columnar endocervical glandular epithelium.[7]

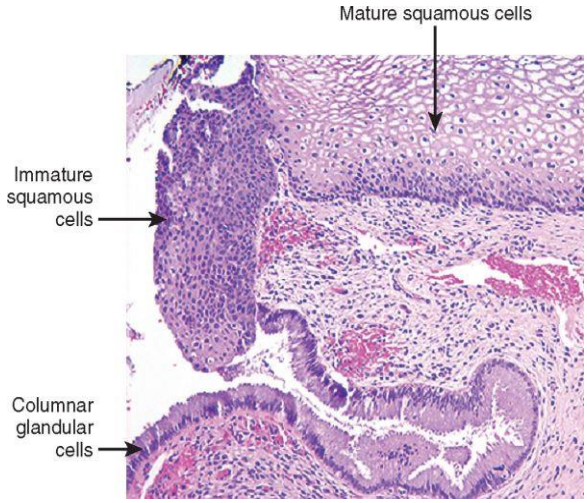


Fig 2:-Microphotograph showing squamo- columnar junction of cervix

Global burden of cervical cancer[15]

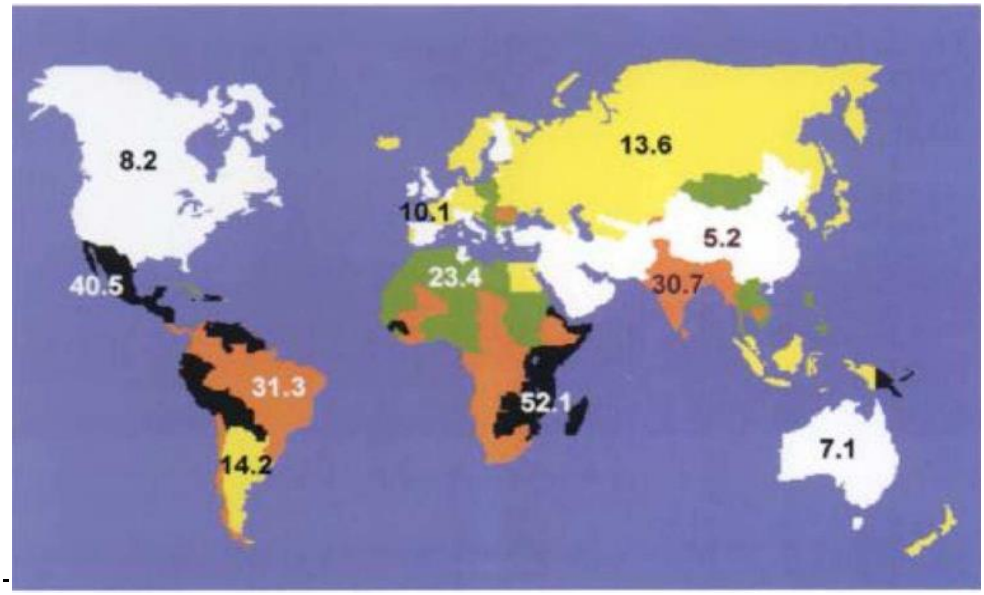


Fig 3:- Global burden of cervical cancer. Age- standardized incidence rates per100,000 population and year.

Etiology

The association between cervical neoplasia and sexual activity is well established and current studies have identified the Human Papilloma Virus (HPV) as the most important factor responsible for this association. HPV therefore, may be a causal agent in cervical neoplasia.[7] An epidemiologic study by Schiffman et al showed that the increased risk of cervical intraepithelial neoplasia (CIN) previously associated with other factors (such as increased number of sex partners, earlier age at first intercourse, lower level of education, lower income, and smoking) is actually a result of HPV infection. The only risk factor studied that was noted to be independent of HPV status was increased parity. Schiffman et al concluded that HPV satisfied all the requirements that designate a cause of cervical neoplasia.[16]

The risk factors for cervical cancer are related to both host and viral characteristics such as HPV exposure, viral oncogenicity and inefficiency of immune response and presence of co-carcinogens. The risk factors include:[7]

1. Multiple sexual partners
2. A male partner with multiple previous or current sexual partners
3. Young age at first intercourse
4. High parity
5. Persistent infection with a high oncogenic risk HPV, e.g., HPV 16 or HPV18
6. Immunosuppression
7. Certain HLA subtypes
8. Use of oral contraceptives
9. Use of nicotine[7]
10. Prenatal exposure to diethylstilbestrol

Conflicting data have been reported for other postulated risk factors for cervical neoplasia, including Herpes simplex virus type 2 infection, vitamin A, vitamin C deficiencies and use of oral contraceptives and.[16]

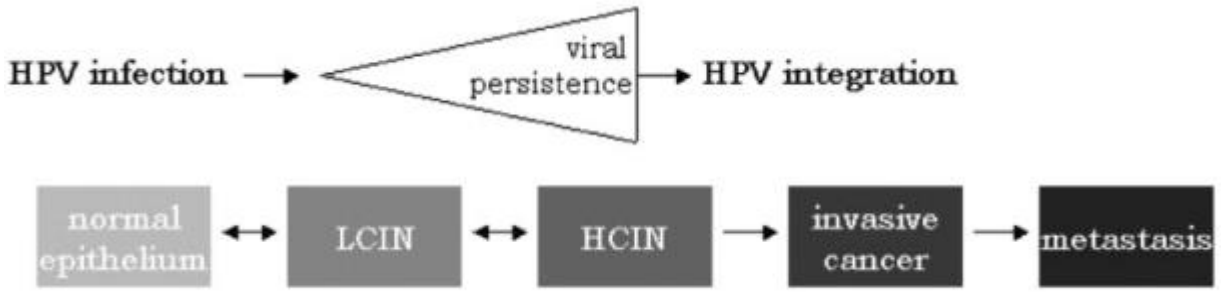
Pathogenesis.

The pathogenesis of cervical carcinoma has been delineated by a series of epidemiological, clinicopathological and molecular genetic studies. Epidemiological data have long implicated a sexually transmitted agent, which is now established to be HPV. Many types of HPV have been isolated in the human genital tract infection with HPV types 16, 18, 45 or 56 have a high correlation with cervical cancer. In a retrospective study by Lorincz et al these high risk types of HPV were present in 74% of cases of invasive cervical cancer and in 53% of cases of moderate to severe dysplasia. HPV 16 is the most prevalent among these types. HPV types 31, 33, 35, 51, 52 and 58 were deemed intermediate risk viruses and were

present in 10% of cases of invasive carcinoma and in 24% of cases of moderate to severe dysplasia.

HPV 16 and HPV 18 are the most important. HPV 16 alone accounts for almost 60% of cervical cancer cases, and HPV 18 accounts for another 10% of cases; other HPV types contribute to less than 5% of cases individually. The duration of the infection is related to HPV type; on average, infections with high oncogenic risk HPVs last longer than infections with low oncogenic risk HPVs, 13 months versus 8 months respectively. Persistent infection increases the risk of development of cervical precancer and subsequent carcinoma.[7] A direct molecular interaction between HIV and HPV has also been described, with HIV gene products causing transaction of HPV proteins.

Chart 1:- <u>HPV- induced cervical cancer, a multistep process</u>



HPVs infect immature basal cells of the squamous epithelium in areas of epithelial breaks, or immature metaplastic squamous cells present at the squamocolumnar junction. HPVs cannot infect the mature superficial squamous cells that cover the ectocervix. Establishing HPV infection in these sites requires damage to the surface epithelium, which gives the virus access to the immature cells in the basal layer of the epithelium. The cervix, with its relatively large areas of immature squamous metaplastic epithelium, is particularly vulnerable to HPV infection as compared to mature squamous epithelium. This difference in epithelial susceptibility to HPV infection accounts for the marked difference in incidence of HPV-related cancers arising in different sites, and explains the high frequency of cervical cancer in women or anal cancer in homosexual men.[7]

Although the virus can infect only the immature squamous cells, replication of HPV occurs in the maturing squamous cells and results in a cytopathic effect, "koilocytic atypia," consisting of nuclear atypia and a cytoplasmic perinuclear halo. To replicate, HPV has to induce DNA synthesis in the host cells. Since HPV replicates in maturing, nonproliferating squamous cells, it must reactivate the mitotic cycle in such cells. Experimental studies have shown that HPV activates the cell cycle by interfering with the function of Rb and p53, two important tumor suppressor genes.[7]

Viral E6 and E7 proteins are critical for the oncogenic effects of HPV. They can promote cell cycle by binding to Rb and up-regulation of cyclin E (E7), interrupt cell death pathways by binding to p53 (E6), induce centrosome duplication and genomic instability (E6, E7) and prevent replicative senescence by up-regulation of telomerase (E6). HPV E6 induces rapid degradation of p53 via ubiquitin-dependent proteolysis, reducing p53 levels by two- to three-fold. E7 complexes with the hypophosphorylated (active) form of Rb, promoting its proteolysis via the proteosome pathway. Because hypophosphorylated Rb normally inhibits S-phase entry via binding to the E2F transcription factor, the two viral oncogenes cooperate to promote DNA synthesis. This interrupts p53-mediated growth arrest and apoptosis of genetically altered cells. Thus, the viral oncogenes are critical in extending the life span of epithelial cells, a necessary component of tumor development.[7]

Chart 2:- Mechanism of human papilloma virus

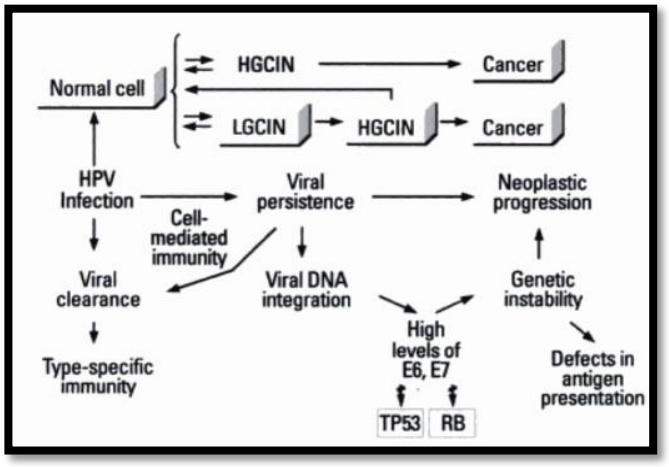
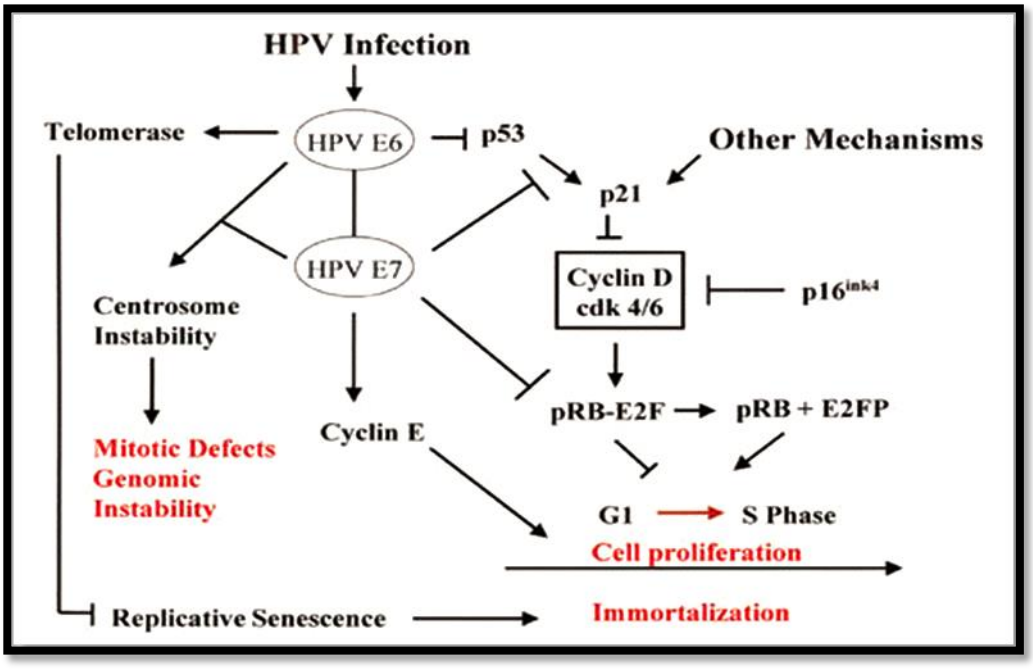


Chart 3:-Diagram of mechanisms by which human papillomaviruses (HPV) influence cell biology, including alterations in cell cycle, centrosome duplication, and increased telomerase expression.



In addition to infecting squamous cells, HPVs may also infect glandular cells or neuroendocrine cells present in the cervical mucosa and cause malignant transformation, resulting in adenocarcinomas,

adenosquamous and neuroendocrine carcinomas. These tumor subtypes, however, are less common since glandular and neuroendocrine cells do not support effective HPV replication.[7]

WHO Histological Classification of Tumors of the Uterine Cervix

Epithelial Tumors

Squamous tumors and precursors

Squamous cell carcinoma, not otherwise specified

Keratinizing

Nonkeratinizing

Basaloid

Verrucous

Warty

Papillary

Lymphoepithelioma like

Squamotransitional

Early invasive (microinvasive) squamous cell carcinoma

Squamous intraepithelial neoplasia

Cervical intraepithelial neoplasia-3 (CIN3)/

Squamous cell carcinoma in situ

Benign squamous cell lesions

Condyloma acuminatum

Squamous papilloma

Fibroepithelial polyp

Glandular tumors and precursors

Adenocarcinoma

--Mucinous adenocarcinoma

Endocervical

Intestinal

Signet-ring cell

Minimal deviation

Villoglandular

--Endometrioid adenocarcinoma

--Clear cell adenocarcinoma

--Serous adenocarcinoma

--Mesonephric adenocarcinoma

Early invasive adenocarcinoma

Adenocarcinoma in situ

Glandular dysplasia

Benign glandular lesions

Müllerian papilloma

Endocervical polyp

Other epithelial tumors

Adenosquamous carcinoma

..Glassy cell carcinoma variant

Adenoid cystic carcinoma

Adenoid basal carcinoma

Neuroendocrine tumors

Carcinoid

Atypical carcinoid

Small cell carcinoma

Large cell neuroendocrine carcinoma

Undifferentiated carcinoma

Mesenchymal Tumors and Tumorlike Conditions

Leiomyosarcoma

Endometrioid stromal sarcoma, low grade

Undifferentiated endocervical sarcoma

Sarcoma botryoides

Alveolar soft part sarcoma

Angiosarcoma

Malignant peripheral nerve sheath tumor

Leiomyoma

Genital rhabdomyoma

Postoperative spindle cell nodule

Mixed Epithelial and Mesenchymal Tumors

Carcinosarcoma (malignant mixed Müllerian tumor)

Adenosarcoma

Wilms tumor

Adenofibroma

Adenomyoma

Melanocytic Tumors

Malignant melanoma

Blue nevus

Miscellaneous Tumors

Tumors of germ cell type

Yolk sac tumor

Dermoid cyst

Mature cystic teratoma

Lymphoid and Hematopoetic Tumors

Malignant lymphoma (specify type)

Leukemia (specify type)

Secondary Tumors

PREMALIGNANT LESIONS OF CERVIX

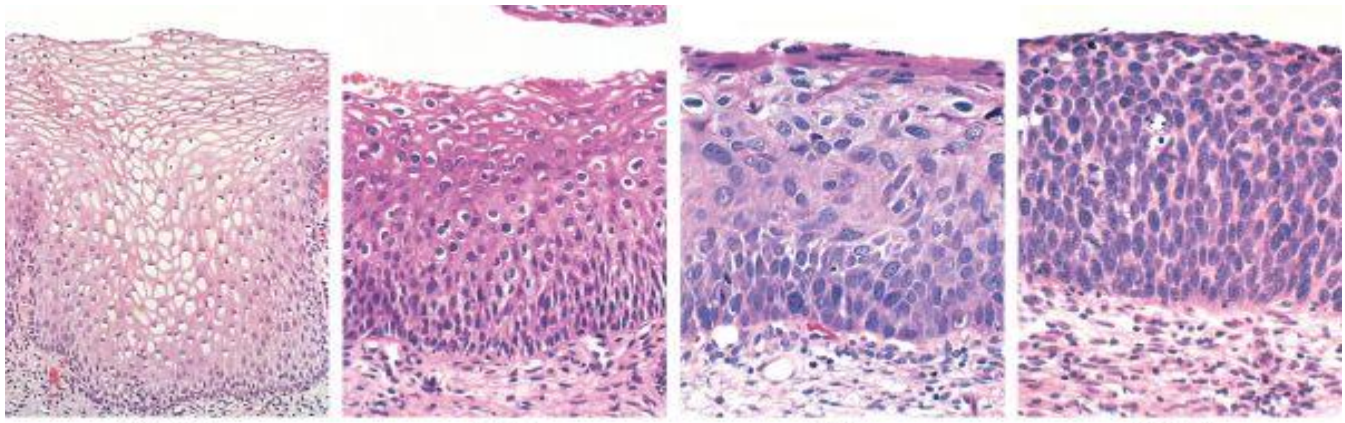
The development of invasive cervical carcinoma traditionally has been viewed as a continuum that begins with mild dysplasia.

The classification of cervical precancerous lesions has evolved over time and the terms from the different classification systems are currently used interchangeably. The oldest classification system classified lesions as having mild dysplasia on one end and severe dysplasia/carcinoma in situ on the other. This was followed by cervical intraepithelial neoplasia (CIN) classification, with mild dysplasia termed CIN I, moderate dysplasia CIN II, and severe dysplasia termed CIN III. Because the decision with regard to patient management is two-tiered (observation versus surgical treatment), the three-tier classification system has been recently simplified to a two-tiered system, with CIN I renamed low-grade squamous intraepithelial lesion (LSIL) and CIN II and CIN III combined into one category referred to as high-grade squamous intraepithelial lesion (HSIL)

Table 1:- Classification Systems for Premalignant Squamous Cervical Lesions

Dysplasia/Carcinoma in Situ	**Cervical Intraepithelial Neoplasia (CIN)**	**Squamous Intraepithelial Lesion (SIL), Current Classification**
Mild dysplasia	CIN I	Low-grade SIL (LSIL)
Moderate dysplasia	CIN II	High-grade SIL (HSIL)
Severe dysplasia	CIN III	High-grade SIL (HSIL)
Carcinoma in situ	CIN III	High-grade SIL (HSIL)

Fig 4:- <u>Spectrum of cervical intraepithelial neoplasia</u>

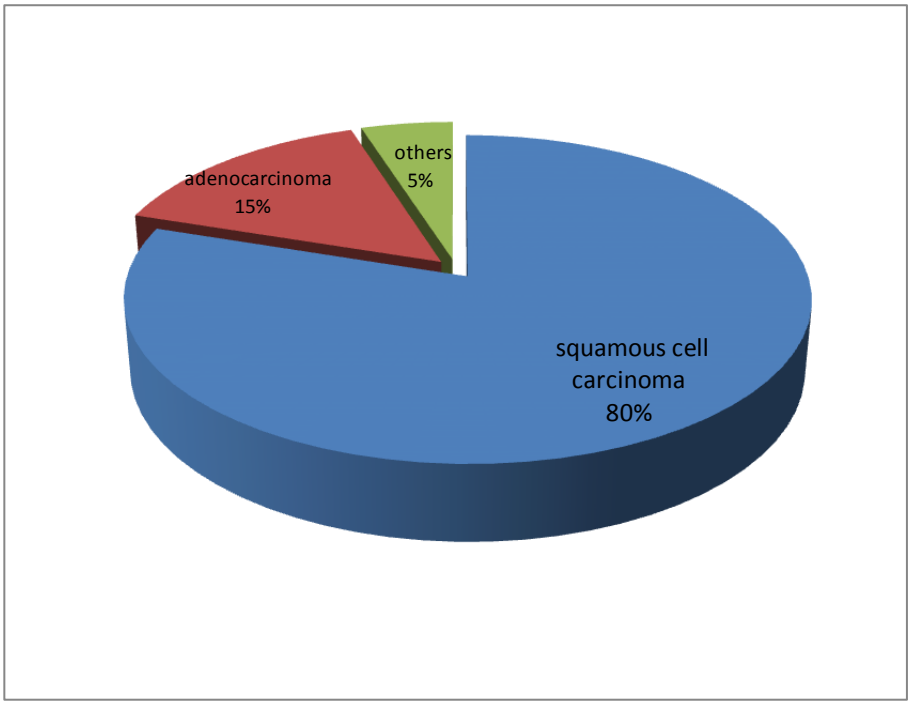


Morphology- The diagnosis of SIL is based on identification of nuclear atypia characterized by nuclear enlargement, hyperchromasia (dark staining), presence of coarse chromatin granules and variation of nuclear sizes and shapes. The nuclear changes may be accompanied by cytoplasmic halos indicating disruption of the cytoskeleton before release of the virus into the environment. Nuclear alterations and perinuclear halo are termed koilocytic atypia. The grading of SIL into low or high grade is based on expansion of the immature cell layer from its normal, basal location. If the atypical, immature squamous cells are confined to the lower one third of the epithelium, the lesion is graded as LSIL; if they expand to two thirds of the epithelial thickness, it is graded as HSIL.[7]

CERVICAL CARCINOMA

Squamous cell carcinoma is the most common histologic subtype of cervical cancer, accounting for approximately 80% of cases. As outlined above, HSIL is an immediate precursor of cervical squamous cell carcinoma. The second most common tumor type is cervical adenocarcinoma, which constitutes about 15% of cervical cancer cases and develops from a precursor lesion called adenocarcinoma in situ. Adenosquamous and neuroendocrine carcinomas are rare cervical tumors that account for the remaining 5% of cases.

Chart 4:- Percentage of various histological types



On histologic examination, squamous cell carcinomas are composed of nests and tongues of malignant squamous epithelium, either keratinizing or nonkeratinizing, invading the underlying cervical stroma. Adenocarcinomas are characterized by proliferation of glandular epithelium composed of malignant endocervical cells with large, hyperchromatic nuclei and relatively mucin-depleted cytoplasm, resulting in dark appearance of the glands, as compared with the normal endocervical epithelium.

Adenosquamous carcinomas are tumors composed of intermixed malignant glandular and malignant squamous epithelium. Neuroendocrine cervical carcinomas typically have an appearance similar to small-cell carcinoma of the lung.

Table 2:- Clinical Staging System of Cervical cancer

Stage 0.	Carcinoma in situ (CIN III, HSIL)
Stage I.	Carcinoma confined to the cervix
	Ia. Preclinical carcinoma, that is, diagnosed only by microscopy
	Ia1. Stromal invasion no deeper than 3 mm and no wider than 7 mm (so-called **microinvasive carcinoma**)
	Ia2. Maximum depth of invasion of stroma deeper than 3 mm and no deeper than 5 mm taken from base of epithelium; horizontal invasion not more than 7 mm
	Ib. Histologically invasive carcinoma confined to the cervix and greater than stage Ia2
Stage II.	Carcinoma extends beyond the cervix but not to the pelvic wall. Carcinoma involves the vagina but not the lower third.
Stage III.	Carcinoma has extended to the pelvic wall. On rectal examination there is no cancer-free space between the tumor and the pelvic wall. The tumor involves the lower third of the vagina.
Stage IV.	Carcinoma has extended beyond the true pelvis or has involved the mucosa of the bladder or rectum. This stage also includes cancers with metastatic dissemination.

Table 3:- Worldwide 5- Year Actuarial Survival Rates for Cervical Carcinoma by FIGO Stage

Stage	Number of Patients	5-Year survival rate (%)
I	12,143	81.6
II	10,285	81.3
III	8,206	36.7
IV	1,378	12.1

IMMUNOHISTOCHEMISTRY

Histopathologic diagnosis of cervical biopsies determines the clinical management of the patient. But there is still poor interobserver reproducibility in histopathological diagnosis. Immunohistochemical staining of biomarkers related to the different stages of cervical carcinogenesis may provide objective standards to reduce diagnostic variability of cervical biopsy evaluations. However systematic, rigorous evaluations of their potential clinical utility are lacking[17].

More than 99% of cervical cancers are positive for high-risk human papilloma viruses (HPVs). E6 and E7 genes encode oncoproteins responsible for virus replication, and also for immortalization and neoplastic transformation of human keratinocytes. The interaction of human papilloma virus oncoproteins E6 and E7 with cell cycle proteins leads to disturbances of the cell cycle mechanism and subsequent alteration in the expression of some proteins, such as p53, pRb and Ki-67. The affinity of these viral proteins for the tumor suppressing gene products differs in relation with the oncogenic potential of HPV. The affinity increases for high-risk viruses and decreases for low risk viruses[2].

p53

The tumor suppressor gene p53, among its many functions, exerts its control on the cell cycle through the activation of p21, an inhibitor of cyclin and cyclin-dependent-kinase complexes. Although in many human cancers p53 appears to be down-regulated by point mutations, in cervical cancer the HPV oncoprotein E6 binds p53 and causes its degradation by the cellular ubiquitin proteolysis system. Its expression by means of immunohistochemical analysis seems to be increased in high-grade SIL (HSIL) and cervical carcinoma compared with low-grade SIL (LSIL) and normal cervix. Several authors have reported higher p53 expression in cervical biopsy specimens of patients with no HPV infection or infection with low-risk HPV[18].

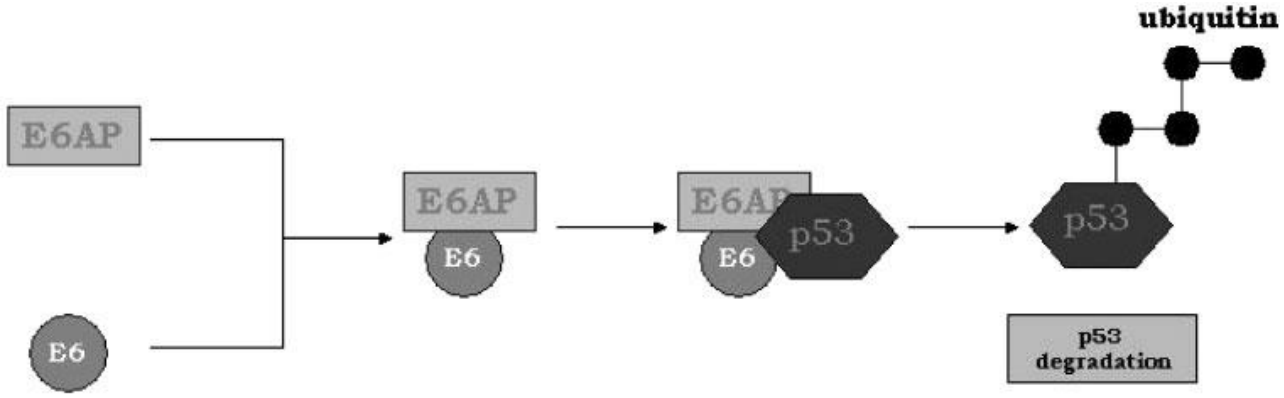
The p53 gene is located on chromosome 17pl3.1 and is thought to act as a tumour suppressor gene. It is involved in the regulation of cell proliferation by stimulating the transcription of other specific

cell cycle control genes. Normally, cells with wild type p53 are able to delay progression from the G1 to the S phase of the cell cycle while abnormal DNA is repaired. Cells with the inactivated, mutant p53 protein cannot, and thus the replication of abnormal DNA is not prevented. Consequently, inactivation of wild type p53 gene product represents the most common genetic alteration in human carcinogenesis. Although the exact sequence of molecular events has not been elucidated fully, loss of p53 function is believed to play an important role in the pathogenesis of carcinomas of the uterine cervix, where it has been shown that the p53 gene product may be inactivated in three principal ways: after (1) somatic point mutation (2) loss of heterozygosity (3) following human papillomavirus (HPV) infection, as the viral oncoprotein E6 is known to bind to, stabilise and ultimately degrade wild type p53.[19]

A single nucleotide polymorphism in the p53 gene resulting in the substitution of arginine (Arg) by proline (Pro) at codon 72 was identified and shown to alter the primary structure of the p53 protein. Biochemical and functional differences between the two p53 forms (p53arg, p53pro) have been identified[20]. Both forms have wild-type biological activity. However, it was recently proposed that the two p53 variants at codon 72 might contribute differently to the development of invasive cervical cancer. Storey et al [1998] showed that the p53arg variant is more efficiently inactivated by the viral oncoprotein E6 of the high risk HPV types than the p53pro variant. In addition, they analyzed cervical specimens for the distribution of the two p53 variants in healthy women and women with cancer. Their findings suggest that women with the arg/arg allotype are at higher risk of HPV-associated cervical cancer than pro/pro or heterozygotes.[21]

The p53 arg/arg polymorphism may be an important determinant of the risk for cervical cancer, but it does not appear to be sufficient for carcinogenesis. Numerous biochemical studies have elucidated the mechanism of action of the high-risk HPV types in cervical carcinogenesis. They have unequivocally proved that the products of two early genes, E6 and E7, play a key role in the induction of pre-malignant and malignant cervical lesions.

Chart 5:- <u>Shows p53 degradation via the ubiquitin pathway.</u>



Both viral proteins have the ability to associate and subsequently inactivate several cellular proteins, including products of tumor suppressor genes. As mentioned before, these HPV mediated events lead to loss of control of fundamental cellular pathways, such as cell cycle and apoptosis. The best characterized E6 activity of the high-risk HPV types (e.g., types 16 and 18) is its ability to induce degradation of the tumor suppressor protein p53 via the ubiquitin pathway. HPV16 E6 binds to a cellular protein, termed E6 associated protein, which functions as an ubiquitin protein ligase. The E6/E6AP complex then binds the central region (also termed the core domain) of p53, which becomes rapidly ubiquitinated and is targeted to proteasomes. Consequently, p53 levels are extremely low in cervical tumor cells. p53 plays a crucial role in safeguarding the integrity of the genome. The cells expressing HPV16 E6 show chromosomal instability. This will greatly increase the probability that HPV infected cells will evolve toward malignancy. The induction of p53 destabilization is an exclusive feature of E6 proteins from the high-risk HPV types[20].

Inactivation of p53 represents a key step in cervical carcinogenesis, similarly to other human cancers, in which the p53 gene is frequently mutated. [20,22] In addition to viral gene products, several cellular proteins are implicated in the inactivation of p53, and could be responsible for p53 inactivation in HPV-negative carcinomas.[23]

In conclusion wild type p53 monitors the integrity of the genome. The mechanism of activation of p53 in response to DNA damage depends on DNA protein kinase.[24] The p53 gene is one of the most important targets of the HPV E6 gene. Two important mechanisms are known as to how p53 causes cervical cancer.

1) E6 protein has the ability to stimulate p53 degradation. E6 binds to E6 associated protein (E6AP), which functions as ubiquitine protein ligase. E6/E6AP complex binds the central region of p53, which becomes rapidly ubiquitinated and is targeted to proteosomes. This leads to low levels of p53 in cervical tumor cells.[20,24,25]

2) Mutation of p53, it is rarely found to cause carcinomas. However mutation can be found in both HPV-positive or negative cervical tumours, indicating that there is no correlation between HPV and p53 status. Mutated p53 suggests that p53 can be functionally inactivated in cervical cancer cells either by association with E6 or mutation of the gene.[20]

Several studies have demonstrated an association between p53 protein accumulation and aggressive behavior of several carcinomas, including a greater propensity toward metastases and shorter patient survival. These data support the idea that the p53 gene is a cellular oncogene of the nuclear protein group, whose role may be implicated in tumor progression .[26]

A significant correlation was found between the expression of p53 and the histological type of the cases examined. p53 expression was seen in epithelium, showing cervical intraepithelial neoplasia (CIN) grade III, to invasive squamous carcinoma and in a few cases of adenocarcinoma but not in epithelium showing CIN grade II or I or in any normal epithelium present[26,27]. p53 protein expression does indeed seem to play a pivotal role in cervical tumor progression as a late event. But despite the increasing expression of p53 in advanced CIN lesion and invasive cervical cancer, there is no evidence to suggest that p53 may be useful as a prognostic indicator[26].

According to Khunamornpong S et al p53 did not correlate with tumor recurrence. Immunohistochemistry for p53 protein appears to provide no prognostic information in patients with early stage cervical cancer treated by surgery[28].

According to Phaik- Leng Cheah et al p53 was detected more frequently in CIN I compared with CIN II/III and invasive carcinoma which may be due to p53 protein degradation following interaction with high risk human papillomavirus E6 protein in CIN II/III and invasive carcinoma[29].

pRb

The retinoblastoma gene was the first tumour suppressor gene identified that was altered not only in retinoblastomas but has also been described in a wide variety of human neoplasms.

The retinoblastoma tumour suppressor gene (Rb) encodes a nuclear phosphoprotein, termed p105Rb or pRb. This, in its hypophosphorylated state, plays an important role in regulating the cell cycle, thus preventing tumour formation. Introduction of pRb into tumour cells lacking the Rb gene in vitro stops cell proliferation, suggesting that pRb acts in normal cells to constrain growth and its loss in cancer cells allows for uncontrolled proliferation[30].

Transcription of Rb gene gives rise to mRNA that encodes a protein (pRb) that plays an important role in regulating the ability of cells to enter S phase, the period when DNA is synthesised. Loss of normal Rb function may allow cells to proliferate in uncontrolled manner, not only to initiate events in tumourigenesis, but also as a step associated with malignant progression and progressive outcome.[4]

The retinoblastoma gene was initially identified as a genetic locus associated with the development of an inherited eye tumor. The realization that it was a loss of function of Rb gene that was associated with disease established the tumor suppressor paradigm. Subsequent work identified the E2F transcription factor activity as a key target for the growth suppressing action of the Rb protein. Additional work demonstrated that Rb function, including the ability to interact with E2F, was regulated by

phosphorylation and that the primary kinase responsible was the D-type cyclin-dependent kinases. Cyclin D/cdk4 activity is induced by growth stimulation, thus initiating the cascade of events that leads to E2F accumulation and S-phase entry.

The Rb/E2F pathway has an important role in the control of cell proliferation. In addition to the control of cell proliferation, it is also clear that this pathway is linked to events that determine cell fate through an induction of apoptosis.[31]

Epidemiological and experimental studies have implicated high-risk humanpapillomavirus (HPV) genotypes 16 and 18 in the development of cervical carcinoma. [32] E7 interacts with the dephosphorylated form of pRb, thereby releasing growth-promoting factors such as E2F-1, cyclins, Cdks and probably Myc. It has also been proposed that HPV-16/E7 induces the degradation of pRb through the ubiquitin pathway. This mechanism appears to be crucial for the development of cervical carcinoma in infected patients with oncogenic HPV strains. In the presence of HPV infection, other cellular oncogene alterations and overexpression could also represent important steps in cervical carcinogenesis.[30]E7 protein was found predominantly in the nucleus and to a minor extent in the cytoplasm in the cervical cancer cell line Ca Ski in vitro and in cervical carcinoma in situ, suggesting that nuclear resident E7 plays a major role in cervical carcinogenesis in humans.[33]

Loss of normal Rb function can also occur due to mutation of Rb gene. Majority of this mutations lead to absence of Rb mRNA and its protein product. The complete absence of nuclear reactivity in all areas of tumour associated with a positive internal control has shown a strong indication of underlying Rb mutations.[4]

Immunohistochemical techniques allow the detection of pRb expression in individual cells and specific cell populations in tissue sections. A low frequency of Rb gene alterations has been found in cervical carcinoma by Southern blot and so far a few reports have been focused on the cell per cell immunohistochemical expression of pRb in normal cervical epithelium.[30]

All the pre-cancerous cervical lesions (SIL) showed numerous pRb positive cells. The percentage of pRb positive cells was higher in SIL than in normal cervical epithelium. In general, the intensity of reaction and the number of pRb positive cells in invasive uterine-cervix lesions were significantly lower than in SIL specimens but similar to normal samples. A relation between pRb expression and clinical stage was not observed. The positive pRb immunostaining was quite heterogeneous some samples showed negative pRb reaction in the majority of cells but slightly positive reaction in the cytoplasm, while other samples contained intense nuclear staining and a few specimens showed nuclear and cytoplasmic immunostaining. In some specimens a weak nuclear immunoreactions was observed.[28]

Nuclear pRb immunoreactivity was also seen in normal endocervical glands and infiltrated lymphocytes which served as internal control.[4]

Among the invasive squamous cell carcinoma, well-differentiated cases showed higher percentage of pRb-positive cells in contrast to poorly differentiated tumors which revealed a lower percentage of pRb positive cells. The adenocarcinoma group showed variable staining proportions[4].

Noraini et al showed overexpression of retinoblastoma protein product (pRb) in majority of premalignant and malignant lesions of the uterine cervix as compared with normal cervical squamous epithelium. Statistical analysis showed significant differences in pRb expression between normal and CIN lesions. Proportions of pRb immunoreactivity are higher in better differentiated cancers and there is complete loss in undifferentiated carcinomas[4].

Marc Fiedler et al in his study reported that pRb appears weakly expressed in uninfected normal cervical epithelium but is strongly up-regulated in parabasal layers of histologically normal looking epithelium adjacent to an LSIL and in LSIL. In more advanced lesions, the pRb expression level decreases and pRb becomes virtually undetectable in Ca cervix.[33]

pRb expression is inversely correlated with E7 expression in HSIL whereas pRb staining almost disappeared in carcinoma , concomitant with an increasing E7 level. The inverse correlation of E7 and pRb degradation occurs in vivo and is likely to be involved in cervical carcinogenesis.[33]

Mutational inactivation of the Rb-related gene RbL2/p130 has been reported as a common and important prognostic factor in human lung cancer.[34]

Ki-67

Ki-67 is a nonhistone protein expressed in the nucleus during the whole cell cycle, except in the G0 and G1 early phases. Ki67 is the gold standard proliferative index and is immunhistochemically detectable throughout the interphase of the cell cycle, reaching its maximal level during mitosis. Immediately after mitosis, the cellular Ki67 antigen content decreases due to the short half life and is not detectable in Go phase.

This protein has a function of growth in human tumor, and expression of its marker could suggest the degree of malignancy. Therefore, it constitutes an efficient marker of proliferating cells and has been associated with an increasing degree of cervical dysplasia and is reported as an additional prognostic marker of cervical cancer.[9,18,35] The grade and pattern of expression of Ki-67 in precursor lesions is still a topic for debate. It also correlates with the histological grade of cervical carcinoma.

Recently, it was found that in patients with high-risk HPV the viral load (detected by hybrid capture II method) is positively correlated with the expression of Ki-67 and CIN grade. Biological behavior of preneoplastic lesions could be predictable by multiple parameter logistic regression models with Ki-67 labeling index. Higher Ki-67 levels were seen in tumors with a lower grade and higher stage at diagnosis, being associated with poorer outcome. Moreover, Ki-67 levels seem to be higher in tumors due to infection with HPV16 and 18 compared with HPV-4.[2]

The antibody Ki-67 reacts with the nuclear Ki-67 antigen, a protein encoded on 10q25. Ki-67 is only present in proliferating cells. In normal ectocervical epithelia, it is only expressed in the suprabasal layer and in CIN cases; it is expressed throughout the different epithelial layers. The Ki-67 labeling index (Ki- 67 LI) increases, according to the degree of squamous cervical neoplasia. The Ki-67 LI can be useful in distinguishing the different grades of dysplasia, though not in predicting their behavior.[36]

Ki-67 immunostaining has been shown to have potential prognostic value in previous studies of malignant disease. Hall et al (1988) found that patients with histologically low grade lymphoma and a relatively high Ki-67 count (>5%) had a worse survival rate than those with a count below 5%. In contrast, those patients with histologically high grade disease with Ki-67 counts of more than 80% had a better survival rate than those below that figure. One explanation for this might be that rapidly proliferating lesions are more vulnerable to chemotherapy.

The fact therefore is that Ki-67 immunostaining appeared to give a grading of these tumors independent of histology, indicating that clinical follow up of such patients might be valuable.[37] Elevated Ki67 expression was associated with lymph node metastasis in cervical cancer patients who underwent radical surgery.[38]

Other biomarkers which have role in cervical carcinoma include p16 , p63, E-cadherin, c-myc, ras and c-erb B-2, the cellular proliferation markers PCNA and other more recently described biomarkers such as nm23-H1, MN protein and metalloproteinase.

p16INK4a

It is a tumor-suppressor protein and cyclin-dependent kinase (cdk) inhibitor that blocks cdk4- and cdk6-mediated pRb phosphorylation to inhibit E2F-dependent transcription and cell-cycle progression . In most cervical carcinomas, the functional inactivation of pRb by HPV E7 results in the overexpression of p16INK4a and the accumulation of the protein in cells. p16INK4a is thus a surrogate marker of HPV E7-mediated pRb catabolism, providing evidence of transformation of the cervical mucosa. Immunohistochemical analysis has demonstrated that diffuse staining for p16INK4a is present in almost all cases of CIN2, CIN3, and squamous cell carcinoma (as well as in endocervical glandular neoplasia). However, it is rarely detected in benign squamous mucosa or CIN1lesions associated with LR-HPV.[9,17,35]

E-cadherin

This is a 120 kD transmembrane adhesion molecule being encoded by a chromosome 16q22. It is mainly located in Ca2+ dependent adherent junctions and concentrates at intercellular contact site, the epidermal growth factor receptor .Through its extracellular domain it is involved in intercellular adhesion by osmophilic Ca2+ dependent interactions, while the intracellular domain binds to actin cytoskeleton via catenins. E-cadherin plays an important role in intercellular adhesion of epithelial cells, in epithelial polarization achievement, differentiation and stratification. E-cadherin immunoexpression studies in cervical squamous lesions are controversial. Various authors have shown varied results in its expression. Although E-cadherin immunoexpression can be maintained, its function may be impaired due to disruption of other components of the intercellular adhesion system, like catenins. So this will lead to abnormal staining pattern which is difficult to interpret.[35]

p63

This is a homologue of the tumor suppressor gene p53 and is expressed in the embryonic, adult murine, and human basal squamous epithelium. Studies have shown that p63 has potential as a marker for grading CIN. Studies have analyzed p63 expression in CIN and reported that p63 expression increases in high-grade CIN.[9]

Cytokeratin (CK)

It is a cytoskeletal intermediate filament protein. The CK isotype depends on the cell type and the localization of CK in the cytoplasm. Several studies have used immunohistochemistry to evaluate the expression of CK17 in the uterine cervix and its prognostic value, but these studies have produced diverse results. In the study of Regauer and Reich, all the CIN patients showed negative CK17 immunohistochemical staining. Martens et al also reported that the CK17 expression in CIN was confined to the basal compartment. CK17 expression was not a prognostic factor for CIN 1 progression.[9]

C-myc

The C-myc oncogenes function as nuclear DNA transcription factors associated with cell cycle progression, immortalisation and differentiation. Adding to its functional complexity, c-myc also has a capacity for transcriptional regression and an additional role in apoptosis. C-myc is expressed in both cervical intraepithelial neoplasia (CIN) and invasive carcinoma, with varying levels of expression according to the extent of the disease.[10]

Ras

The Ras family of oncogenes code for transducing proteins. One such product is the membrane-associated protein p21 which possesses guanine triphosphatase (GTPase) activity. The putative role of mutant Ras is to convert human papillomavirus (HPV)-immortalised keratinocytes to the tumorigenic state. Compared with normal tissue and lower grades of dysplasia, Ras over-expression is found more commonly in CIN3 and invasive carcinoma.[10]

Epidermal growth factor receptor (EGFR)

The EGFR is an oncogene-encoded 170 kD membrane bound glycoprotein of importance in the regulation of normal and neoplastic cellular proliferation. Activation of the receptor's intracellular tyrosine kinase domain functions to transmit intracellular signals leading to regulation of cell growth. EGFR expression has been examined in a relatively few cases of normal and dysplastic epithelium with contradictory results. The situation is not clear in invasive carcinoma, with reports demonstrating lack of significance of tumour EGFR expression for survival, countered by poor survival in positive cases.[10]

C-erb B-2

The C-erb B-2 proto-oncogene codes for a transmembrane tyrosine kinase 185 kD oncoprotein which is related to the epidermal growth factor receptor (EGFR). It has been suggested that higher levels of CerbB-2 expression are seen at later stages of carcinoma. A relatively high rate of IHC positivity is

seen in the late stage of the disease. But both lower and higher positivity rates have been described in early stage cases.[10,36]

nm23-HI

The nm23-HI gene is a metastasis suppressor gene, the product of which is identical to human nucleotide diphosphate (NDP) kinase A. The function of this protein remains unclear. It has been suggested that it modulates intracellular signal transduction by phosphorylation of GTP binding proteins, or plays a role in cell attachment and detachment to the extracellular matrix. A possible intriguing association of nm23-HI expression with a high incidence of lymph node involvement and poor prognosis has been described in the adenocarcinoma subtype of tumours only. [10]

Cathepsin

The relatively scant IHC studies in cervical carcinoma of this acid proteinase- cathepsin has produced conflicting results. Kristensen et al found positive staining in SCC to correlate with lymph node metastases and lower relapse free survival, with the immunohistochemical result achieving independent prognostic significance.

This is in disagreement with the finding of Mitchell et al for invasive carcinoma despite a similar percentage of positive immunostaining. As cathespin acts to degrade extra-cellular matrix and activates other proteinase involved in early invasion, dysplasia and microinvasive stage 1 malignancies may be more fruitful areas for investigation. Lack of high levels of expression in established invasive malignancy may highlight the supervening importance of other co-factors required for the development of metastases, or be related to levels at which the proteinase is detectable in tissue sections.[10]

MN antigen

The membrane-associated MN antigen (Ag) has homology with carbonic anhydrase. Expression of this protein has only recently been detected in cervical neoplasia, and may correlate with loss of a tumor suppressor gene on chromosome 11, possibly at an early stage of oncogenesis.

Although MN protein expression in cervix carcinoma is ubiquitous, there is quantitative variation, with low expression found to correlate with poor differentiation, the adenosquamous histologic subtype, deep stromal invasion, regional lymph node metastases and HPV negativity. The role of this biomarker protein as a prognostic marker in cervical carcinoma is yet to be determined.[10]

Metalloproteinase (MP)

This may help to degrade basement membrane type IV collagen, assisting the early phases of tumour invasion, and thereby potential for metastasis. It has been demonstrated that there is a significant increase in IHC positivity in microinvasive squamous cell carcinoma compared with CIN lesions. Increased staining positivity in CIN also related directly to the severity of cellular atypia. A significant relationship has been claimed between the tumour MP index and presence of nodal metastasis, as well as the number of positive nodes and recurrencerisk. Overall early results seem promising but will require additional confirmatory evidence.[10]

Cyclin D1

Cyclin D1 forms a complex with cyclin-dependent kinase 4 or 6 to carry out the phosphorylation of pRb. Phosphorylated pRb causes the release of the active E2F transcriptional factor, which then can induce the expression of genes whose protein products are required for cells to enter the DNA synthesis phase (S phase) of the cell cycle. Overexpression of cyclin D1 has been found to be an independent factor associated with poor prognosis in cervical carcinoma.[18,36]

ProEx C

Recently, transcriptional profiling studies have identified 2 cell cycle–related proteins, minichromosome maintenance protein-2 (MCM2) and topoisomerase II-α (TOP2A), whose genes are overexpressed in cervical cancer and probably exert their function in early S phase, allowing recognition of replication origin and DNA unwinding. ProEx C is a novel immunohistochemical marker for the detection of MCM2 and TOP2A proteins, and its increased expression seems to be associated with HSIL.[18]

MATERIALS AND METHODS

The present study emphasises on the role of immunohistochemistry in premalignant and malignant cervical lesions.

We used 120 paraffin blocks (cases & controls) obtained from biopsy and hysterectomy specimens which were sent from the Department of OBG at R.L.Jalappa Hospital and Research Centre attached to Sri Devaraj Urs Medical College, Tamaka, Kolar.

This study was done from the period of October 2010 to March 2012. Prior to the study ethical clearance was obtained from the institutional ethical board.

Inclusion Criteria:

1. All patients who come to obstetrics & gynaecology OPD for routine cervical cancer screening, with complaints of WDPV, bleeding per vagina and mass per vagina and patients suspected to have dysplasia on application of acetic acid or Lugol's iodine.
2. Hysterectomy specimen, if there is dysplasia and malignancy will be included.

Exclusion Criteria:

1. Pregnancy.
2. Patients receiving radiotherapy , chemotherapy or recurrence.

Method of collection of data:

- Patients attending the OPD of Department of OBG for cervical cancer screening as well as with complaints of white discharge per vagina, bleeding per vagina and mass per vagina.
- Clinical data was obtained in each case – name, age, history of present illness, personal history and any other associated complaints.

- Consent from the patient was taken.
- Biopsy was taken from the suspected area after application of 5% acetic acid or Lugol's iodine.
- Biopsy was routinely processed and stained with H&E staining. The sections were examined for dysplasia, carcinoma in situ and malignancy.

  Steps followed for tissue processing and staining-

  - Dehydration
  - Clearing
  - Impregnation with wax
  - Embedding with paraffin wax
  - Trimming and cutting the tissue blocks using microtome.
  - Sections are stained using Haematoxylin & Eosin stains.
  - The sections are examined for dysplasia, carcinoma in situ and malignancy.

- All cervical biopsies and hysterectomy specimens received in pathology department were screened. Light microscopy showing dysplasia and malignancy were selected for the study.
- Out of 120 samples, 100 were neoplastic cervical lesions. Neoplastic lesions of cervix included cervical intraepithelial lesions and invasive carcinoma of cervix. Histologically, there were 20 cases of normal cervix, 21 of LSIL and 18 of HSIL. Among the invasive carcinoma groups, there were 13 cases of well differentiated squamous cell carcinoma, 28 of moderately differentiated squamous cell carcinoma, 7 of poorly differentiated squamous cell carcinoma, 8 Non-keratinising squamous cell carcinoma, 3 of invasive adenocarcinoma and 1 each of Adenosquamous and microinvasive carcinoma respectively. Classification of the carcinoma is based on Modified World Health Organisation criteria.

Immunohistochemical technique

The immunohistochemistry (IHC) was performed on 3-µm thick sections from 10% formalin-fixed paraffin-embedded tissues, according to horse-peroxidase method was used for a panel of three antibodies (p53, Ki-67, pRb) details are shown in table below.

Table 4:- Antibodies used in the study batch

Antigen	Clone	Species	Producer	Dilution	Control	Stain
P53	BP53-12	Mouse	Biogenex	Undiluted	Breast cancer	Nuclear
Ki-67	BGX-297	Mouse	Biogenex	Undiluted		Nuclear
PRb	Rb (IF8): sc-102	Mouse	Bio-tech	1:50	Colon cancer	Nuclear/ cytoplasmic

The IHC procedure includes following step—

- Sections are 3-4mm thickness, floated on to organosialine coated slide and left on hot plate at 60^0 C over night.
- **Deparaffinization** using Xylene I and II—15 min each
- **Dexylinisation** using absolute alcohol I and II—1 min each
- **Dealcoholisation** using 90% and 70% alcohol—1 min each
- Washing with distilled water.
- **Antigen Retrieval technique:** Microwave power 10 for 6 minutes in Citrate buffer of pH-6.0 for 3 cycles (applicable for all 3 antibodies). Distilled water rinsing for 5 minutes.Transfer to TBS (Tris buffer solution pH- 7.6)- 5minutes x 2 times-wash.

- **Peroxidase block**- 10-15minutes to block endogenous peroxidase enzyme. TBS buffer for 5 minutes washing for 3 times. TBS buffer for 5minutes washing for 3 times
- **Power block**- 10-15 minutes to block non- specific reaction with other tissue antigen.
- Cover sections with targeted antibody (primary)- 2hrs. TBS buffer- 5min x 3 times
- **Super Enhancer**- 30 minutes to enhance the reaction between primary and secondary antibodies.
- TBS buffer- 5min x 3 times
- Super sensitive poly- HRP(secondary antibody)- 30 min
- TBS buffer- 5min x 3 times
- **Color development** with working color development solution (DAB) - 5-8 min
- TBS wash- 5min x 3 times
- Counter stain with Haematoxylin- 2 sec
- Tap water wash for 5 minutes.
- Dehydrate, clear and mount
- Mount with DPX.
- All the slides were examined.

To ensure the reliability of the experimental study, internal quality control of immunohistochemical techniques was performed as a part of an implemented and certified quality assurance system.

Immunohistochemical Analysis-

Cells were noted as positive when they showed nuclear/ Cytoplasmic immunoreactivity (brown precipitate). Only the neoplastic region of each tissue section was evaluated. To assess the stained nuclei, the slides were reviewed at x40 magnification. To evaluate the marker positivity, we counted at least1000 cells per case, in a blind manner. We made a quantification of the results by determining the nuclear positivity (number of cells marked by the antibody divided by the number of cells counted per sample). Positivity was nuclear for p53, Ki-67 and nuclear and cytoplasmic for pRb, in light microscopy. The percentage of positive cells in each tissue section was estimated on a semi-quantitative scale where: 0(0%), +1 (1-24%), +2 (25-49%), +3 (50-74%), +4 (75-100%).

The staining intensity was also assessed based on the following category as mild, moderate and high or grade I to III. This is mainly based on subjective assessment.

Fig 5:- Photograph showing pH meter with buffer solution

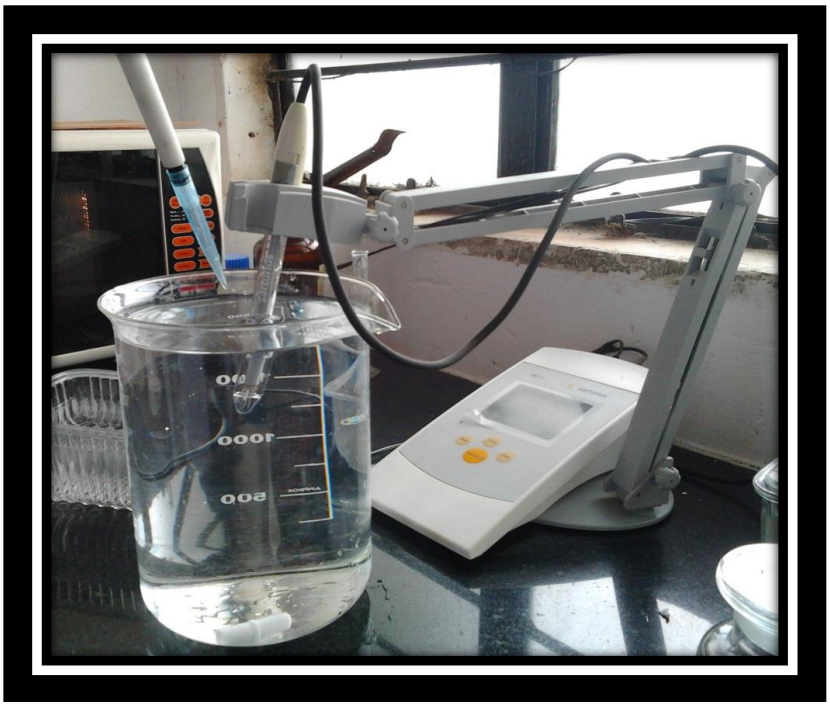


Fig 6:- Photograph showing slide rack with other ingredients used for IHC

STATISTICAL ANALYSIS:

The data was suitably arranged into tables for discussion under different headings. Descriptive statistical analysis was carried out on this data. Results on continuous measurements are presented as mean ± standard deviation and results on categorical measurements are presented in number %. Significance was assessed at 5% level of significance. The mean value of p53, pRb and Ki-67 was compared between normal and various premalignant and malignant lesions separately using Indepentent 't' test.

RESULTS

Table 5:- Age distribution and histopathological diagnosis

HPE Type	AGE IN YEARS					
	30-39	40-49	50-59	60-69	70-79	TOTAL
Normal	5	10	5	0	0	20
LSIL	6	10	4	1	0	21
HSIL	4	9	4	1	0	18
MI	0	0	1	0	0	1
WDSCC	1	3	6	3	0	13
MDSCC	0	13	9	5	1	28
PDSCC	0	2	4	0	1	7
S-NKSCC	0	3	0	0	0	3
L-NKSCC	1	1	2	0	1	5
AC-VG	0	0	2	0	0	2
AC-EN	0	1	0	0	0	1
AS	0	0	1	0	0	1
TOTAL	17	52	38	10	3	120

In our study 120 patients were considered. The age group range from 30-79yrs. The highest malignancy rate belongs to age group of 40-49yrs.

A total of 120 cervical tissues were selected for the study. Of the 120 cases, 20 were histologically diagnosed as normal and 39 cases were diagnosed as Squamous Intraepithelial lesions (21 LSIL and 18 HSIL). The remaining 61 cases were of invasive carcinomas consisting of 1 case of microinvasive carcinoma, 48 cases of keratinising squamous cell carcinoma (WDSCC, MDSCC & PDSCC), 8 cases of non-keratinising squamous cell carcinoma, 3 cases of adenocarcinoma and 1 of adenosquamous carcinoma.

Table 6:- p53 immunoscoring in various histological types

HPE Type	0	+1 (1-24%)	+2 (25-49%)	+3 (50-74%)	+4 (75-100%)	TOTAL
Normal n (%)	0	15(75)	5(25)	0	0	20
LSIL n (%)	3(14.3)	7 (33.3)	7(33.3)	2 (9.5)	2(9.5)	21
HSIL n (%)	1(5.5)	13(72.2)	0	0	4(22.2)	18
MI n (%)	0	0	0	0	1(100)	1
WDSCC n (%)	0	1(7.6)	2(15.4)	4(30.7)	6(46.2)	13
MDSCC n (%)	1(3.5)	6(21.4)	7(25)	4(14.3)	10(35.7)	28
PDSCC n (%)	0	1(14.3)	1(14.3)	3(42.8)	2(28.6)	7
S-NKSCC n (%)	0	1(33.3)	0	1(33.3)	1(33.3)	3
L-NKSCC n (%)	1(20)	1(20)	1(20)	1(20)	1(20)	5
AC-VG n (%)	0	0	1(50)	0	1(50)	2
AC- EN n (%)	0	0	0	0	1(100)	1
AS n (%)	0	1(100)	0	0	0	1

Out of the 20 normal cases 15 of them showed +1 scoring and 5 of them showed +2 scoring. In premalignant lesions (LSIL & HSIL) majority of them showed +1 scoring. In cases of invasive carcinoma (WDSCC, MDSCC & PDSCC) maximum number of cases showed +3 and +4 scoring. Out of 8 non-keratinising squamous cell carcinoma 4 cases showed +3 and +4 scoring, 2 of 3 adenocarcinoma cases showed +4 scoring.

Table 7:- pRb (n) immunoscoring in various histological types

HPE Type	0	+1 (1-24%)	+2 (25-49%)	+3 (50-74%)	+4 (75-100%)	TOTAL
Norma l n(%)	2(10)	16(80)	2(10)	0	0	20
LSIL n(%)	1(4.7)	6(28.6)	9(42.8)	5(23.80)	0	21
HSIL n(%)	3(16.6)	6(33.3)	2(11.1)	6(33.3)	1(5.5)	18
MI n(%)	0	0	1(100)	0	0	1
WDSCC n(%)	1(7.7)	7(53.8)	3(23.07)	1(7.7)	1(7.7)	13
MDSCC n(%)	5(17.8)	19(67.8)	4(14.3)	0	0	28
PDSCC n(%)	3(42.8)	4(57.1)	0	0	0	7
S-NKSCC n(%)	1(33.3)	2(66.6)	0	0	0	3
L-NKSCC n(%)	1(20)	4(80)	0	0	0	5
AC-VG n(%)	0	2(100)	0	0	0	2
AC- EN n(%)	1(100)	0	0	0	0	1
AS n(%)	1(100)	0	0	0	0	1

Out of the 20 normal cases 16 of them showed +1 scoring, 2 of them showed +2 scoring and 2 of them were negative. In case of LSIL, 9 out of 21 cases showed +2 scoring. Out of 18 cases of HSIL, 6 each showed +1 and +3 scoring. In case of invasive carcinoma (WDSCC, MDSCC & PDSCC), maximum number of cases showed +1 scoring. In case of MDSCC, 5 of 28 cases were negative; similarly 3 out of 7 cases of PDSCC were also negative. Out of 8 non-keratinising squamous cell carcinoma 6 cases showed +1 scoring and 2 of them were negative. 2 of 3 adenocarcinoma cases showed +1 scoring.

Table 8:- pRb-(c) immunoscoring in various histological types

HPE Type	0	+1 (1-24%)	+2 (25-49%)	+3 (50-74%)	+4 (75-100%)	TOTAL
Normal n(%)	20(100)	0	0	0	0	20
LSIL n(%)	21(100)	0	0	0	0	21
HSIL n(%)	13(72.2)	3(16.6)	1(5.5)	1(5.5)	0	18
MI n(%)	0	1(100)	0	0	0	1
WDSCC n(%)	4(30.7)	5(38.5)	2(15.4)	1(7.7)	1(7.7)	13
MDSCC n(%)	5(17.8)	8(28.6)	5(17.8)	8(28.6)	2(7.1)	28
PDSCC n(%)	4(57.1)	2(28.6)	1(14.3)	0	0	7
S-NKSCC n(%)	1(33.3)	0	0	1(33.3)	1(33.3)	3
L-NKSCC n(%)	1(20)	1(20)	1(20)	2(40)	0	5
AC-VG n(%)	1(50)	0	0	0	1(50)	2
AC- EN n(%)	0	0	1(100)	0	0	1
AS n(%)	0	1(100)	0	0	0	1

Out of the 20 normal cases none of them showed cytoplasmic staining. Majority of premalignant lesions (LSIL & HSIL) also showed no cytoplasmic staining. In case of WDSCC, 5 out of 13 showed +1 scoring and 4 cases had no cytoplasmic staining. In MDSCC, out of 28 cases 8 each showed +1 and +3 scoring, 5 each showed +2 scoring and no staining respectively. Out of 7 PDSCC cases 2 of them showed +1 scoring and 4 of them had no staining. Out of 8 non-keratinising squamous cell carcinoma 3 cases showed +3 scoring.

Table 9:- Ki-67 immunoscoring in various histological types

HPE Type	0	+1 (1-24%)	+2 (25-49%)	+3 (50-74%)	+4 (75-100%)	TOTAL
Normal n(%)	2(10)	18(90)	0	0	0	20
LSIL n(%)	0	18(85.7)	2(9.5)	0	1(4.7)	21
HSIL n(%)	1(5.5)	12(66.6)	1(5.5)	2(11.1)	2(11.1)	18
MI n(%)	0	0	0	1(100)	0	1
WDSCC n(%)	0	0	1(7.7)	1(7.7)	11(84.6)	13
MDSCC n(%)	1(3.6)	1(3.6)	1(3.6)	9(32.1)	16(57.1)	28
PDSCC n(%)	0	0	4(57.1)	2(28.5)	1(14.2)	7
S-NKSCC n(%)	0	0	0	1(33.3)	2(66.6)	3
L-NKSCC n(%)	0	0	1(20)	1(20)	3(60)	5
AC-VG n(%)	0	0	0	0	2(100)	2
AC- EN n(%)	0	0	0	0	1(100)	1
AS n(%)	0	1(100)	0	0	0	1

Out of the 20 normal cases 18 (90%) of them showed +1 scoring and 2 of them showed no scoring. In premalignant lesions (LSIL & HSIL) 59% of cases showed +1 scoring. In cases of invasive carcinoma (WDSCC & MDSCC) maximum number of cases showed +4 scoring, whereas 4 of 7 cases of PDSCC showed +2 scoring. Out of 8 non-keratinising squamous cell carcinoma 5 cases showed +4 scoring, 2 of 3 adenocarcinoma cases showed +4 scoring.

Table 10:- Association of immunoscoring between normal and low grade squamous intraepithelial lesions

	HPE	Number of cases	Mean±SD	p- value
P53	Normal	20	14.95±9.30	0.023
	LSIL	21	26.97±20.76	
Ki-67	Normal	20	5.5±7.55	0.012
	LSIL	21	18.29±20.53	
pRb (n)	Normal	20	8.35±10.22	<0.001
	LSIL	21	29.52±21.56	
pRb (c)	Normal	20	0	
	LSIL	21	0	

Mean p53 expression was significantly higher among LSIL cases as compared to normal cervical epithelium. The mean p53 value among LSIL cases was 26.97±20.76 as compared to normal which was 14.95±9.30. This difference was found to be statistically significant (p=0.023). Mean Ki-67 expression was significantly higher among LSIL cases as compared to normal cervical epithelium. The mean Ki-67 value among LSIL cases was 18.29±20.53 as compared to normal which was 5.5±7.55. This difference was found to be statistically significant (p=0.012). Mean pRb (n) expressions were significantly higher among LSIL cases as compared to normal cervical epithelium. The mean pRb (n) value among LSIL cases was 29.52±21.56 as compared to normal which was 8.35±10.22. This difference was found to be statistically significant (p<0.001).

Table 11:- Association of immunoscoring between normal and high grade squamous intraepithelial lesions

	HPE	Number of cases	Mean±SD	p- value
P53	Normal	20	14.95±9.30	0.09
	HSIL	18	26.78±28.76	
Ki-67	Normal	20	5.5±7.55	<0.001
	HSIL	18	22.78±18.63	
pRb (n)	Normal	20	8.35±10.22	0.001
	HSIL	18	28.28±23.25	
pRb (c)	Normal	20	0	0.03
	HSIL	18	6.94±13.63	

Mean p53 expressions were low among HSIL (26.78±28.76) cases as compared to normal cervical epithelium. This difference was found to be statistically insignificant. Mean Ki-67 expression was significantly higher among HSIL cases as compared to normal cervical epithelium. The mean Ki-67 value among HSIL cases was 22.78±18.63 as compared to normal which was 5.5±7.55. This difference was found to be statistically significant ($p<0.001$). Mean pRb (n) expressions were significantly higher among HSIL cases as compared to normal cervical epithelium. The mean pRb (n) value among HSIL cases was 28.28±23.25 as compared to normal which was 8.35±10.22. This difference was found to be statistically significant ($p=0.001$).Mean pRb(c) expressions were significantly higher among HSIL cases as compared to normal cervical epithelium. The mean pRb(c) value among HSIL cases was 6.94±13.63 as compared to normal which was 0. This difference was found to be statistically significant ($p=0.03$).

Table 12:- Association of immunoscoring between normal and squamous cell carcinoma

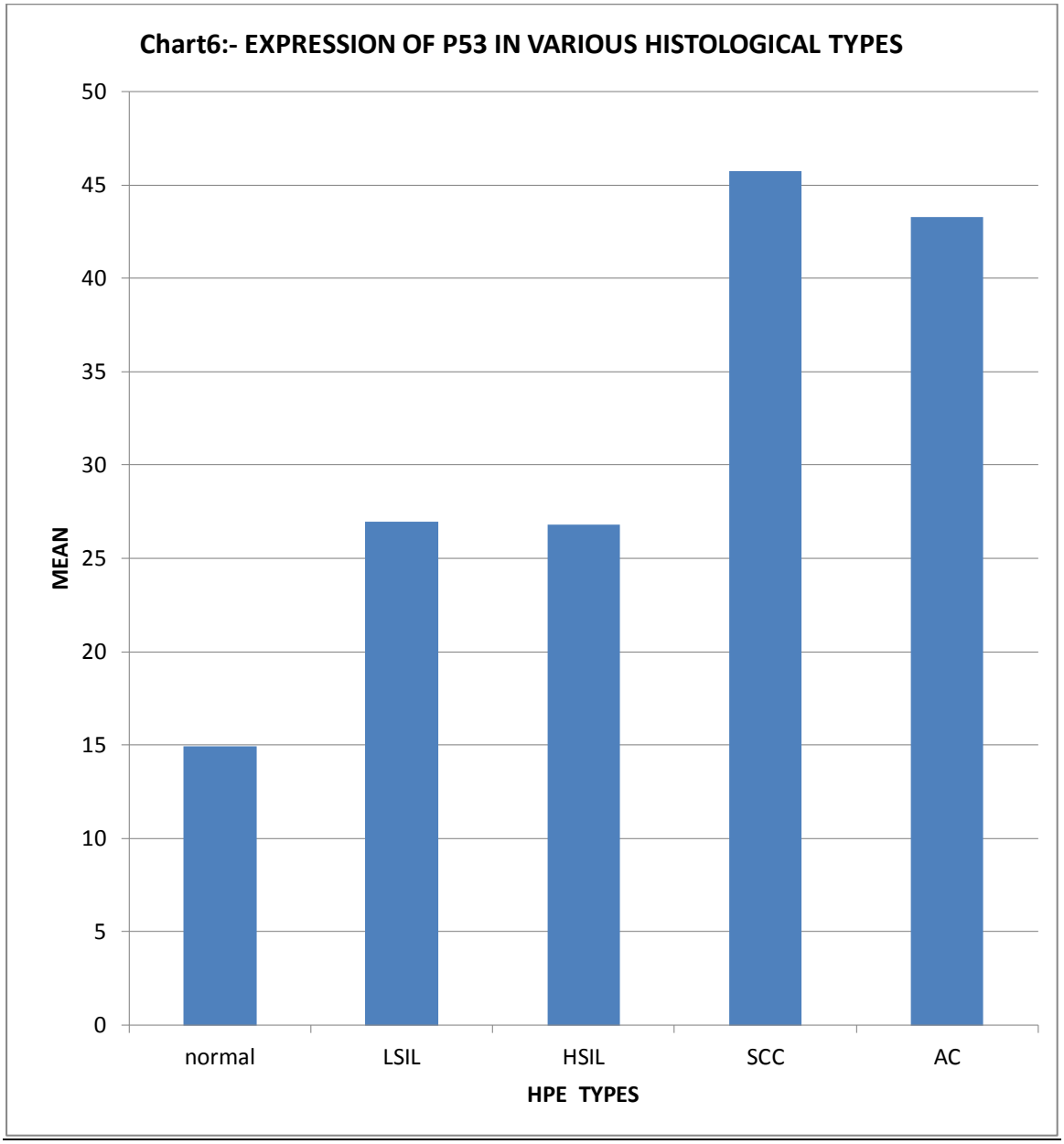
	HPE	Number of cases	Mean±SD	p- value
P53	Normal	20	14.95±9.30	<0.001
	SCC	57	45.74±25.85	
Ki-67	Normal	20	5.5±7.55	<0.001
	SCC	57	64.07±25.68	
pRb (n)	Normal	20	8.35±10.22	0.207
	SCC	57	13.14±15.69	
pRb (c)	Normal	20	0	<0.001
	SCC	57	27±26.80	

Mean p53 expression was significantly higher among SCC cases as compared to normal cervical epithelium. The mean p53 value among SCC cases was 45.74±25.85 as compared to normal which was 14.95±9.30. This difference was found to be statistically significant ($p<0.001$). Mean Ki-67 expression was significantly higher among SCC cases as compared to normal cervical epithelium. The mean Ki-67 value among SCC cases was 64.07±25.68 as compared to normal which was 5.5±7.55. This difference was found to be statistically significant ($p<0.001$). Mean pRb(n) expressions were low among SCC (13.14±15.69) cases as compared to normal cervical epithelium. This difference was found to be statistically insignificant. Mean pRb(c) expressions were significantly higher among SCC cases as compared to normal cervical epithelium. The mean pRb(c) value among SCC cases was 27±26.80 as compared to normal which was 0. This difference was found to be statistically significant ($p<0.001$).

Table 13:- Associationof immunoscoring between normal and adenocarcinoma

	HPE	Number of cases	Mean±SD	p- value
P53	Normal	20	14.95±9.30	0.004
	Adenocarcinoma	4	43.25±37.48	
Ki-67	Normal	20	5.5±7.55	<0.001
	Adenocarcinoma	4	69.50±37.42	
pRb (n)	Normal	20	8.35±10.22	0.322
	Adenocarcinoma	4	3.00±4.76	
pRb (c)	Normal	20	0	<0.001
	Adenocarcinoma	4	18.75±21.22	

Mean p53 expression was significantly higher among AC cases as compared to normal cervical epithelium. The mean p53 value among AC cases was 43.25±37.48 as compared to normal which was 14.95±9.30. This difference was found to be statistically significant (p=0.004). Mean Ki-67 expression was significantly higher among AC cases as compared to normal cervical epithelium. The mean Ki-67 value among AC cases was 69.50±37.42 as compared to normal which was 5.5±7.55. This difference was found to be statistically significant (p<0.001). Mean pRb (n) expressions were significantly lower among AC cases as compared to normal cervical epithelium. The mean pRb (n) value among AC cases was 3.00±4.76 as compared to normal which was 8.35±10.22. This difference was found to be statistically insignificant. Mean pRb (c) expressions were significantly higher among AC cases as compared to normal cervical epithelium. The mean pRb(c) value among AC cases was 18.75±21.22 as compared to normal which was 0. This difference was found to be statistically significant (p<0.001).



This graphical representation shows expression of p53 in various grades of cervical neoplasm, from normal to invasive carcinoma

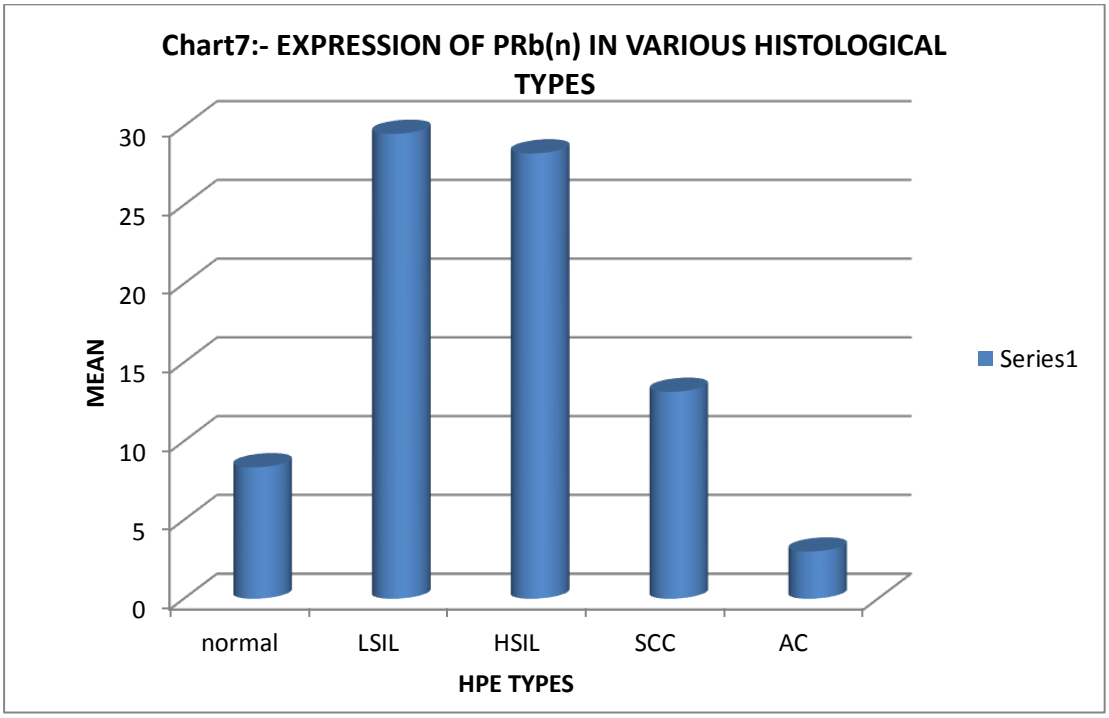


Chart7:- EXPRESSION OF PRb(n) IN VARIOUS HISTOLOGICAL TYPES
30
25
20
15
10
5
0
MEAN
normal
LSIL
HSIL
SCC
AC
HPE TYPES
Series1

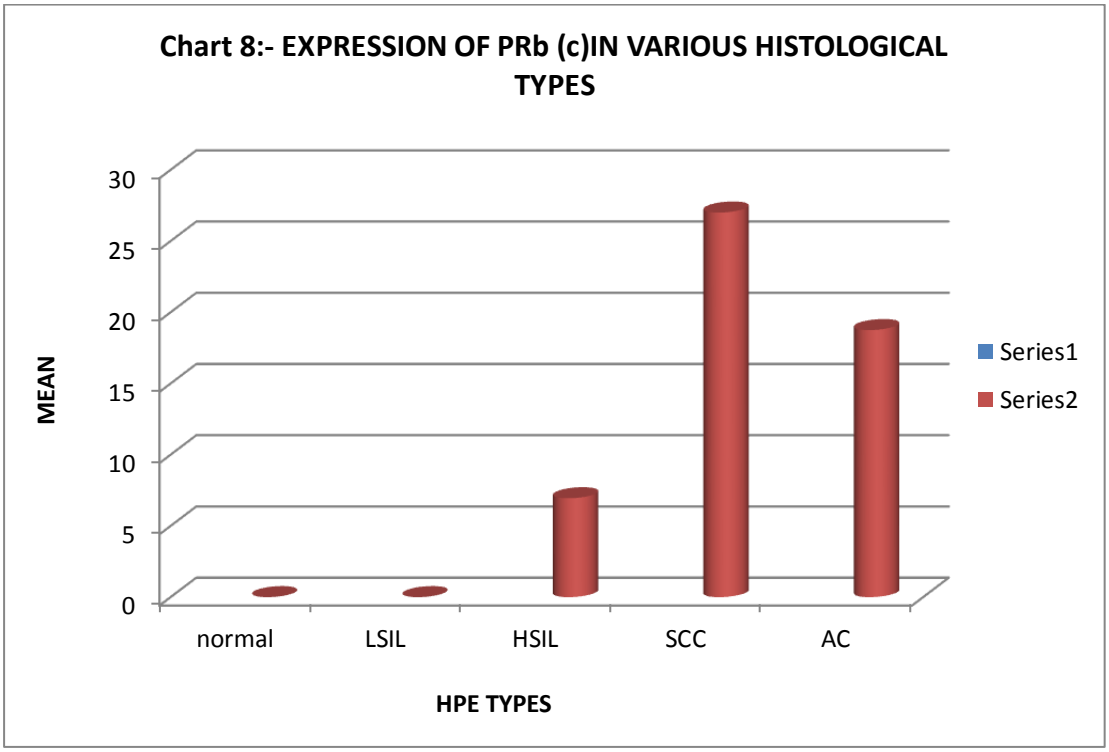


Chart 8:- EXPRESSION OF PRb (c)IN VARIOUS HISTOLOGICAL TYPES
30
25
20
15
10
5
0
MEAN
normal
LSIL
HSIL
SCC
AC
HPE TYPES
Series1
Series2

Chart 9:- EXPRESSION OF KI-67 IN VARIOUS HISTOLOGICAL TYPES

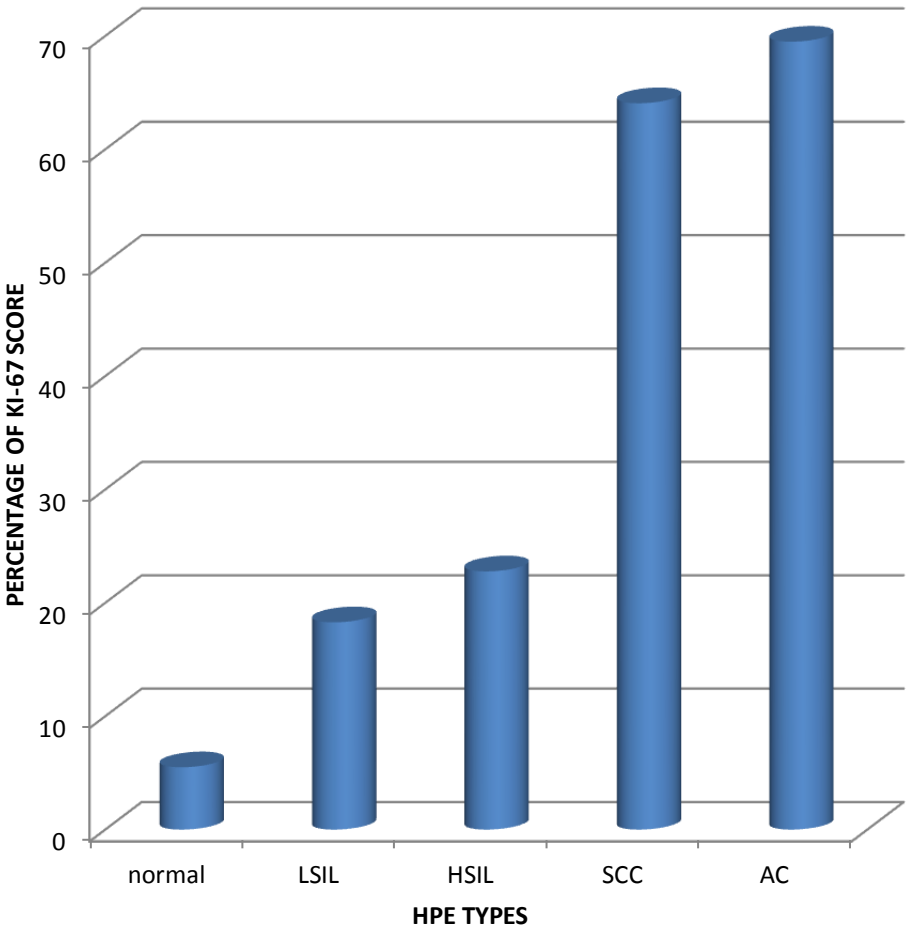


Table14:- Staining intensity of p53 in various histological types

HPE Type	0	I	II	III
Normal	0	18	2	0
LSIL	2	13	3	3
HSIL	2	13	2	1
MI	1	1	0	0
WDSCC	1	5	6	1
MDSCC	0	10	17	0
PDSCC	0	5	2	0
S-NKSCC	1	2	1	0
L-NKSCC	0	3	1	0
AC-VG	0	1	1	0
AC- EN	0	0	1	0
AS	0	1	0	0
TOTAL	7	72	36	5

Majority of the normal and premalignant (LSIL & HSIL) cases showed grade I staining intensity. Maximum number of invasive carcinoma (WDSCC & MDSCC) cases had grade II intensity. Out of 8 non keratinizing squamous carcinoma 5 had grade I staining intensity. 2 out of 3 adenocarcinoma cases showed grade II staining intensity.

Table 15:- Staining intensity of pRb(n) in various histological types

HPE Type	0	I	II	III
Normal	2	18	0	0
LSIL	2	10	9	0
HSIL	3	11	4	0
MI	0	0	1	0
WDSCC	1	9	3	0
MDSCC	6	19	3	0
PDSCC	3	4	0	0
S-NKSCC	1	2	0	0
L-NKSCC	2	3	0	0
AC-VG	0	2	0	0
AC- EN	0	0	1	0
AS	1	0	0	0
TOTAL	21	78	21	0

Majority of the normal and premalignant (LSIL & HSIL) cases showed grade I staining intensity. Maximum number of invasive carcinoma (WDSCC, MDSCC & PDSCC) cases showed grade I intensity. Out of 28 cases of MDSCC 6 showed no staining. Out of 8 non keratinizing squamous carcinoma, 5 showed grade I staining intensity, 3 showed no staining. 2 out of 3 adenocarcinoma cases showed grade I staining intensity.

Table 16:- Staining intensity of pRb(c) in various histological types

HPE Type	0	I	II	III
Normal	20	0	0	0
LSIL	20	0	1	0
HSIL	12	5	1	0
MI	0	1	0	0
WDSCC	3	5	5	0
MDSCC	5	15	7	1
PDSCC	4	2	1	0
S-NKSCC	1	1	1	0
L-NKSCC	2	2	1	0
AC-VG	1	0	1	0
AC- EN	1	0	0	0
AS	0	1	0	0
TOTAL	69	32	18	1

Majority of the normal and premalignant (LSIL & HSIL) cases showed grade 0 i.e there was no staining. In case of WDSCC out of 13 cases 5 each showed grade I & II staining intensity, whereas majority of MDSCC showed grade I staining intensity. 4 out of 7 cases of PDSCC showed no staining. Out of 8 non keratinizing squamous carcinoma 3 each showed grade 0 &I staining intensity respectively. 2 out of 3 adenocarcinoma cases showed grade 0 staining intensity.

Table 17:- Staining intensity of ki-67 in various histological types

HPE Type	0	I	II	III
Normal	2	13	5	0
LSIL	0	6	9	6
HSIL	2	2	10	4
MI	0	0	1	0
WDSCC	0	0	5	8
MDSCC	1	2	18	7
PDSCC	0	0	5	2
S-NKSCC	0	0	1	2
L-NKSCC	0	0	3	2
AC-VG	0	0	1	1
AC- EN	0	0	0	1
AS	0	0	1	0
TOTAL	5	23	59	33

Majority of the normal cases showed grade I staining intensity. 9 out of 21 cases of LSIL showed grade II staining intensity and 6 each showed grade I and III staining intensity respectively. In cases of HSIL, out of 18 cases 10 of them showed grade II staining intensity. In cases of WDSCC majority of them showed grade III intensity. Out of 28 cases of MDSCC 18 showed grade II intensity. Out of 8 non keratinizing squamous carcinoma 4 each showed grade II and III staining intensity respectively. 2 out of 3 adenocarcinoma cases showed grade III staining intensity.

Table 18:- Correlation of clinical staging and IHC staining

	Clinical Stage	Number of cases	Mean±SD	p- value
P53	I	4	33.25±34.24	0.287
	II	8	42.00±30.49	
	III	49	53.61±29.18	
Ki-67	I	4	48.25±19.47	0.177
	II	8	78.13±27.57	
	III	49	71.57±26.64	
pRb (n)	I	4	15.25±29.84	0.472
	II	8	20.75±13.96	
	III	49	12.82±16.27	
pRb (c)	I	4	6.25±9.46	0.125
	II	8	40.88±35.04	
	III	49	27.41±26.89	

There was no correlation between immunohistochemical staining using p53, Ki-67 and pRb with the clinical staging.

DISCUSSION

Cervical carcinoma is one of the most common malignancies affecting women.[2] It is one of the major contributors to cancer related morbidity and mortality worldwide. By extensive epidemiological and molecular biological studies, the HPV is known to be the most important cause of cervical cancer.[39]

HPV are small double stranded DNA viruses which can be divided into two groups, the 'low risk' (type 6 and 11) and the 'high risk' (type 16 and 18). High risk viruses are implicated in the pathogenesis of cervical neoplasm. HPV-induced cervical cancer is a multistep process. But additional risk factors also play a role in progression of HPV-induced disease, most likely influencing the immune surveillance or acting as additional carcinogens.[20] A small percentage of infections induce the development of low- and/or high-grade cervical intraepithelial neoplasia (CIN), which can still regress or progress to an invasive cervical carcinoma after a long period of latency. The HPV genome encodes proteins that are able to induce unscheduled proliferation and prevent apoptosis.[40]

E6 and E7, the viral oncoproteins causes inactivation of host tumor suppressor genes (p53 & pRb). This will result in loss of normal maturation sequence, representing persistent, proliferative HPV infection.[39]

The multistep process of carcinogenesis is based directly or indirectly on cell proliferation. Ki-67 is a nuclear proliferation associated antigen and a well known cell proliferation marker.[9] Cell proliferation has been described as an additional parameter useful in the prognostic evaluation of cervical cancer.[41,42]

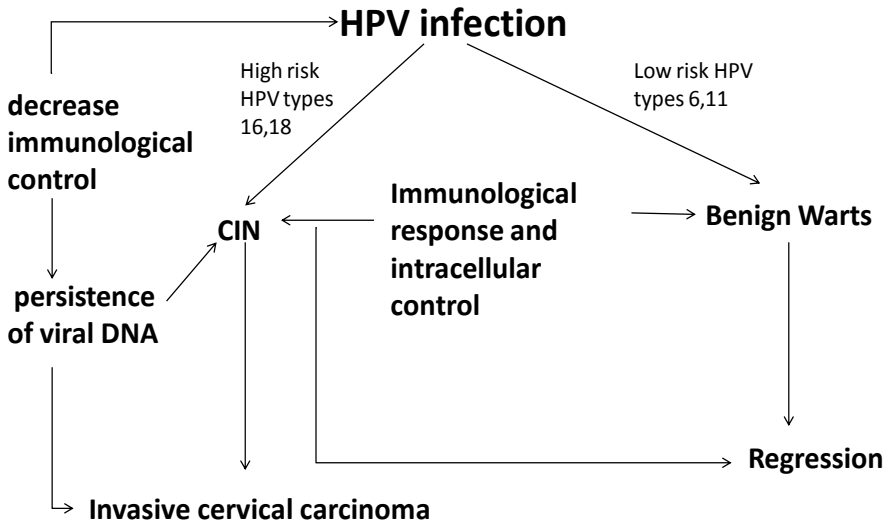


Chart 10:- Host response to HPV infection

The current study was done to know the role of the above mentioned (biomarkers) cellular proteins in various grades of dysplasia and malignancy.

According to our study as shown in the table-5, maximum frequency of premalignant and malignant cervical lesions were found in the age group of 40-49yrs. Misra et al[3] observed maximum frequency of cases in older women beyond 40yrs of age (SIL-10.71%, carcinoma cervix-1.3%). Kalyani et al[1] had got a similar age distribution in their study. Aswathy et al[43] had found that the most common age group involved in carcinoma cervix ranged from35-50yrs.

About 90% of cervical cancers are squamous cell carcinomas and rest 10% are adenocarcinoma .Similar results are seen in the studies conducted by Das BC et al[5] and Misra et al[3] where around 85-90% of cases are squamous cell carcinoma and rest of them are adenocarcinoma. Kalyani et al[1] in her study showed that majority of cervical carcinoma cases were histologically squamous cell carcinoma followed by adenocarcinoma.

Alterations of tumour suppression genes and their role in the process of carcinogenesis have been extensively described. The suppressor gene p53 plays an important role in protection against the development of cancer.

Howayda ABD EL ALL et al[44], in his work showed that p53 immunoreactivity starts in SIL. The expression of p53 began to appear in a few cells in the basal layer of LSIL and increase in parallel to the extension of neoplastic cells in HSIL and carcinoma in situ and was more extensive in invasive lesions. Bosari et al[45] documented positive immunoreactivity confined to basal layer in 74% of normal cervical epithelium and in all cases of low grade cervical intraepithelial lesions (CIN I). He also showed that there was suprabasal p53 immunoreactivity in 25% of high grade squamous intraepithelial lesions (CIN II & CIN III) and 72% of invasive squamous cell carcinoma showed positivity for p53. Holm et al[46] identified p53 protein in 7% of HSIL, 62% of invasive squamous cell carcinoma and 11% of adenocarcinoma. Miwa Akasofu et al[47] showed that no staining was observed in the normal cervix, CIN I or CIN II for p53 but 62.1% of CIN III and 73.9% of SCC showed positivity. Betty L S et al[48], showed positivity for p53 in 2.6% of normal cervical epithelium, 1.2% of LSIL and 3.6% of HSIL**.** Dimitrakakis et al[26] showed that immunostaining for p53 was not detected in any of the cases of the normal epithelium, condyloma, and CIN I and II, while 15% of CIN III, 29% of invasive squamous carcinoma and 11% of the adenocarcinoma cases were positive for p53 staining. The staining was nuclear, strong, and uniform. Abeer et al [6] showed that 18.4% of HSIL and 44.2% of SCC were positive for p53. Tan GC et al[25] showed that 47.6% of LSIL, 78.7% of HSIL, 72.2% of SCC and 42.9% of AC were positive for p53.

Table 19:- Shows expression of p53 in cervical lesions compared with other studies

Various studies	Normal (%)	LSIL (%)	HSIL (%)	SCC (%)	AC (%)
Howayda ABD EL ALL et al (1993)	--	Basal layer positivity	Full thickness positivity		
Bosari et al (1993)	74	100	25	72	
Holm et al (1993)	---	0	7	62	11
Miwa Akasofu et al (1995)	0	0	62.1	73.9	---
Betty L S et al (1998)	2.6	1.2	3.6	---	----
Dimitrakakis et al (2000)	0	0	15	29	11
Abeer et al (2007)	0	0	18.4	44.2	
Tan GC et al (2007)	--	47.6	78.7	72.2	42.9
Present study (2012)	100 basal layer positivity	86 suprabasal positivity	94.5 full thickness positivity	94.6	100

In the present study, p53 was detected in all cases of normal cervical epithelium confined to the basal layer. Among premalignant lesions, 86% of LSIL cases showed positivity in basal and suprabasal region. 94.4% of HSIL cases showed suprabasal/ full thickness positivity. On the contrary, when LSIL cases were compared with normal cases, significant association was found ($p=0.023$). But this was not true in case of HSIL where no significant association was found.

In our study 94.6% of invasive squamous cell carcinoma showed p53 expression. In comparison with normal cervical epithelium, significant association was found ($p<0.001$). Although the rates of p53 positivity in cervical carcinoma differ compared to the previous reports and the

present study, all of them at least indicate that p53 immunoreactivity was commonly detected in ISCC.(chart 6)

Conflicting results on this topic have been published. Positive p53 immunoreactivity which was detected in normal cervical epithelium can be explained as a result of overexpression or stabilization of the wild type, or as an effect of the microwave oven heating in the absence of mutations.[25] This could probably be the reason of overexpression of p53 in normal cases in our study also. Some studies showed a significant correlation of p53 expression with HSIL compared with normal, whereas others showed no significant association.[18]

The possible explanation for overexpression of p53 in SCC may imply several things: p53 gene may be randomly mutated, abnormal accumulation of non-mutant p53 protein due to altered p53 homeostasis in tumour cells rather than p53 mutation, conformational changes in wild type p53 protein causing it to change from suppressor to promoter, or mutant p53 and/or detected p53 may be normal p53 that is abnormally stabilised or increased in amount.

We all know adenocarcinoma and adenosquamous carcinoma has bad prognosis when compared to squamous cell carcinoma. Therefore p53 expression would be expected to be greater in the former two. In our study, p53 expression was found to be greater in adenocarcinoma. This finding does not reflect the true population as there were only 4 cases available for evaluation. Also association with normal does not carry much value even though it was found to be significant. When the intensity of staining was evaluated in our study, both normal and premalignant cases showed grade I (mild) intensity. However malignant cases showed grade II to III (moderate to intense) intensity. According to Tan G.C et al[25], majority of CIN showed negative to grade I intensity, whereas 65.2% of malignant cases showed grade III intensity.

The protein product of the Rb gene (pRb) is a nuclear phosphoprotein that plays an important role in regulating the cell cycle.

Amortegui AJ et al[49] showed higher pRb expression in premalignant lesions when compared to normal cervical epithelium. Chetty et al [50], in his study, showed positivity in 86.6% of SCC cases and 66.6% of AC cases. Mauricio et al[28], in his study, found that pRb immunoreactivity was found in all cases of normal cervical epithelium but confined to parabasal cell layer. All most all pre-cancerous lesions showed positivity, staining was confined to basal and parabasal layers of the epithelium. But he observed that pRb staining was lower in invasive carcinoma than SIL, suggesting that Rb gene downregulation could be involved in cervical carcinogenesis. According to Noraini et al[4], pRb expression was found in almost all the cases of normal cervical epithelium and premalignant lesions. In case of normal cervical epithelium, in majority of cases staining was confined to parabasal cell layer or basal third of the epithelium. Majority of premalignant cases staining was seen in basal two-thirds of the epithelium or full thickness and all malignant lesions showed positive pRb(n) immunoreactivity. In the study conducted by Barbara et al[51], pRb expression was detected in 99.1% sections of normal squamous epithelium, 94.4% of CIN I , 91.3% of CIN II/III, 94.4% of SCC and 66.7% of AC cases.

Ceccarelli et al[52] in his study found loss or reduction of pRb expression in aggressive behaviour of breast cancer.

Table 20:- Shows expression of pRb in cervical lesions compared with other studies

Various studies		Normal (%)	LSIL (%)	HSIL (%)	SCC (%)	AV (%)
Chetty R et al (1997)		--	---	---	86.6	66.6
Mauricio et al(2002)		100 parabasal layer	100 parabasal layer	100 basal & parabasal		---
Noraini et al (2003)		100 parabasal layer	100 basal & parabasal	100 basal& parabasal	100	100
Barbara et al (2004)		99.1	94.4	91.3	94.4	66.7
Present study (2012)	pRb(n)	90 basal layer	95 basal & parabasal	83.5 full thickness	80.5	50
	pRb(c)	0	0	27.8	73.2	75

In our study, pRb immunoreactivity was seen in 90% of normal cervical epithelium in basal layer. In case of premalignant lesions, 95% of LSIL cases showed positive immunoreactivity in basal and parabasal layer. 83.5% of HSIL cases showed positivity in basal & parabasal layer/ full thickness. One more finding which we would like to highlight here was the appearance of cytoplasmic stain in HSIL (28%) cases. When pRb(n) immunoscoring was compared between normal cervical epithelium and LSIL, significant association was found ($p<0.001$). But no association was found for pRb(c). We also found significant association between normal cervical epithelium and HSIL cases for both pRb(n)($p=0.001$) and pRb(c)($p=0.03$).

In our study, 80% of malignant lesions showed positive pRb(n) immunoreactivity. The percentage of immunoreactivity in case of PDSCC was low when compared to WDSCC & MDSCC. The percentage of pRb positive cells was higher in SIL than in normal cervical epithelium. Increased pRb expression in premalignant lesions when compared to normal may be due to an increased proportion of proliferating cells (chart 7). This is supported by the fact that hyperphosphorylated pRb is increased during G2/M phases.[4,45]

In case of pRb(c) immunoreactivity, 73% of malignant lesions showed positivity. When pRb(n) immunoscoring was compared between normal and invasive squamous cell carcinoma, no significant association was found, whereas for pRb(c), significant association was found ($p<0.001$).

In case of adenocarcinoma 50% of cases showed positivity. Since the number of cases was very less, commenting on its immunoreactivity was not possible. An additional finding was the presence of cytoplasmic staining, which appeared in HSIL and increased in proportion in WDSCC and MDSCC, but decreased in PDSCC (chart 8). This finding has been observed in many other studies but the reason is still not known. It was thought to be an artifact by some investigators or it could be related to Rb gene mutation. [30] One more reason could be the dispersion of gene products from the nucleus to the cytoplasm which will take up the brown stain. Studies have shown that E7 protein was found predominantly in the nucleus and to a minor extent in the cytoplasm in cervical carcinoma. As we all know, HPV is one of the most common risk factors for cervical carcinogenesis. The oncoprotein in HPV, that is E7, binds with Rb and forms a complex. This complex could be present in the cytoplasm which will also take up the stain. Further studies should be done to support these finding.

Mauricio et al,[30] in his study, also observed cytoplasmic immunoreactivity in mitotic cells, indicating that in the absence of nuclear envelope, pRb diffuses to other cellular locations.

The staining intensity of pRb(n) varied from grade 1 to 2 in both premalignant and malignant lesions, whereas for pRb(c) it varied from grade 0 to 1. This variation in staining probably resulted from asynchronous progression of the cells through the cell cycle.

Ki67 is a cell cycle associated protein present in the peri-chromosomal region, expression of which is associated with cell proliferation to measure growth fraction of cells in human tumors.

According to A.L. Silva-Filho et al[42], 6.7% of normal cases showed positivity for Ki-67and the expression was significantly higher in patients with invasive squamous cell carcinoma (93.3%), Carreras R et al[36], in his study, showed Ki-67 expression in 25% of CIN I cases, 70% of CIN III

cases and 65.5% of SCC cases. These data are in accordance with the literature. The Ki-67 expression can be useful in distinguishing the different grades of dysplasia, though not predicting their behavior. Eun Ji Nam et al[53] showed that Ki-67 expression was seen in 58.3% of CIN I cases and 50% of CIN III cases had strong Ki-67 expression. The higher the CIN grade, the stronger the Ki-67 expression observed ($p=0.003$). Pablo C Z et al,[18] in his study, showed that positive Ki-67 expression was detected in 23% of normal cervical epithelium, 48% of LSIL cases, 89% HSIL cases ($p<0.05$) and 100% of invasive carcinoma (SCC & AC). This showed that Ki-67 expression has a tendency to correlate positively with the histologic grade. Natalia G.M et al[35] showed Ki-67 positivity in 6.6% of normal cases, 35.6% of LSIL cases, 46.5% of HSIL cases and 58% of SCC cases. According to Su Mi Kim et al[9], Ki-67 expression was greater in CIN samples than in normal/metaplastic epithelium and Ki-67 staining was stronger in high-grade CIN than in low-grade CIN. Therefore, Ki-67 can used to differentiate and grade CIN.

Table 21:- Shows expression of Ki-67 in cervical lesions compared with other studies

Various studies	Normal (%)	LSIL (%)	HSIL (%)	SCC (%)	Adenocarcinoma (%)
A.L. Silva-Filho et al (2004)	6.7	---	---	93.3	
Carreras. R et al (2007)	--	25	68	65.5	----
Eun Ji Nam et al(2008)	---	58.3	50	---	-----
Pablo C Z et al (2009)	23	48	89	100	----
Natália G M et al (2009)	6.6	35.6	46.5	57.8	----
Su Mi Kim et al (2011)	--	51.5	--	--	---
Present study (2012)	90 basal layer	100 basal,suprabasal layer	94.5 full thickness	98.2	100

In our study, 90% of normal cases showed positivity for Ki-67 only in the basal layer. All the LSIL (100%) cases showed Ki-67 positivity in basal and suprabasal layer, but a few cases (5%) showed higher expression. In case of HSIL, 95% of cases showed Ki-67 expression (full thickness). To conclude, the expression of Ki-67 increases with the grade of dysplasia. As shown in the Table-9, the expression of Ki-67 was significantly higher in invasive squamous cell carcinoma cases. Around 98.2% of cases showed positivity. In our study we also found a significant association when compared with normal cervical epithelium ($p<0.001$). This finding shows that Ki-67 expression correlates with the histological grade of cervical neoplasia. We also found that all adenocarcinoma cases showed Ki-67 positivity. A significant association was found between

normal cervical epithelium and adenocarcinoma cases as well ($p<0.001$). Since the number of cases was very less commenting on its results has less value. (Chart 9)

An attempt to grade the intensity of staining was made, which showed that normal cervical epithelium cases (13 out of 20) showed mild (grade I) staining. In premalignant lesions, moderate (grade II) staining was noted. In case of invasive carcinomas, the intensity ranges from grade II – III.

An effort was also made to know if there was any significant association between the markers and clinical stages. Unfortunately, no significant association was found between them. According to Surapan K et al[54], there was no correlation of p53 expression with tumour size, histologic grade, depth of invasion and lymphovascular space invasion in early stage cervical carcinomas.

CONCLUSION

Squamous cell carcinoma is the commonest histological type of carcinoma cervix which is commonly seen in 4^{th} decade of life in women. p53 expression progressively increases from normal cervical epithelium to intraepithelial lesion to malignant lesion. However pRb (n) expression was maximum in intraepithelial lesion compared to normal cervical epithelium and malignant lesion. pRb(c) expression as p53 protein progressively increases from intraepithelial lesion to frank malignancy. Ki-67 expression is directly proportional to the degree of dysplasia to malignant lesion. However further studies is required to exactly analyze the cytogenetic aberration in p53 and pRb genes. The present study can be used as a base line study of the expression of p53 and pRb protein in cervical neoplasia. p53 and pRb(c) can be used as marker for early detection of cervical intraepithelial neoplasia.

SUMMARY

1) In the present study, both premalignant and malignant cervical lesions are most commonly seen in the age group of 40-49yrs. Among all cervical malignancies, squamous cell carcinoma was the most prevalent subtype.

2) p53 expression, though present in all cases including normal cervical epithelium, showed increased mean expression as the lesion progressed from normal to malignant. Squamous cell carcinoma showed the highest expression. Both normal and premalignant cases had grade I intensity of staining whereas malignant cases had grade III intensity of staining.

3) pRb(n) expression was seen in all cases inclusive of normal cervical epithelium. However, a higher degree of expression was seen in SIL cases (LSIL > HSIL) than normal cervical epithelium and malignant lesions. The staining intensity varied from grade I to II in premalignant and malignant lesions.

4) pRb(c) expression was negligible/absent in cases of normal cervical epithelium and LSIL, but showed minimal expression in cases of HSIL and increased expression in malignant lesions. SCC showed highest expression of pRb(c). In premalignant and malignant lesions it varied from grade 0 to I.

5) Ki-67 expression was seen in all cases including normal cervical epithelium. The imunoscoring was proportional to the degree of dysplasia, the highest being in malignant lesions. Adenocarcinoma cervix showed highest expression of Ki-67. The Ki-67 expression can be useful in distinguishing the different grades of dysplasia. The intensity of staining also increased as the lesion progressed to malignancy.

6) The current study showed no correlation between the clinical staging of the disease with the immunohistochemical staining results for p53, Ki-67 or pRb.

7) p53 and pRb(c) can be used as markers for early detection of cervical intraepithelial neoplasia.

Fig 7:- Microphotograph showing case of normal cervical epithelium

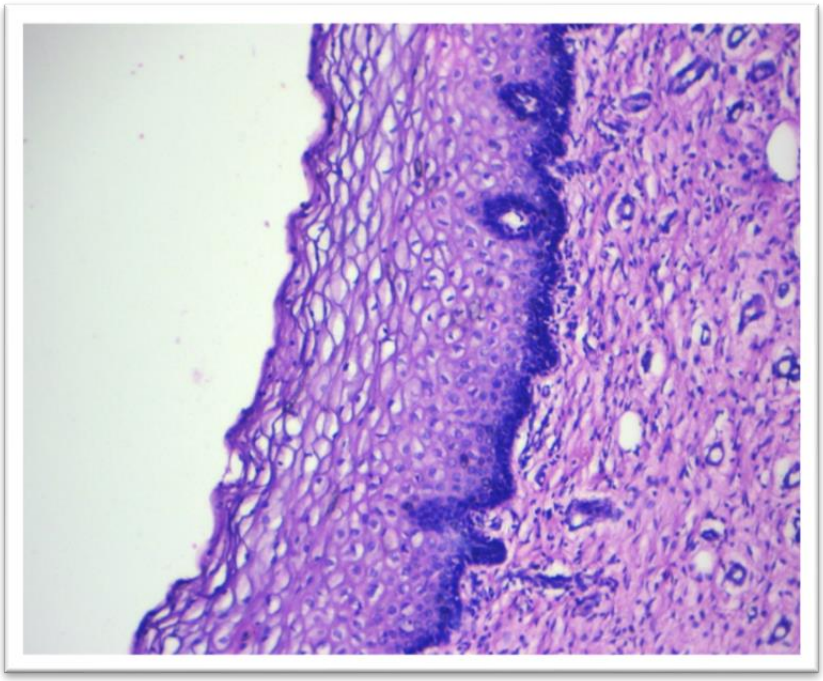


Fig 8:- Microphotograph showing p53 expression in case of normal cervical epithelium

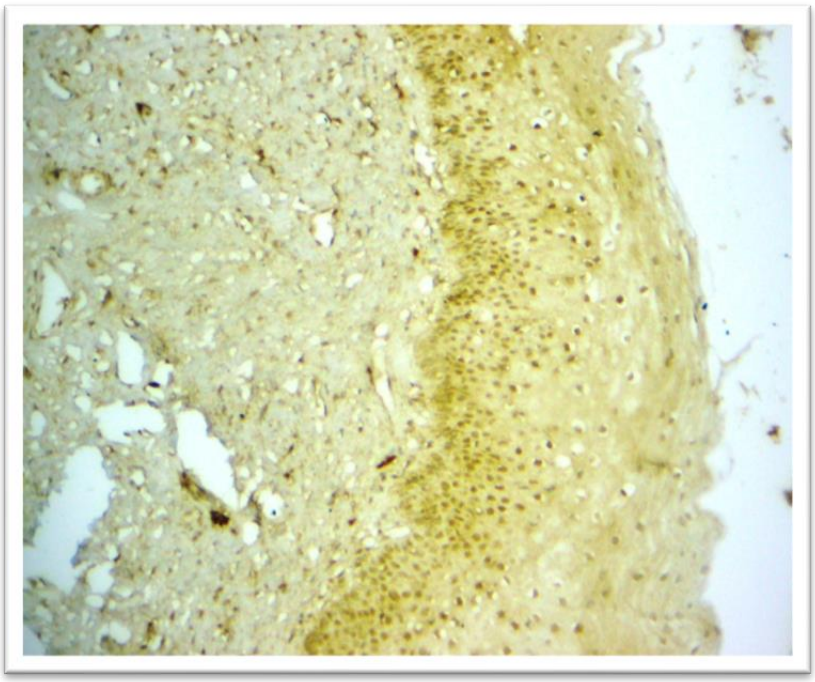


Fig 9:- Microphotograph showing basal expression of Ki-67 in case of normal cervical epithelium

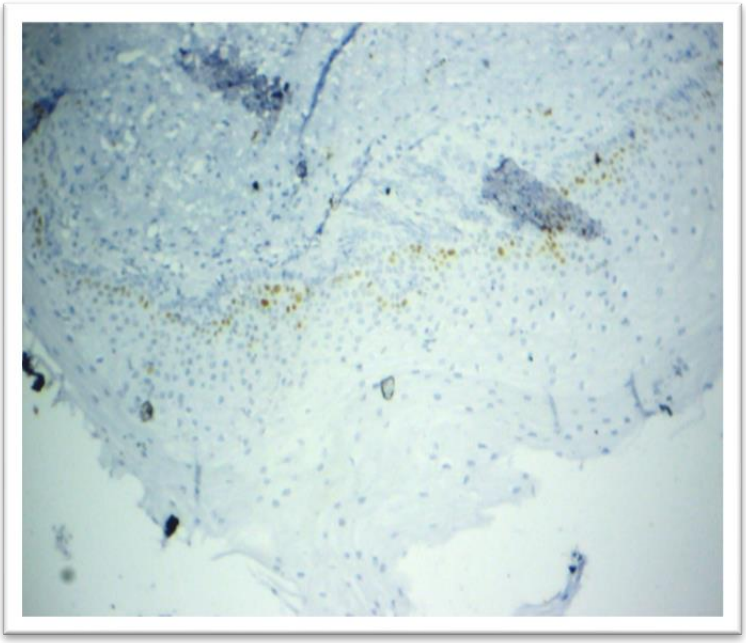


Fig 10:- Microphotograph showing mild expression of pRb in case of normal cervical epithelium

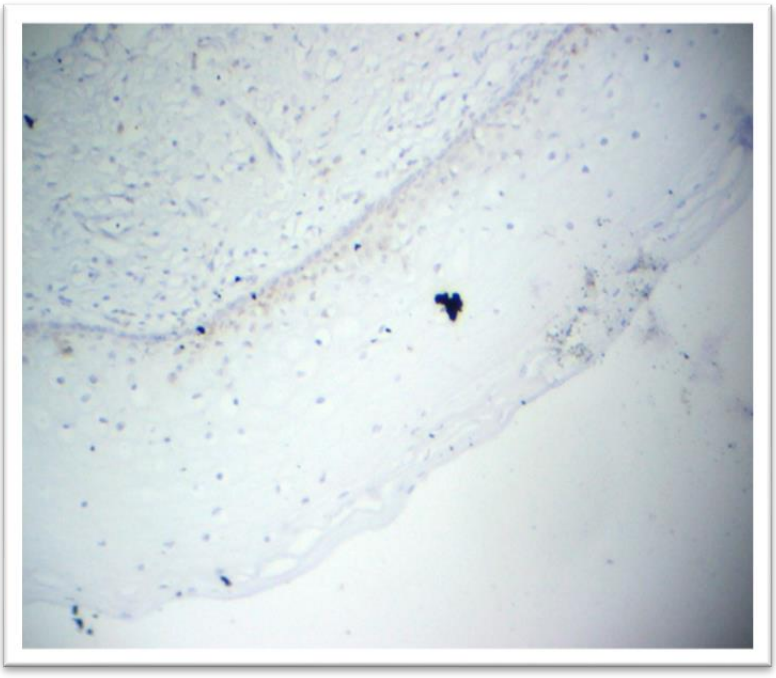


Fig 11:- Microphotograph showing low grade squamous intraepithelial lesion (100x)

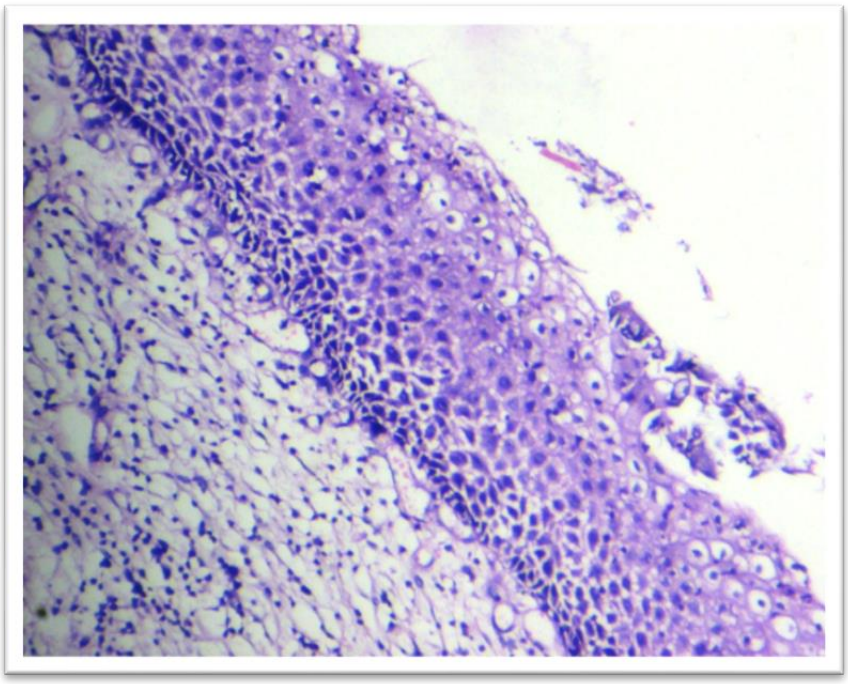


Fig 12:- Microphotograph showing p53 expression in basal & suprabasal layer of cells in case of LSIL (100X)

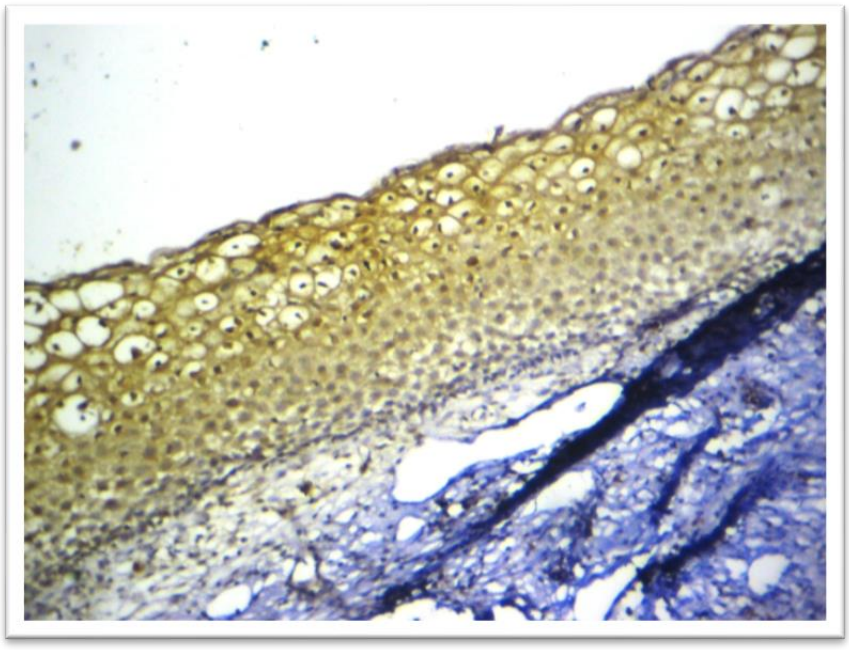


Fig 13:- Microphotograph showing Ki-67 positivity in basal layer of cells in LSIL (100X)

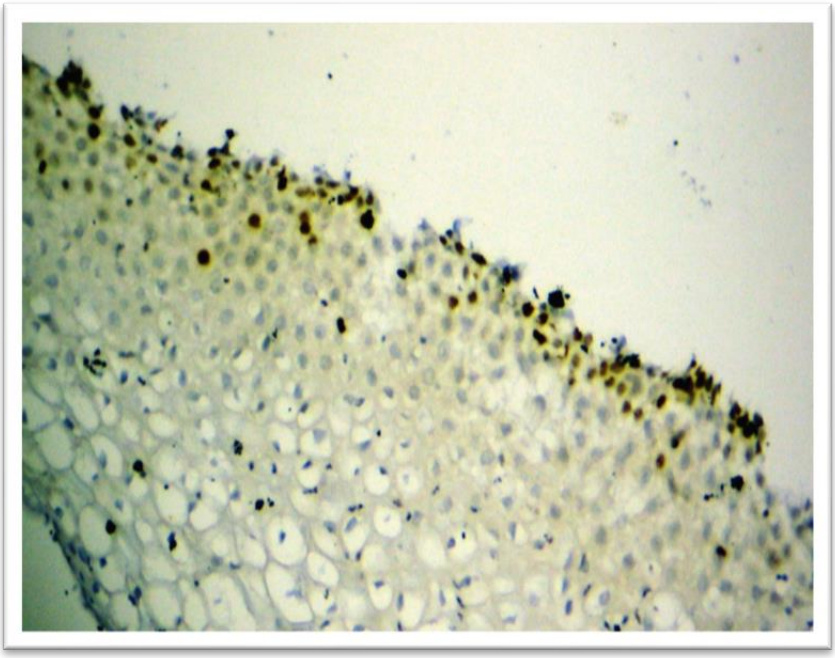


Fig14:- Microphotograph showing basal and few cells of suprabasal layer in case of LSIL. No cytoplasmic stain is seen. (100x)

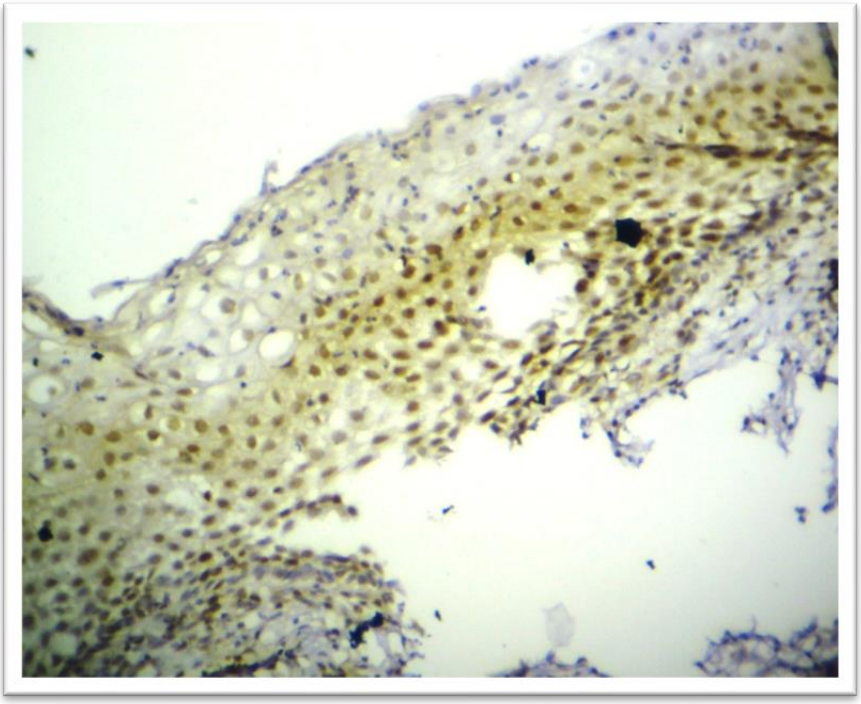


Fig 15:-Microphotograph showing high grade squamous intraepithelial lesion (H & E 100X)

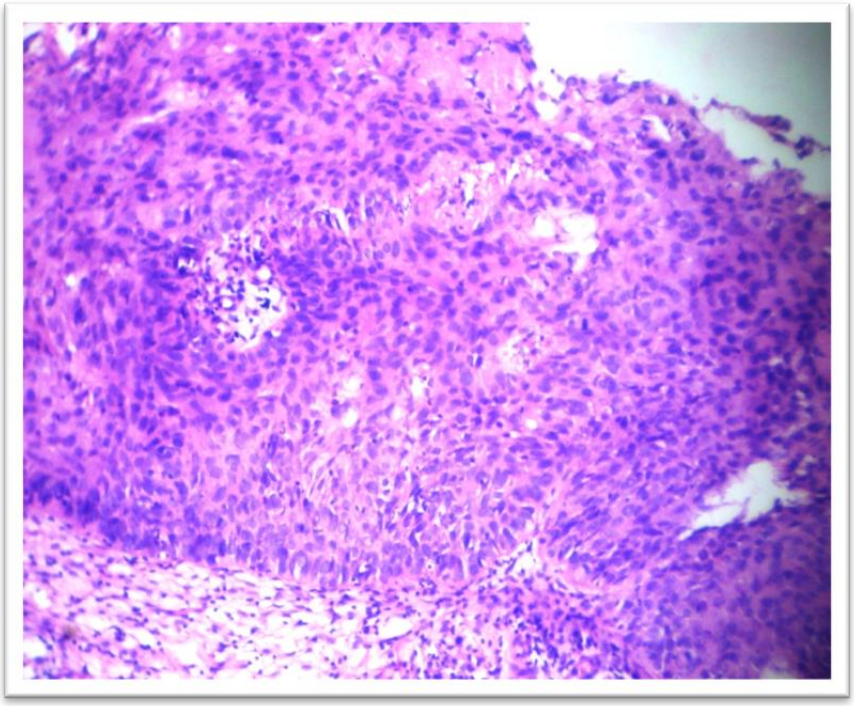


Fig 16:- Microphotograph showing p53 expression in basal and suprabasal layer of cells in case of HSIL. (100x)

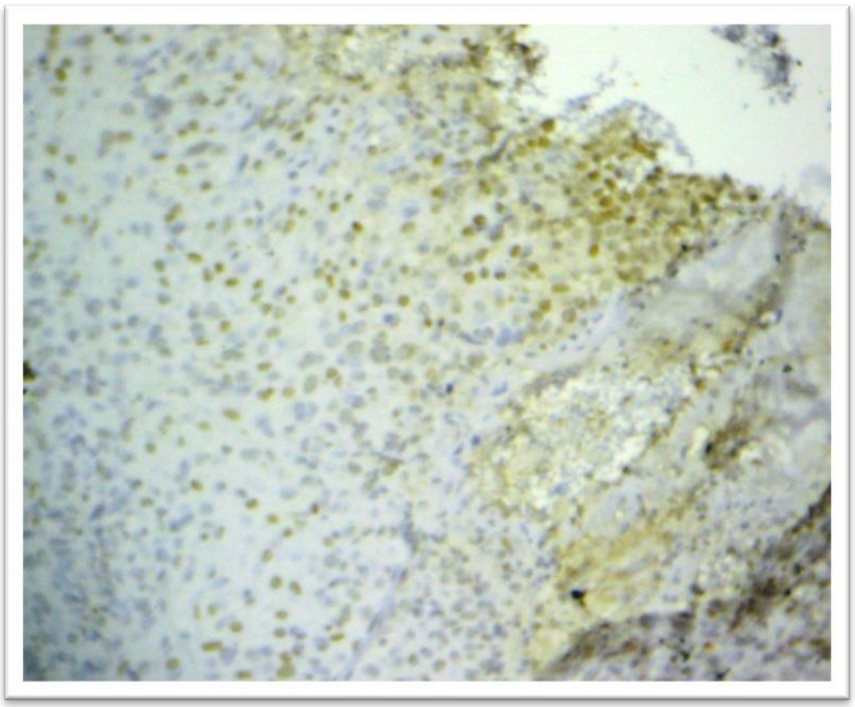


Fig 17:- Microphotograph showing positivity of Ki-67 in basal & few cells of Suprabasal cell layer in case of HSIL (100x)

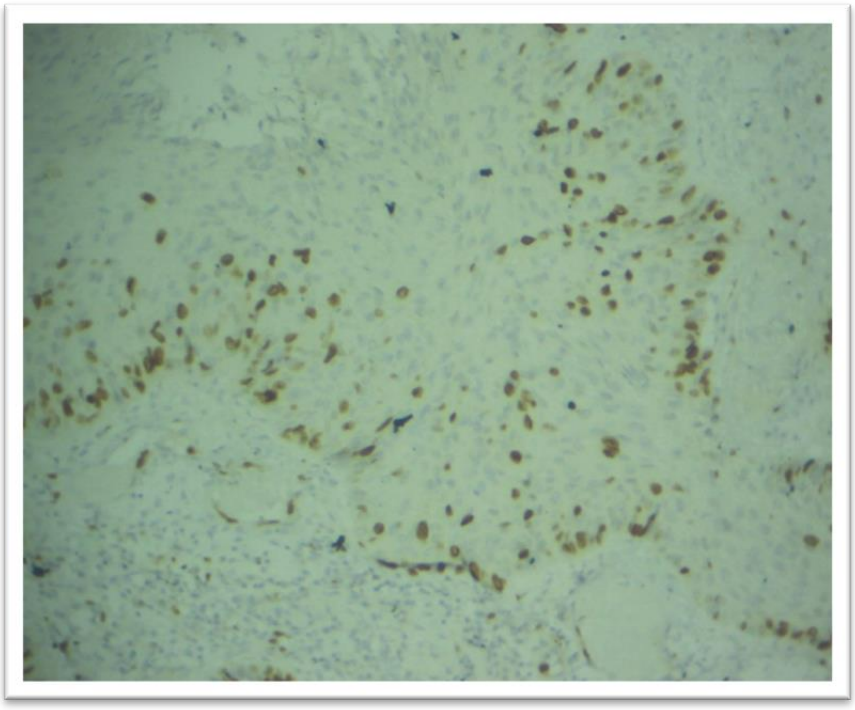


Fig 18:- Microphotograph showing pRb expression showing nuclear positivity with few cells showing cytoplasmic stain in case of HSIL.(100x)

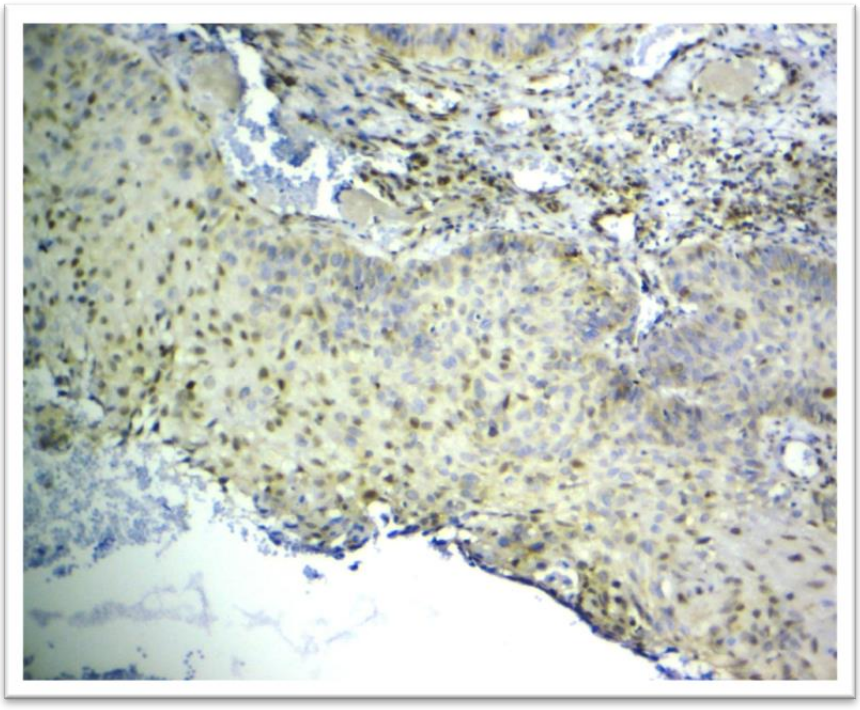


Fig 19:- Microphotograph showing well differentiated squamous cell carcinoma (H & E 100X)

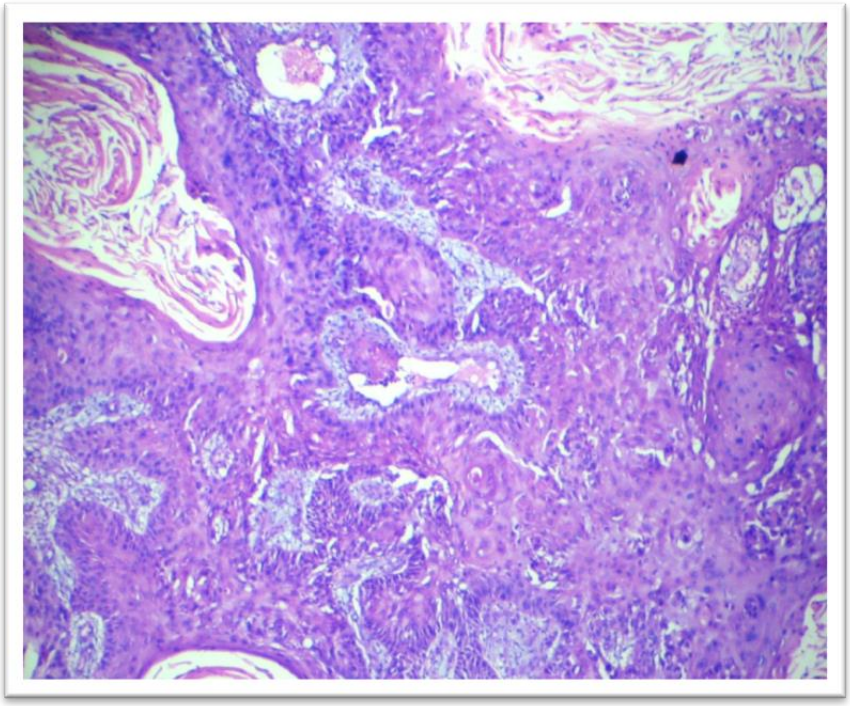


Fig 20:- Microphotograph showing p53 expression in well differentiated squamous cell carcinoma(100x)

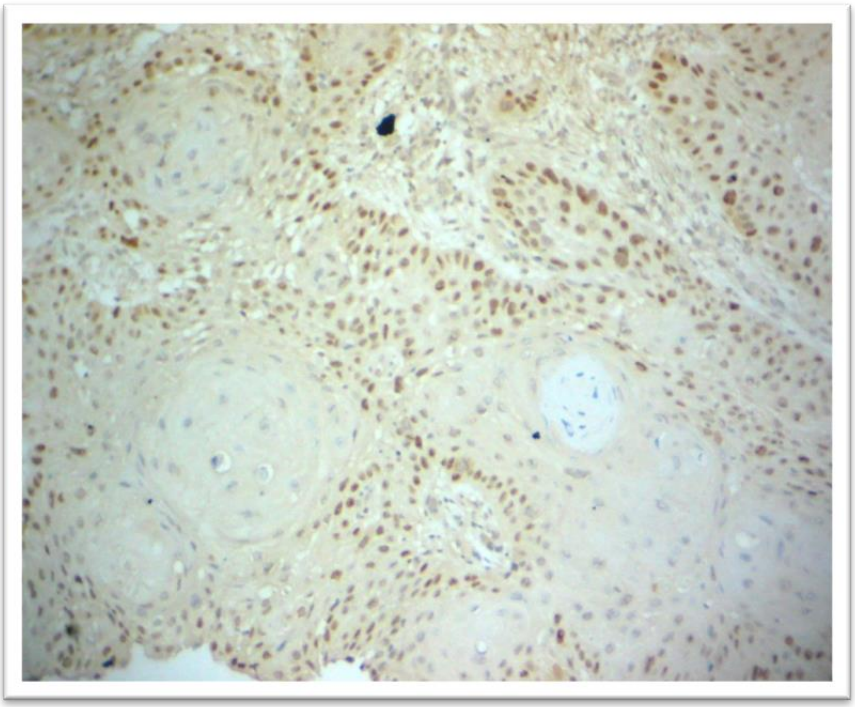


Fig 21:- Microphotograph shows Ki-67 expression in well differentiated carcinoma. Almost all the tumor cell nuclei are stained with strong intensity. (100X)

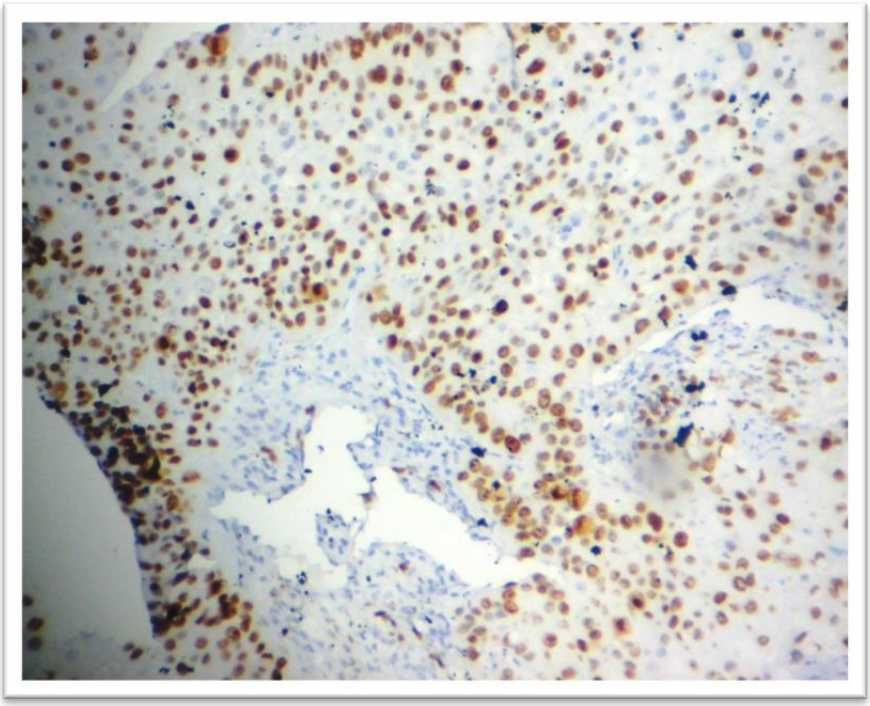


Fig 22:- Microphotograph is showing pRb immunoexpression in well differentiated squamous cell carcinoma. Note the cytoplasmic staining. (100X)

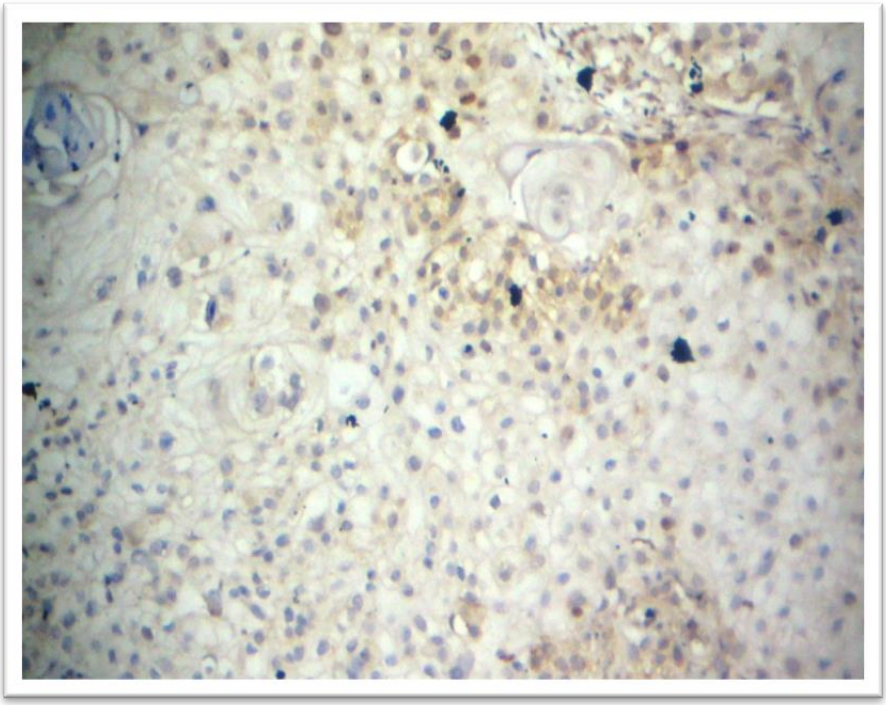


Fig 23:- Microphotograph showing moderately differentiated squamous cell carcinoma. (H and E 100X)

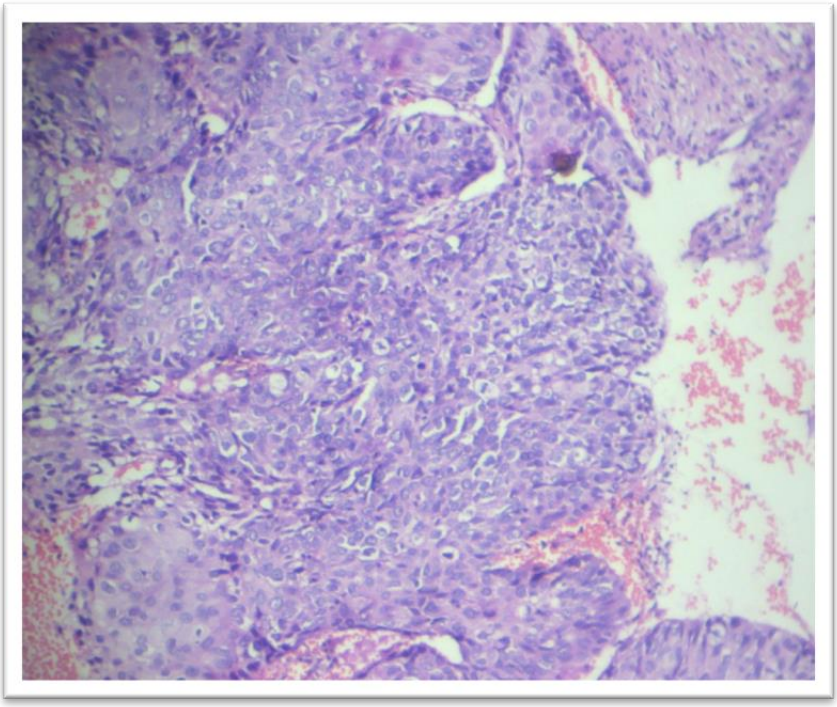


Fig 24:- Microphotograph showing p53 expression in moderately differentiated squamous cell carcinoma.(100X)

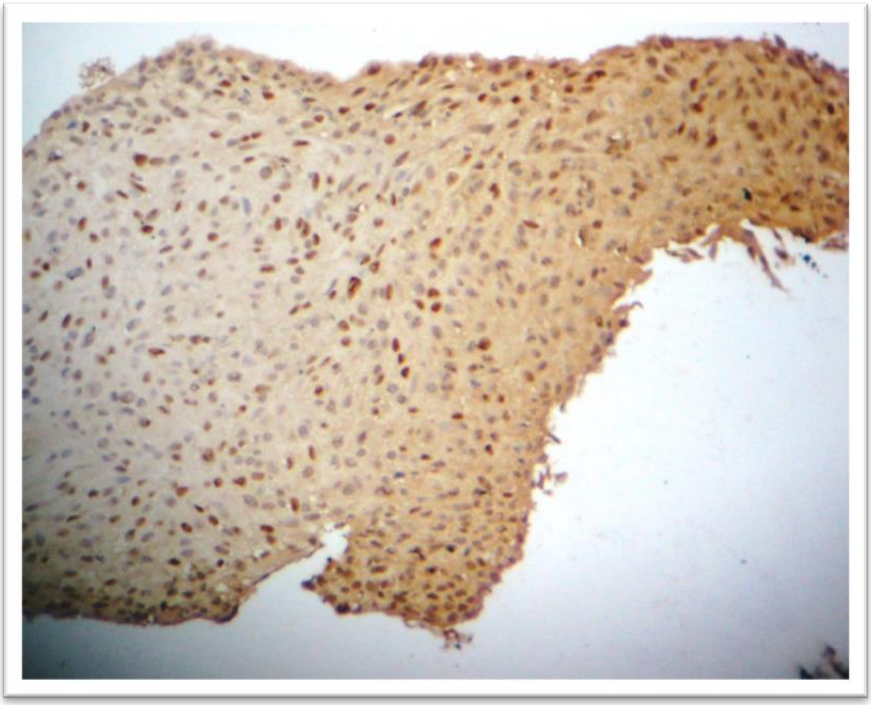


Fig 25:- Microphotograph showing strong immunoreactivity of Ki-67 in moderately differentiated squamous cell carcinoma (100X)

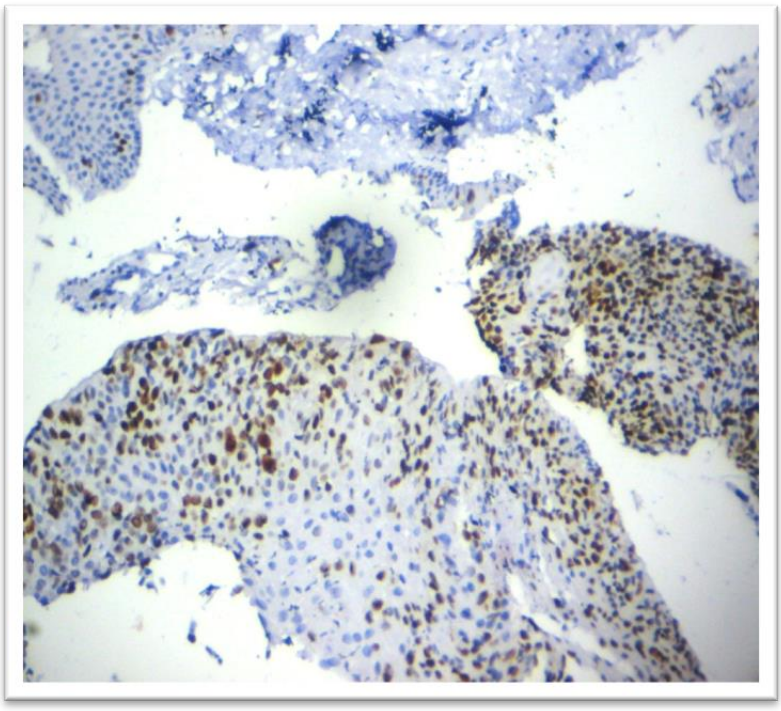


Fig 26:- Microphotograph showing pRb expression in moderately differentiated squamous cell carcinoma. Note the cytoplasmic staining.(100X)

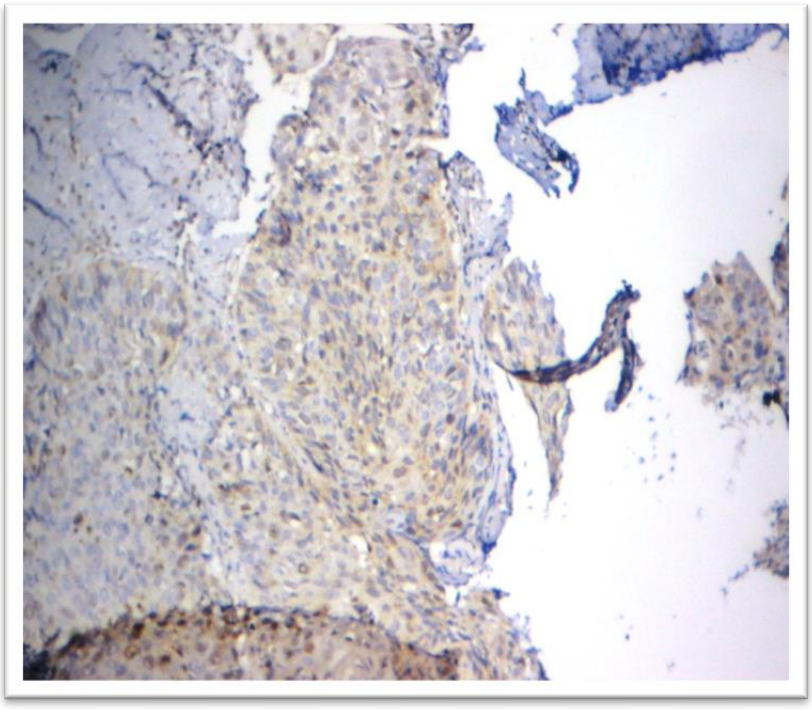


Fig 27:- Microphotograph showing poorly differentiated squamous cell carcinoma (H and E 100X)

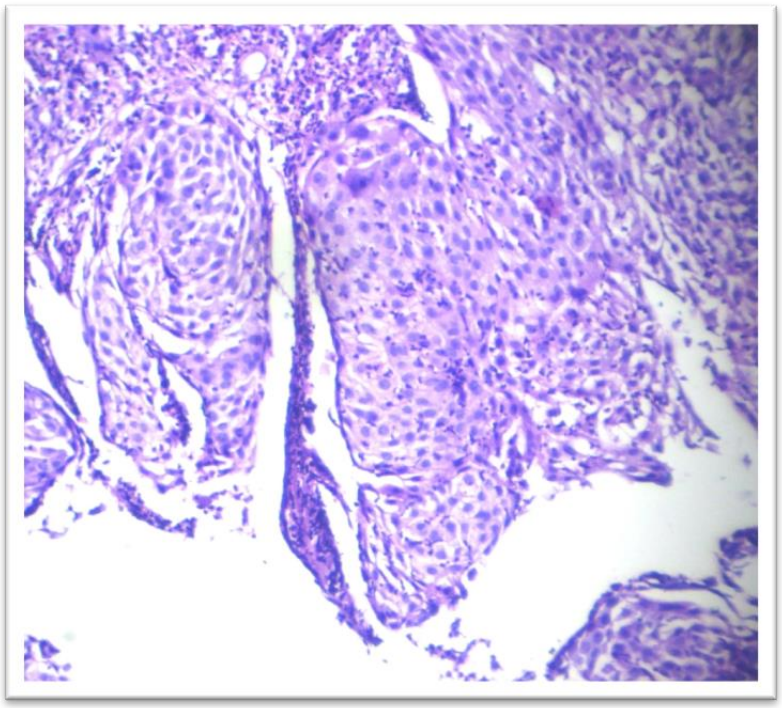


Fig 28:- Microphotograph showing tumor cells positive for p53 in a case of poorly differentiated squamous cell carcinoma.(100X)

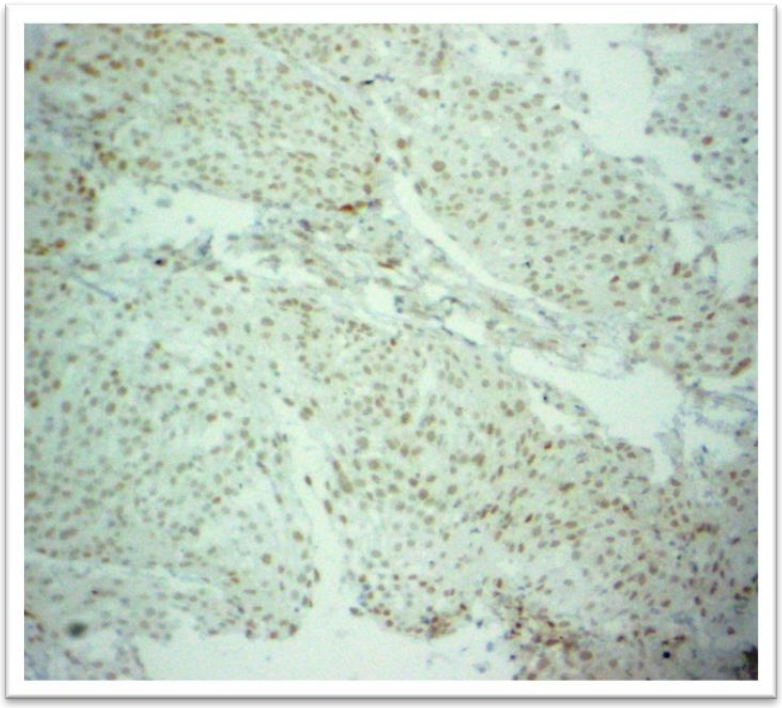


Fig29:- Microphotograph showing strong immunoreactivity of Ki-67 in case of PDSCC (100X). Inset showing higher magnification.

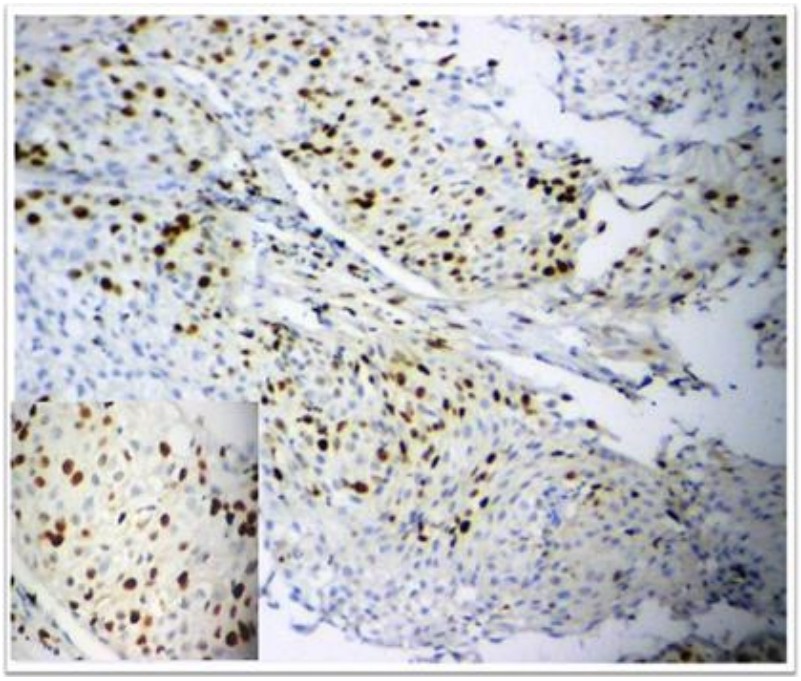
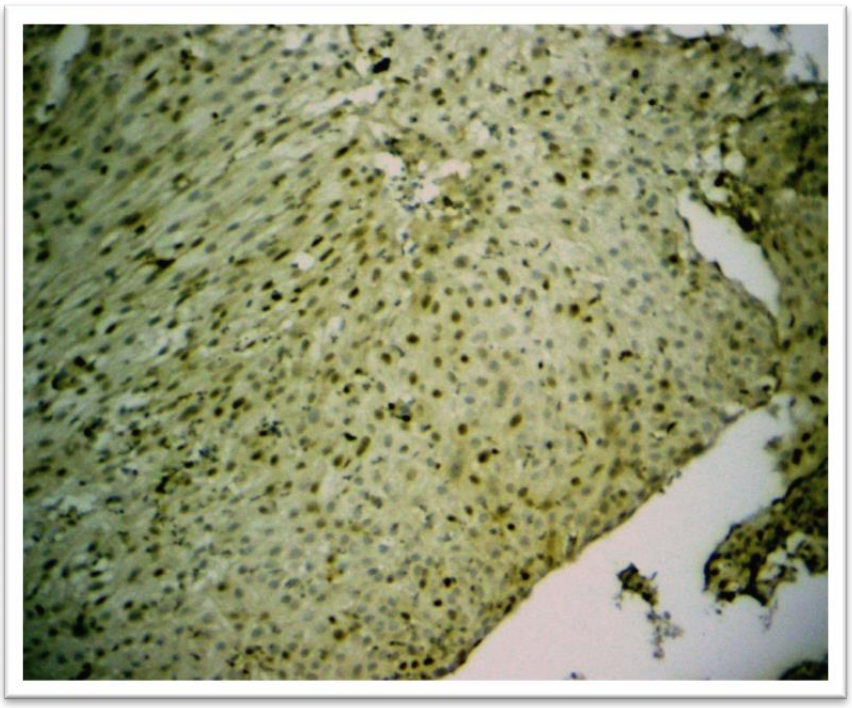


Fig 30:- Microphotograph showing pRb expression in PDSCC (100X).Note more of nuclear staining compared to cytoplasmic staining.



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ANNEXURE I:

PROFORMA FOR "STUDY AND ANALYSIS OF P53, PRB AND KI-67 EXPRESSION IN DYSPLASTIC AND MALIGNANT CERVICAL LESIONS"

NAME : I.P.NO:

AGE : D.O.A:

OCCUPATION : D.O.D:

ADDRESS :

HUSBAND'S OCCUPATION :

SOCIO-ECONOMIC STATUS :

INFORMANT :

CLINICAL HISTORY--

H/O BLEEDING PER VAGINA

H/O WHITE DISCHARGE PER VAGINA

H/O FOUL SMELLING DISCHARGE

H/O POST COITAL BLEEDING

H/O PREMENSTRUAL BLEEDING

H/O ABDOMINALPAIN/PELVIC PAIN

H/O LOSS OF APPETITE/WT LOSS

H/O URINARY COMPLAINTS

H/O POST MENOPAUSAL BLEEDING

MENSTRUAL HISTORY --

AGE OF MENARCHE :

PAST MENSTRUAL HISTORY:

ATTAINED MENOPAUSE :

OBSTETRIC HISTORY—

AGE OF MARRIAGE :

MARRIED LIFE :

NO. OF CHILDREN :

LAST CHILD BIRTH :

STERILIZATION :

GYNECOLOGICAL HISTORY—

H/O ULCER OVER GENITALIA:

PAST HISTORY :

PREVIOUS SURGERIES/MEDICAL :

FAMILY HISTORY--

H/O GENITAL MALIGNANCY/ KOCHS:

PERSONAL HISTORY—

SLEEP : DIET :

APPETITE : BOWEL & BLADDER :

G.P.E—

BUILD : NOURISHMENT:

PALLOR : ICTERUS :

B.P. : RESP. RATE :

PULSE :

TEMP :

SYSTEMIC EXAMINATION—

- CVS :
- RS :
- CNS :
- ABDOMINAL EXAMINATION :

LOCAL EXAMINATION---

EXTERNAL GENITALIA:

P/S

VAGINA DISCHARGE-

SMELL-

EXTENSION OF GROWTH-

P/V :

P/R :

CLINICAL DIAGNOSIS:

INVESTIGATIONS :

HB%:

TC:

DC:

ESR:

BL. GP & RH TYPING :

URINE EXAMINATION:

BLOOD SUGAR:

ULTRA SOUND (PELVIC & ABDOMINAL):

MRI

CT

HISTOPATHOLOGY REPORT—

GROSS—

MICROSCOPY-

KEY TO MASTER CHART

SI No: Serial Number

IP No: In Patient Number

HPE Report: Histopathological examination report

(S): Score

(I): Intensity

DUB: Disfunctional Uterine Bleeding

UV: Uterovaginal

PID: Pelvic Inflammatory Disease

Ca. Cx: Carcinoma Cervix

WDPV: White Discharge Per Vagina

LSIL: Low Grade Squamous Intraepithelial Lesion

HSIL: High Grade Squamous Intraepithelial Lesion

WDSCC: Well Differentiated Squamous Cell Carcinoma

MDSCC: Moderately Differentiated Squamous Cell Carcinoma

PDSCC: Poorly Differentiated Squamous Cell Carcinoma

L- NKSCC: Large cell Non- keratinized Squamous Cell Carcinoma

S-NKSCC: Small cell Non- keratinized Squamous Cell Carcinoma

AC: Adenocarcinoma

AS: Adenosquamous

CASES

SI NO	NAME	AGE	IP NO	BIOPSY NO	CLINICAL DIAGNOSIS	HPE REPORT	p53 (S)	p53 (I)	KI-67 (S)	ki-67 (I)	PRb-N (S)	pRb-N (I)	PRb-C (S)	pRb-C(I)
1	mallika	35yrs	638686	B/2235/10	DUB	HSIL	10%	I	10%	I	0%	0	10%	I
2	khanimunnisa	45yrs	638314	B/2234/10	Ca Cx stage IIb	MDSCC	80%	II	85%	III	45%	II	0%	0
3	lakshmamma	40yrs	676593	B/132/11	Ca Cx stage IIIb	MDSCC	90%	II	75%	II	25%	I	0%	0
4	Shamalamma	40yrs	721177	B/1666/11	DUB	LSIL	10%	III	45%	I	60%	II	0%	0
5	rathnamma	50yrs	725480	B/1620/11	DUB	MI	80%	I	55%	II	30%	II	10%	I
6	anjanamma	45yrs	726945	B/1759/12	DUB	HSIL	20%	I	20%	I	15%	I	0%	0
7	narayanamma	55yrs	733323	B/1814/11	DUB	LSIL	35%	I	1%	I	38%	I	0%	0
8	Sahana begem	40yrs	733373	B/1813/11	DUB	LSIL	45%	I	20%	I	25%	I	0%	0
9	munirathnamma	35yrs	728573	B/1694/11	DUB	LSIL	10%	III	45%	I	60%	II	0%	0
10	rajeshwari	48yrs	644641	B/2389/10	III UV descent	LSIL	70%	I	90%	II	35%	II	70%	0
11	lakshmidevamma	45yrs	272040	B/2570/10	Ca Cx stage IIIb	WDSCC	63%	II	75%	III	28%	I	0%	0
12	anusuyamma	55yrs	671443	B/195/11	II UV descent	LSIL	45%	III	20%	I	25%	II	0%	0
13	Shanthamma	40yrs	663847	B/2811/10	Ca Cx stage IIIb	MDSCC	0	0	55%	II	5%	I	15%	I
14	venkateshamma	35yrs	274180	B/2703/10	Ca Cx stage IIIb	L-NKSCC	65%	I	90%	III	10%	I	40%	I
15	rajamma	50yrs	287466	B/2600/10	Ca Cx stage IIIb	PDSCC	65%	I	45%	III	20%	I	30%	II
16	Venkatamma	50yrs	267236	B/2294/10	Ca Cx stage Ib	PDSCC	25%	I	40%	II	0%	0	5%	I
17	anusuyamma	45yrs	636778	B/2271/10	WDPV	HSIL	0%	0	10%	II	30%	I	10%	I
18	shanihamma	40yrs	675432	B/2371/10	Ca Cx stage IIIa	MDSCC	53%	I	80%	II	15%	I	0%	0
19	lakshmi	50yrs	646178	B/2440/10	Ca Cx stage IIIb	MDSCC	80%	I	0	0	0	0	52%	I
20	muniyamma	45yrs	651918	B/2589/10	Ca Cx stage IIIb	MDSCC	75%	I	85%	II	0	0	70%	II
21	narayanamma	30yrs	633617	B/2610/10	DUB	LSIL	34%	I	1%	II	38%	II	0%	0
22	Meenakshamma	35yrs	655076	B/2627/10	Ca Cx stage II	HSIL	10%	I	15%	II	18%	I	0%	0
23	chinamma	50yrs	691066	B/569/11	Ca Cx stage IIIb	MDSCC	32%	I	98%	II	20%	I	40%	I

CASES

SI NO	NAME	AGE	IP NO	BIOPSY NO	CLINICAL DIAGNOSIS	HPE REPORT	p53(S)	p53 (I)	KI-67 (S)	ki-67 (I)	PRb-N (S)	pRb-N (I)	PRb-C (S)	pRb-C(I)
24	lakshminarayanamma	55yrs	676471	B/377/11	Cervical polyp	LSIL	36.40%	II	1%	I	38%	II	0	0
25	parvathamma	40yrs	678621	B/358/11	DUB	HSIL	20%	I	20%	0	15%	I	0%	0
26	thimmakka	40yrs	679073	B/320/11	Ca Cx stage IIIb	MDSCC	90%	II	3%	II	0	0	10%	I
27	lakshmamma	40yrs	676593	B/303/11	Ca Cx stage IIIb	MDSCC	66%	II	65%	III	1%	I	10%	I
28	narayanamma	50yrs	676734	B/283/11	Ca Cx stage Ib	PDSCC	5%	I	25%	II	1%	I	0	0
29	Gowramma	40yrs	676277	B/275/11	Ca Cx stage Ib	PDSCC	83%	II	68%	II	0	0	0	0
30	manjula	35yrs	672096	B/208/11	DUB	LSIL	80%	I	20%	II	60%	II	0%	0
31	thimakka	40yrs	679073	B/2268/10	Ca Cx stage IIIa	MDSCC	32%	I	98%	II	20%	I	40%	0
32	bibijan	55yrs	269107	B/2402/10	Ca Cx stage IIIb	HSIL	88%	III	44%	III	40%	II	25%	II
33	rathnamma	50yrs	646234	B/2447/10	Ca Cx stage IIIb	L-NKSCC	0%	0	70%	II	0	0	60%	II
34	mubeen jai	55yrs	566959	B/2480/10	Ca Cx	PDSCC	60%	I	50%	II	0%	0	1%	I
35	parvathamma	70yrs	703161	B/947/11	Ca Cx stage IIIb	L-NKSCC	90%	II	35%	II	1%	I	0%	0
36	Meenakshamma	70yrs	694610	B/687/11	Ca Cx stage IIIb	MDSCC	60%	II	50%	II	0%	0	0%	0
37	rajamma	50yrs	658775	B/811/11	Ca Cx stage IIIb	L-NKSCC	15%	I	98%	III	2%	I	10%	I
38	narayanamma	45yrs	700071	B/820/11	Ca Cx stage IIb	MDSCC	38%	II	95%	I	5%	I	70%	III
39	ahalya	33yrs	719093	B/1421/11	WDPV	HSIL	2%	I	0	0	1%	I	0	0
40	rajamma	55yrs	645904	B/2427/10	Ca Cx stage IIIb	WDSCC	87%	III	80%	II	0%	0	0%	0
41	byamma	60yrs	660344	B/2730/10	Ca Cx stage IIIa	WDSCC	60%	0	70%	II	10%	I	15%	I
42	manikyamma	55yrs	651239	B/2561/10	Ca Cx stage IIIB	AS	5%	I	15%	II	0%	0	1%	I
43	rajamma	45yrs	665548	B/16/11	WDPV	HSIL	15%	I	10%	II	60%	I	50%	I
44	Sakamma	45yrs	666790	B/36/11	WDPV	LSIL	0%	0	15%	II	30%	II	0%	0
45	munivenkatamma	45yrs	670817	B/222/11	DUB	HSIL	76%	I	12%	II	88%	I	0%	0
46	Gowramma	40yrs	699825	B/813/11	WDPV	LSIL	0%	0	8%	II	15%	II	0%	0

CASES

SI NO	NAME	AGE	IP NO	BIOPSY NO	CLINICAL DIAGNOSIS	HPE REPORT	p53(S)	p53 (I)	KI-67 (S)	ki-67 (I)	PRb-N (S)	pRb-N (I)	PRb-C (S)	pRb-C(I)
47	Jayamma	50Yrs	754194	B/2355/11	WDPV	HSIL	10%	I	15%	III	50%	II	0	0
48	Shanthamma	40yrs	701570	B/863/11	WDPV	HSIL	90%	II	13%	II	50%	II	0%	0
49	lakshmidevamma	35yrs	668728	B/89/11	WDPV	HSIL	5%	I	10%	II	0%	0	0%	0
50	Tanamma	50Yrs	653443	B/2672/10	Ca Cx stage IIb	MDSCC	80%	I	90%	II	15%	I	75%	I
51	Jayamma	40yrs	642403	B/2345/10	Ca Cx stage IIIb	L-NKSCC	85%	II	50%	II	0%	0	0%	0
52	Amaravathi	35yrs	649903	B/2522/10	Ca Cx stage IIIb	WDSCC	83%	II	30%	II	10%	I	30%	II
53	venkatamma	50yrs	640007	B/2293/10	Ca Cx stage IIIb	MDSCC	90%	II	65%	II	15%	I	30%	I
54	narayanamma	50yrs	600002	B/429/11	Ca Cx stage IIIb	MDSCC	75%	II	35%	I	10%	II	35%	II
55	narayanamma	40yrs	698064	B/667/12	Ca Cx stage IIIb	WDSCC	20%	II	82%	III	10%	I	20%	II
56	munivenkatamma	55yrs	543281	B/31/12	Ca Cx stage IIIb	WDSCC	28%	I	98%	III	30%	II	80%	II
57	Radhamma	35yrs	654379	B/45/12	DUB	LSIL	50%	II	15%	III	5%	I	0%	0
58	changamma	55yrs	324567	B/121/12	?Ca cervix	AC-VG	30%	II	98%	III	10%	I	80%	II
59	nagalaksmi	45yrs	685347	B/318/12	Ca Cx stage IIb	S-NKSCC	68%	I	98%	III	20%	I	75%	II
60	subhamma	42yrs	598743	B/202/12	DUB	LSIL	20%	I	15%	III	30%	I	0%	0
61	Rukamma	50yrs	612890	B/320/12	Ca Cx stage IIIb	MDSCC	75%	I	98%	III	10%	I	75%	II
62	narayanamma	45yrs	783465	B/383/11	Ca Cx stage IIIb	MDSCC	5%	I	52%	II	5%	I	1%	I
63	muniyamma	70yrs	701348	B/390/12	Ca Cx	PDSCC	90%	II	40%	III	10%	I	0%	0
64	venkatamma	45yrs	600078	B/446/12	Ca Cx	PDSCC	55%	I	90%	II	5%	I	0%	0
65	Venkatamma	55yrs	699982	B/479/12	Ca Cx stage IIIb	WDSCC	90%	II	90%	II	10%	I	10%	I
66	Eeramma	55yrs	677739	B/485/12	Ca Cx stage IIIb	MDSCC	75%	II	85%	III	20%	I	60%	II
67	Shanthamma	45yrs	650123	B/505/12	WDPV	HSIL	20%	I	88%	III	0%	0	0%	0
68	Venkatamma	50yrs	721409	B/80/12	Ca Cx stage IIIb	WDSCC	60%	II	95%	III	70%	II	20%	I
69	Venkatamma	60yrs	792350	B/189/12	Ca Cx stage IIIb	WDSCC	60%	I	98%	III	40%	II	60%	I
70	kupamma	40yrs	638910	B/190/12	Ca Cx stage IIIb	S-NKSCC	10%	I	95%	III	10%	I	60%	I

CASES

SI NO	NAME	AGE	IP NO	BIOPSY NO	CLINICAL DIAGNOSIS	HPE REPORT	p53(S)	p53 (I)	KI-67 (S)	ki-67 (I)	PRb-N (S)	pRb-N (I)	PRb-C (S)	pRb-C(I)
71	kanthamma	49yrs	764390	B/233/12	?Ca cervix	HSIL	20%	I	60%	III	60%	II	20%	I
72	narayanamma	53yrs	613890	B/1014/12	Cervical polyp	AC-VG	88%	I	98%	II	2%	I	0%	0
73	pushpa	35yrs	728910	B/1023/12	Chronic PID	LSIL	45%	I	15%	II	5%	I	0%	0
74	Venkatamma	60yrs	730217	B/1890/11	?Ca cervix	MDSCC	5%	I	85%	II	3%	I	15%	I
75	manjulamma	58yrs	719203	B/807/12	Ca Cx stage IIIb	WDSCC	30%	I	85%	II	1%	I	5%	I
76	Shantamma	60yrs	581730	B/734/12	Ca Cx stage IIIb	MDSCC	45%	II	60%	II	5%	I	60%	I
77	lakshmamma	55yrs	672019	B/732/12	Ca Cx stage IIIb	MDSCC	30%	II	70%	II	0%	0	10%	I
78	Venkatamma	60yrs	642839	B/1577/11	Ca Cx stage IIIb	MDSCC	20%	I	98%	III	15%	I	74%	II
79	lakshmidevamma	45yrs	732109	B/1485/11	Ca Cx stage IIIb	MDSCC	52%	II	95%	III	20%	I	74%	II
80	Mangamma	40yrs	507916	B/548/12	Ca Cx stage IIIb	S-NKSCC	48%	I	88%	II	10%	0	50%	0
81	Venkatamma	45yrs	698123	B/2402/11	Ca Cx stage IIIb	WDSCC	80%	I	88%	III	10%	I	40%	II
82	lakshmamma	30yrs	510923	B/1128/12	?Ca cervix	LSIL	5%	II	2%	III	1%	I	0%	II
83	Sarithamma	60yrs	681023	B/1046/12	III UV Prolapse	LSIL	15%	I	10%	II	55%	I	0%	0
84	parvathamma	40yrs	791023	B/968/12	DUB	AC-EN	98%	II	75%	III	0%	II	38%	0
85	Shakeera	60yrs	517829	B/1339/12	Ca Cx stage IIIb	MDSCC	42%	II	64%	II	2%	0	0%	I
86	lakshmidevamma	50yrs	687231	B/22/1	DUB	HSIL	20%	I	60%	II	60%	I	20%	0
87	subhamma	45yrs	622219	B/2524/10	DUB	LSIL	0%	I	5%	II	15%	0	0%	0
88	rajeshwari	48yrs	781630	B/1684/11	DUB	HSIL	20%	0	88%	II	0	I	0	0
89	Mangamma	40yrs	581203	B/1299/12	Ca Cx stage IIIb	MDSCC	15%	II	98%	II	15%	I	30%	I
90	Naseema	68yrs	510732	B/1287/12	Ca Cx stage IIIb	WDSCC	80%	I	83%	III	78%	I	0%	II
91	Basamma	40yrs	611195	B/1235/12	Ca Cx stage	MDSCC	20%	II	93%	III	30%	II	72%	II
92	nagalaksmi	45yrs	633393	B/158/11	Ca Cx stage	MDSCC	35%	II	64%	II	30%	I	20%	I
93	narayanamma	50yrs	589231	B/133/11	Ca Cx stage	MDSCC	3%	II	98%	II	5%	I	20%	I
94	Umadevi	46yrs	781045	B/79/11	DUB	HSIL	10	I	10%	II	10%	I	0	I

CASES

SI NO	NAME	AGE	IP NO	BIOPSY NO	CLINICAL DIAGNOSIS	HPE REPORT	p53(S)	p53 (I)	KI-67 (S)	ki-67 (I)	PRb-N (S)	pRb-N (I)	PRb-C (S)	pRb-C(I)
95	Devamma	48yrs	721049	B/1107/12	DUB	LSIL	86%	I	15%	III	70%	I	0%	0
96	Shantamma	50yrs	520413	B/619/12	DUB	HSIL	90%	II	13%	II	50%	I	0	0
97	Venkatamma	44yrs	570821	B/2530/11	DUB	LSIL	5%	I	20%	III	2%	I	0%	0
98	lakshmidevamma	46yrs	690802	B/2553/11	DUB	LSIL	45%	I	20%	III	25%	I	0%	0
99	Prema	45yrs	680201	B/2542/11	DUB	LSIL	10%	I	5%	II	0%	0	0%	0
100	Narasamma	50yrs	666551	B/1492/12	Ca Cx stage IIIb	WDSCC	75%	II	98%	III	5%	I	0%	0

CONTROLS

SI NO	NAME	AGE	IP NO	BIOPSY NO	CLINICAL DIAGNOSIS	HPE REPORT	P53(S)	p53 (I)	KI-67 (S)	ki-67 (I)	PRb-N (S)	pRb-N (I)	PRb-C (S)	pRb-C(I)
1	Venkatalakshmamma	40yrs	668822	B/819/12	DUB	Normal cervical epithelium	15%	I	1%	I	1%	I	0	0
2	Boothayamma	48yrs	557201	B/513/12	DUB	Normal cervical epithelium	18%	I	20%	II	30%	I	0	0
3	kamalamma	36yrs	557621	B/820/12	DUB	Normal cervical epithelium	20%	I	2%	I	2%	I	0	0
4	nagalaksmi	45yrs	672091	B/806/12	DUB	Normal cervical epithelium	7%	I	4%	I	4%	I	0	0
5	subhamma	45yrs	687120	B/180/12	DUB	Normal cervical epithelium	3%	I	1%	I	1%	I	0	0
6	Rukamma	35yrs	523189	B/818/12	DUB	Normal cervical epithelium	3%	I	1%	I	2%	I	0	0
7	narayanamma	38yrs	589210	B/824/12	DUB	Normal cervical epithelium	8%	I	0	0	0	0	0	0
8	Venkatamma	50yrs	677881	B/20/12	IIUV Prolapse	Normal cervical epithelium	2%	I	1%	I	0	0	0	0
9	kupamma	35yrs	699001	B/860/12	DUB	Normal cervical epithelium	28%	II	8%	II	3%	I	0	0
10	kanthamma	35yrs	544229	B/859/12	DUB	Normal cervical epithelium	28%	II	1%	I	2%	I	0	0
11	narayanamma	50yrs	652081	B/199/12	IIUV Prolapse	Normal cervical epithelium	7%	I	1%	I	1%	I	0	0
12	thimakka	50yrs	739105	B/514/12	IIUV Prolapse	Normal cervical epithelium	22%	I	4%	I	22%	I	0	0
13	bibijan	46yrs	775922	B/551/12	DUB	Normal cervical epithelium	5%	I	2%	I	5%	I	0	0
14	rathnamma	45yrs	711054	B/157/12	DUB	Normal cervical epithelium	30%	I	20%	II	20%	I	0	0
15	mubeen jai	45yrs	766210	B/823/12	I UV Prolapse	Normal cervical epithelium	23%	I	1%	I	3%	I	0	0
16	lakshmidevamma	43yrs	663321	B/805/12	DUB	Normal cervical epithelium	15%	I	3%	I	18%	I	0	0
17	subhamma	40yrs	716920	B/1758/12	DUB	Normal cervical epithelium	30%	I	20%	II	20%	I	0	0
18	rajeshwari	52yrs	722910	B/1760/12	III UV Prolapse	Normal cervical epithelium	13%	I	0	0	8%	I	0	0
19	Umadevi	53yrs	562409	B/1754/12	III UV Prolapse	Normal cervical epithelium	18%	I	20%	I	30%	I	0	0
20	Devamma	44yrs	732891	B/201/12	DUB	Normal cervical epithelium	28%	I	8%		3%	I	0	0