SIGNIFICANCE OF APOPTOTIC INDEX AND P16IN NK4 APROTEIN EXPRESSION IN CERVICAL INTRAEPIYHELIAL NEOPLASIA AND SQUAMOUS CELL CARCINOMA OF CERVIX

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Dissertation submitted to

BLDE (Deemed to be University) Vijayapur, Karnataka

In partial fulfillment of the requirements for the degree of

DOCTOROFMEDICINE IN

PATHOLOGY

Under the guidance of

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PROFESSOR

DEPARTMENTOF PATHOLOGY

BLDE (Deemed to be University)

SHRIB.M.PATILMEDICALCOLLEGE

HOSPITAL & RESEARCH CENTRE, VIJAYAPUR

KARNATAKA

2020

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LIST OF ABBREVATIONS USED

LSIL – Low grade squamous intra epithelial lesion

HSIL – High grade squamous intra epithelial lesion

SCC- Squamous cell carcinoma

CIN – Cervical intra epithelial neoplasia

AI – Apoptotic index

IHC- Immunohistochemistry

ABSTRACT

INTRODUCTION

Cervical cancer is a leading cause of cancer related death among women. P16INK4A expression correlates excellently with grade of cervical intraepithelial neoplasia (CIN) and squamous cell carcinoma(SCC) & can be used for screening cervical malignancies. Apoptosis play an important role in tumor progression and development of tumors and apoptotic bodies helps to grade dysplasia. Hence p16INK4A expression and apoptotic index (AI) in CIN and SCC cervix has been studied.

OBJECTIVES

To study the apoptotic index (AI) & p16INK4A expression in cervical intraepithelial neoplasia and squamous cell carcinoma of cervix & to correlate with non-neoplastic lesions of the cervix.

MATERIALS AND METHODS

73 cervical biopsy specimens diagnosed as CIN, invasive SCC and also non neoplastic cervical lesions as controls are studied. One section stained with H&E for histomorphologic diagnosis and apoptotic index. Other section is subjected to p16INK4A staining.

RESULTS

Apoptotic Index (AI) increased progressively from normal to carcinoma but decreased with decreased differentiation of tumor. Expression of P16INK4a noted in 100% cases of carcinoma cervix, 100% cases of HSIL and 100% cases of LSIL with p value 0.0001.

CONCLUSION

p16INK4a expression and AI improves the accuracy of diagnosing premalignant and malignant cervical lesions while also separating them from non-neoplastic cervical lesions to avoid needless surgical operations.

KEYWORDS -

Apoptotic Index, P16INK4a, carcinoma cervix.

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INTRODUCTION

Recent data from the Atlas Cancer of India project by the Indian Council of Medical Research (ICMR) shows that cervical cancer is the second most prevalent cancer in women in most urban population-based registries in India, after breast cancer. Cervical cancer, especially in women from lower socioeconomic groups, is the primary cause of cancer-related death in India.[1]

With an average incidence of about 25 per 100,000 women, the age-adjusted incidence rates for cervical cancer in India ranged from 10.9 to 65.4 among different registries. 7.9% of women in the general population are known to have cervical HPV infection. [1,2]

The primary cause of cervical cancer is the human papillomavirus. More than 100 different varieties of HPV have been found. Of these, HPV types 16 and 18 falls under the category of high-risk types. Cervical HPV infection is sexually transmitted. The risk of HPV infection and preinvasive cervical neoplasia is highest in young, sexually active women.[3] The risk of HPV infection declines as a patient's age increases, while the chance of cancer increases. It is well established that intraepithelial neoplasia, a premalignant lesion, is the precursor of cervical cancer. On average, 5 to 15 years pass before invasive carcinoma develops. The analysis of the step-in pathogenesis of the HPV cell cycle is of vital importance for determining precancerous lesions and indirectly assessing the presence of HPV infection. When the HPV genome is integrated into the host's cellular DNA, the cervical mucosa undergoes malignant transformation and develops invasive carcinoma.[4]

As histomorphological features alone are not sufficient for more standardized and reproducibility of CIN grading, identification of biomarkers in cervical neoplasia is necessary to distinguish from nonneoplastic cervical lesions and to prevent unnecessary surgical interventions[2]

The cyclin-dependent kinase CDK4/6 inhibitor family includes p16INK4A. INK4A, a tumor suppressor gene, encodes it. It plays a significant role in the CDK-Rb-E2F regulation pathway. The gene's protein product, p16INK4A, inhibits the cyclin-dependent kinase CDK4/6 to inhibit pRb from being phosphorylated. pRb continues to bind E2F transcription factors, inhibits the cell in the G1 phase, and prevents DNA from being passed for replication.[5]

When pRb interacts with and inactivates HPV-derived oncoprotein E7 in cervical lesions, the regulatory pathway CDK-Rb-E2F is disrupted. Without being inhibited, inactive pRb allows the cell cycle to continue to the G1/S checkpoint. P16INK4A is overexpressed as a result of this occurrence. Thus, for both malignant and premalignant cells, the protein p16INK4A can be used as a marker.[4]

The effectiveness of the p16INK4A marker can be used to screen for cervical cancer, especially in underdeveloped nations, and as a surrogate marker of HPV infection. This can be emphasized to promote the use of the HPV vaccine. p16INK4A expression correlates excellently with a grade of CIN and squamous cell carcinoma & can be used for screening cervical malignancies in both developing & developed countries[6]

Apoptosis, also known as programmed cell death, is the intrinsic death mechanism of the cell that controls a variety of physiological and pathological processes. The p53 pathway recognises DNA or chromosomal defects, which leads to repair or, in cases where the repair process fails, such as in neoplasias, produces apoptosis. As a result, apoptosis contributes to the suppression of malignant transformation by removing cells with genomic defects. Additionally, apoptosis

delays the emergence of aneuploidy, other genetic anomalies frequently found in cancer cells, and the advancement of neoplasia. A cancer cell's tendency for apoptosis may have significant effects on the development of the tumour and how it responds to treatment.

Apoptotic bodies aid in the more accurate grading of dysplasia, and both apoptosis and cellular proliferation have been shown to be crucial in the development and progression of malignancies. The apoptosis index (AI) is determined by expressing the number of apoptotic cells and apoptotic bodies as a proportion of all the tumor cells counted. Measuring the Apoptotic index help in identifying the individuals who are at a greater risk of developing carcinomas, carry a significant prognostic value and also represent a good model of tumor development[7]

Hence the present study will be taken up to study p16INK4A expression and apoptosis index (AI) in cervical intraepithelial neoplasia and carcinoma cervix.

OBJECTIVES OF THE STUDY:

1. To study the apoptotic index (AI) & p16INK4A expression in cervical intraepithelial neoplasia and squamous cell carcinoma of the cervix & to correlate with non-neoplastic lesions of the cervix.

2. To correlate the AI with p16INK4A expression in cervical intraepithelial neoplasia and squamous cell carcinoma of the cervix.

REVIEW OF LITERATURE

The Normal Uterine Cervix

Anatomy

Cervix, isthmus, and corpus constitute the uterus. The most inferior part of the uterus that protrudes into the upper vagina is called the cervix. The base and entrance to the uterine cavity, which is located at the top of the vagina, are formed by the uterine cervix. The adult nulligravida has a cervix that is 2.5–3 cm long and inclined backward and downward. Sizes and forms may differ depending on the parity of women.

Ectocervix, also known as the portiovaginalis region of the cervix, and endocervix, also known as the supravaginal portion. The ecto cervix is convex and flattened anteroposteriorly, and the vaginal mucosa's reflection covers it.. It comprises of fornices surrounding the anterior and posterior lips. Into the vaginal vault, the protruding cervix creates anterior, posterior, and lateral fornices. The posterior fornix is deeper than the anterior one. A small fusiform canal with mucosal ridges extending in the anterior, posterior, and oblique orientations is known as the endocervix or endocervical canal. The external os is the ectocervix's vaginal aperture. It is circular in nulliparous women and slit-like in multiparous women. The transition from the endocervix to the endometrium is indicated by an internal os. The lateral aspects of the parametrium are attached to the cervix and contain uterine vessels and ureters.[8]

At the embryologic junction of the cervix and the urogenital sinus, the endocervix is lined by columnar epithelium, while the ectocervix is lined by non-keratinized squamous epithelium. In the uterine cervix, the location of this squamocolumnar junction varies depending on a number of variables, includes pathogenic diseases, age, hormonal factors, and

reproductive status. In the endocervical canal, the squamocolumnar junction is frequently elevated. in childhood, but it is typically lower during the reproductive years, frequently resulting in ectropion or eversion of the endocervix. Because of a squamous metaplasia over the reproductive years, the junction returns up into the endocervical canal, with endocervical glands under the mature squamous mucosa serving as microscopic evidence of the old endocervical mucosa inside the transformation zone.[9,10]

HISTOLOGY OF CERVIX[11]

Similar to the vagina, the ectocervix is lined by non-keratinizing stratified squamous epithelium. The basal zone, midzone, and superficial zone constitute its three layers.

The basal zone is one cell thick and made up of basal cells with a high nuclear-cytoplasmic ratio (N:C). The multilayered midzone is made up of intermediate cells.

The suprabasal layer of the midzone, which is located directly above the basal layer, is thought to contain the cervical squamous epithelium's true stem cells. These suprabasal cells exhibit greater cellular proliferation and are actively engaged in mitosis.

The cells in superficial layers are stratified and organized so that their long axis is parallel to the basement membrane. During normal maturation, the nuclear-cytoplasmic ratio(N:C) gradually reduces from the basal cells to the surface layer.

Tissue polypeptide antigen (TPA) and Low molecular weight keratin are immunohistochemically detected in the basal layer cells, but the cells above the basal layer contain involucrin markers and high molecular weight keratin. Estrogen receptors are immunoreactive in basal cells.[12,13]

Age-related alterations in the morphology of these different layers may occur, but they shouldn't be mistaken for cervical intraepithelial neoplasia.

A single layer of mucus-secreting columnar cells lines the endocervix. Their nucleus is positioned basally. On cross-section, the complex infolding of the surface epithelium looks like glands or a fissure. They are supported by a generally inconspicuous layer of subcolumnar "reserve" cells. Squamous metaplasia, cervical intraepithelial neoplasia, and cancer are caused by "reserve" cells, which are found close to the squamo-columnar junction.[14]

Squamous metaplasia, which occurs across the transformation zone in the cervical lining, is the process that changes columnar epithelium into squamous epithelium. This zone corresponds to the external os in low estrogenic situations and to the cervix on the vaginal vault in high estrogenic conditions.

The majority of invasive and preinvasive cervix lesions occur in the Transformation zone.[15] Fibrous tissue, elastic fibres, and a few scatted smooth muscle fibres make up the stroma of the cervix. Contrary to the stroma of the endometrium, which exhibits a dominant CD10 phenotype it has a dominant CD34 immunophenotype.. This variation might make it easier to identify the primary tumor involvement place.

PREMALIGNANT LESIONS OF THE CERVIX

Premalignant cervix lesions are currently divided into various categories. The oldest classification method for cervical dysplasia is the dysplasia carcinoma in situ (CIS) system, which classifies the dysplasia into mild, moderate, and severe categories. After it, the CIN classification was added as a replacement. Mild dysplasia is designated as CIN 1 in this classification, while moderate and severe dysplasia are designated as CIN 2 and CIN 3, respectively. Recent classification reduced these entities to two-tier system: low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL). LSIL for CIN1 and HSIL for CIN2 and CIN3.

Table 1: Classifying systems for Cervical intraepithelial lesions

Dysplasia/ carcinoma in situ	Cervical intraepithelial neoplasia (CIN)	Squamous intraepithelial lesion (SIL), current classification
Mild dysplasia	CIN 1	Low-grade SIL (LSIL)
Moderate dysplasia	CIN 2	High-grade SIL (HSIL)
Severe dysplasia	CIN 3	High-grade SIL (HSIL)
Carcinoma insitu	CIN 3	High-grade SIL (HSIL)

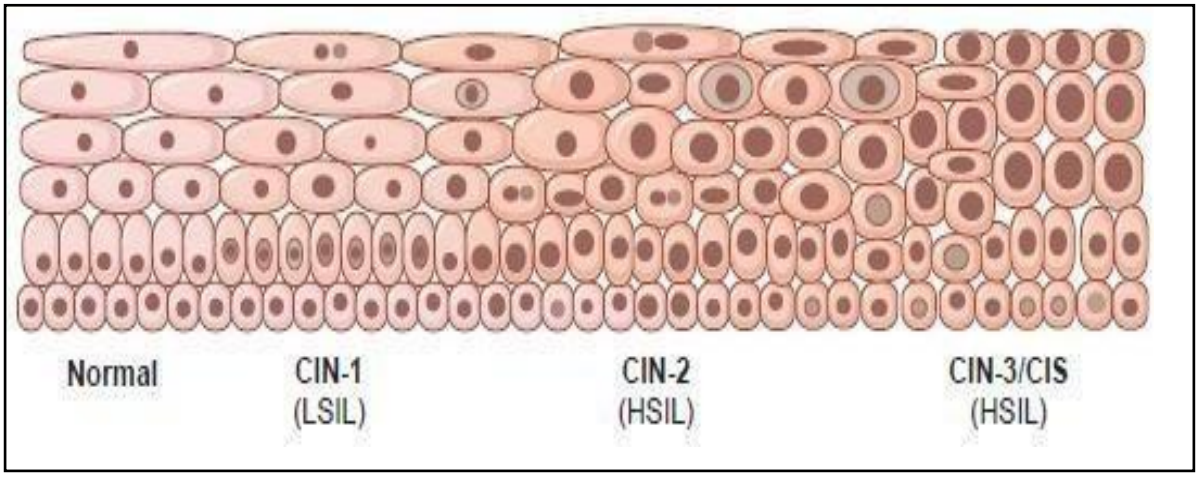
Histology

In a mucosa with a SIL, abnormal cells can be found in the epithelial layers. From the basal layers to the surface, differentiation signs appear in an inverse relationship to the degree of the lesion. An intraepithelial lesion caused by HPV severity is correlated with the morphology of the cells that make up the uppermost layers of the lesion. The epithelial lining cells' abnormal differentiation, excessively big and cytologically atypical nuclei, and various levels of cytoplasmic maturation are characteristics of SIL.

The whole cascade of maturation phases that occur throughout the epithelium are reflected in the shape of cells seen in the upper layers of the mucosa. Only the basal cells in the typical cervical squamous mucosa are able to divide cells. However, these basal cells are where HPV infection is expected to first manifest, the episomal replication of the virus occurs in the non-mitotically active suprabasal cells, that is why these basal cells are where HPV infection is expected to first present. In these circumstances, the expression of the viral DNA causes the

infected cell's cytoplasm to synthesise new viral particles, which are then released into the peeling upper epithelial layers. Nuclear expansion, hyperchromasia, and binucelation with cytoplasmic koilocytic vacuolization are considered the morphologic expression of this viral takeover of the host cell. In other words, the typical cytologic alterations associated with a SIL.

FIGURE1: TRANSITIONS IN HISTOPATHOLOGY STARTING WITH NORMAL, LSIL (CIN-1) THEN HSIL (CIN-2/3).



Low-grade cervical squamous lesions (LSIL) can be divided into three categories. The most common type is a condyloma that is flat and acanthotic with well-preserved basal layers, prominent nuclear atypia, and perinuclear halos that are directed toward the surface. Atypical basal layer growth, which should be restricted to the lowest third of the epithelium, is typically present to some extent. Mitoses should be kept to the lower portion of the body. Less frequently encountered is the traditional proliferative papillomatous condyloma. An endophytic "inverted" condyloma, the third form, most likely results from the dysplastic squamous epithelium spreading into the endocervical glands. Lesions that are more frequently observed on the cervix and vagina and less frequently observed on the surface epithelium of the vulva are flat and endophytic condylomata. These condylomatous lesions were formerly classified as dysplasias due to the lack of knowledge regarding the function of HPV in cervical neoplasia. Meisels and

colleagues hypothesised that the sexually transmitted viral disease condyloma acuminatum was a precursor to cervical neoplasia because it had been demonstrated in a few cases to transform into a malignant condition and had epidemiological characteristics that were similar to those of cervix cancer.[16] It is now known these low-grade lesions are commonly a sign of an HPV infection, which usually goes away with time and has a minimal chance of developing into cervical cancer. There has been an improvement in our knowledge of the epidemiology of HPV infection and the relative risk of these lesions progressing.[17]

There is a greater stratification disturbance in HSIL (CIN 2 and CIN 3). Only the upper 1/3 of the epithelium in CIN 2 still exhibits signs of superficial and intermediate cell size stratification. Flat squamous cells still comprise the outermost layers, while nuclei is larger and slightly hyperchromatic. On rare occasions, the surface layers become keratinized, losing their nuclei and forming a granular layer. The epithelium may display nuclear abnormalities throughout, but especially in the basal layers. Up to two-thirds of the epithelium's thickness is seen as immature abnormal basilar-type cells. Mitoses are more prevalent and can sometimes extend into the middle portion of the epithelium. According to the accepted definition, these CIN 2 lesions should exhibit intense, diffuse block-like positive p16 staining that starts at the basal layer and penetrates at least 1/3 of the thickness of the mucosa.

All three layers of the epithelium have a significantly disorganized arrangement of cells in HSIL (CIN 3). Stratification only appears in the topmost layers. Cells have a lower level of maturation across the entire epithelium, with a loss in cytoplasmic volume and an increase in nuclear size. The size, shape, and often irregular form of cells and nuclei are all variable. The ability of the cells to differentiate may entirely disappear into intermediate or superficial squamous-type cells, and abnormal, immature cells may completely take their place. Chromatin in nuclei is hyperchromatic, unevenly dispersed, and coarsely granular. All

epithelial layers may contain mitoses. The abnormal changes may mimic invasion because they frequently penetrate the endocervical glands deeply.

Precancerous Disease

Squamous Intraepithelial Lesions/Cervical Intraepithelial Neoplasia

Cervical intraepithelial neoplasia (CIN), dysplasia, and squamous intraepithelial lesions are all squamous abnormalities in the transformation zone associated with HPV infection. Because it has become apparent that HPV infection can result in a morphologic continuum of squamous alterations, all of these entities will be presented within the single classification system of squamous intraepithelial lesions, which follows recommendations from the recent 'Lower Anogenital Squamous Terminology' (LAST) project.[18]

In this classification, low-grade squamous intraepithelial lesions encompass flat and exophytic, immature and mature condylomata, and lesions graded as CIN I classification scheme. High-grade squamous intraepithelial lesions correspond to CIN II and III. The lower genital tract employs this two-tiered strategy throughout. It brings standardisation and uniformity of nomenclature that reflects the underlying pathology, with low-grade squamous intraepithelial lesions being a self-limited, temporary but productive viral infection and high-grade squamous intraepithelial lesions representing precancerous change with the potential to progress to invasive cancer.

Clinical Features

Squamous intraepithelial neoplasia may be identified in any age group following the onset of sexual activity. It is now well established that HPV infection is causally related to cervical neoplasia, both a preinvasive and invasive disease. A single HPV type that is persistently infected is strongly related to cervical neoplasia, either concurrently or later. HPV

infection is common in young, sexually active women, with a peak frequency of infection in this age range.[19-23]

Numerous HPV types are known to infect the genital tract, particularly the cervix. Based on their association with invasive cervical cancer, they were divided into low-risk and high-risk groups. HPV 6 and 11 are the prototypic low-risk viruses because they are rarely associated with cervical carcinoma or high-grade squamous precancers. In contrast, HPV type 16 is considered high risk because this virus is associated with 50% of invasive squamous cell carcinomas and high-grade squamous precancers.[24]

Other risk factors for cervical squamous intraepithelial neoplasia, besides HPV infection, include: (1) age (young, sexually active women are most at risk for preinvasive cervical neoplasia) (2) defects in immunity, such as human immunodeficiency virus (HIV) infection or therapeutic immunosuppression; and (3) tobacco use.[25-29]

Squamous Intraepithelial Lesion, Low Grade(LSIL)

Three morphological subsets of low-grade squamous intraepithelial lesions exist: condyloma acuminatum (exophytic condyloma), immature condyloma (squamous papilloma, immature papillary metaplasia), and flat condyloma (CIN I).

Exophytic condyloma has a verruciform growth pattern, acanthosis, and superficial koilocytotic atypia as its defining features (nuclear hyperchromasia, karyomegaly, binucleation, an irregular nuclear contour). In the cervix, these exophytic lesions, which are strongly associated with HPV types 6 and 11, are not very common.

Squamous epithelium lining the slender, filiform papillae of an immature condyloma shows relatively mild nuclear crowding and superficial koilocytotic atypia, but little keratinocyte maturation (resembling squamous metaplasia). This limited development in these lesions, which is dependent on the viral cytopathic effect, is likely the cause of the relative lack

of koilocytotic atypia. Immature condylomas share HPV types 6 and 11, which supports their classification as condylomas together with exophytic condylomas. This category also includes squamous papillomas and papillary immature metaplasia, which are histologically comparable to immature condylomas and also carry HPV strains 6 and 11.[30,31] Although flat condyloma lacks an exophytic growth pattern, it has many morphologic characteristics with condyloma acuminatum..

Although the degree of koilocytotic atypia can vary, it is restricted to the upper part of the epithelium and has no effect on basal and parabasal nuclear atypia. These lesions are typically linked to HPV strains that carry a medium risk.[24]

Squamous Intraepithelial Lesion, High Grade(HSIL)

Nuclear atypia at all epithelial layers and varying degrees of surface maturation are characteristics of high-grade squamous intraepithelial lesions. Lesions may show minimal to no epithelial maturation, koilocytotic surface alteration, or both. These lesions can be differentiated from low-grade squamous intraepithelial lesions by the following characteristics: (1) the presence of nuclear atypia in the lower layers; (2) an increased mitotic index with mitoses in the upper half of the epithelium; (3) loss of cell polarity; and, in some cases, (4) abnormal mitotic figures; and (5) the presence of pronounced atypical, bizzare cells. These lesions are connected to HPV strains at high risk, notably, type 16.

p16 ink4, a cyclin-dependent kinase inhibitor, appears to be a surrogate marker of HPV infection, being strongly expressed (positivity defined as strong cytoplasmic and nuclear staining in the lesional cells) in lesions associated with intermediate-risk and high-risk HPV. Intermediate-risk and high-risk HPVs may be associated with low-grade and high-grade squamous intraepithelial lesions; therefore, this marker cannot distinguish between the two. However, it may be helpful in supporting the diagnosis of a squamous intraepithelial lesion.[32]

Papillomavirus (HPV) with Cervical Squamous Lesions

HPV Epidemiology

Almost all squamous cervical malignancies and most of endocervical cancers are now recognized to be directly and almost exclusively caused by HPV. Epidemiological research from the late 1970s suggested that the transmission of a sexually transmitted factor may be the cause of cervical cancer.[33-35] Since then, studies have repeatedly demonstrated that having multiple sexual partners is the single most significant risk factor for getting cervical cancer..[36-38] Smoking and immune-compromised conditions, including HIV infection and organ transplantation, are additional risk factors. **Zur Hausen** first identified the potential etiological involvement of HPV in cervical cancer in 1973.[39] Numerous epidemiological and clinicopathologic studies that used molecular methods later confirmed the role of HPV in the emergence of carcinoma cervix and its premalignant lesions.[38]

Cervical cancer is the second most prevalent malignancy in women worldwide. In underdeveloped nations, this type of cancer is the most prevalent, while it is the tenth most prevalent in developed nations. Since the 1950s, cervical cancer incidence and fatality rates have decreased in the majority of affluent nations. In order to avoid cervix cancer, cervical smears are crucial.[40]

HPV Biology in the Female Genital System

With a genome size of 8000 base pairs and seven to eight early (E) and two late (L) open reading frames (ORFs), HPV; a circular double-stranded DNA virus that encodes more than 20 mRNAs and all viral gene products through alternative splicing. In addition, regulatory sequences that control the ORFs' expression are found in a non-coding area known as the upstream regulatory region or long control region. The virus is non-enveloped and replicates in the nucleus of the host cell. Its icosahedral protein capsule is formed by L1 and L2 coding. In

squamous mature cells that have been infected and consequently, in warts and low-grade lesions, the most frequently expressed mRNA and protein are encoded by E4; the protein modifies the cytoplasmic keratin structure of the host cell to create Koss's koilocyte shape.[41,43] Only the ORFs E6 and E7 exhibit conserved expression in all HPV-associated disease, including low-grade lesions and invasive malignancy. Both proteins have the capacity to replace the host cell's regular growth cycle regulation, leading to the transformation of some lesions into cells with the potential to become cancerous; E6 prevents normal cell DNA repair and/or apoptosis by utilising the ubiquitin pathway to specifically target the TP53 tumour suppressor gene product for eradication.[41,42] Retinoblastoma family proteins are bound by E7, which then targets them for destruction and releases the Rb-bound E2F transcription factor, advancing S-phase progression. P16, a protein overexpressed in the presence of HPV that, has been utilized as a diagnostic for this cell cycle dysregulation.[44]

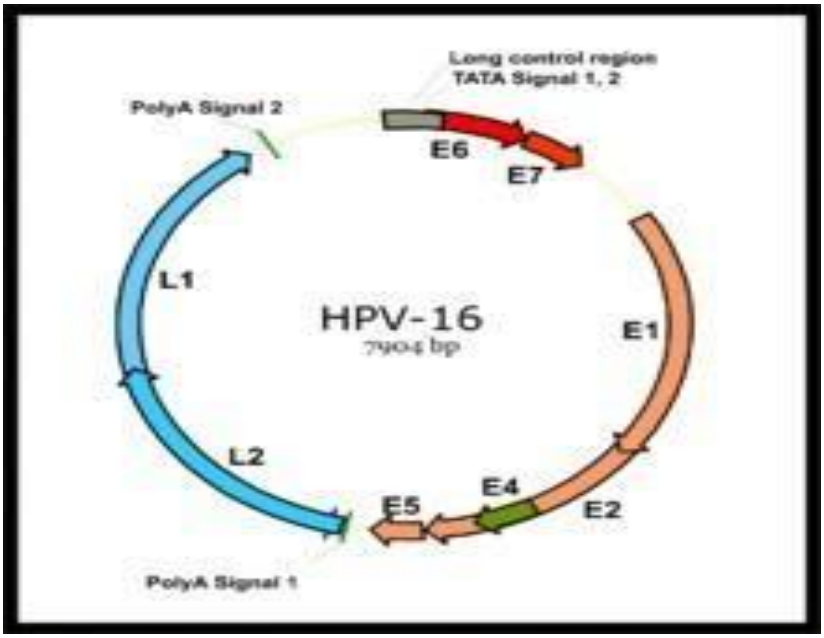


FIGURE 2 : HPV LIFE CYCLE

Although extensively studied[45], there is still debate regarding the cell that is the target of viral infection and the mechanism of cell entry. As previously mentioned, the

squamocolumnar junction is a commonly involved site of HSIL of the uterine cervix. While LSIL can happen anywhere, it usually occurs in the adult ectocervix or transformation zone. However, when the cell matures, the expression of the L1 and L2 capsid components completes the life

Depending on the technique of detection, with the severity of the lesion, high-risk HPV genotype prevalence rises. The most sensitive method used in epidemiologic research for many years was polymerase chain reaction (PCR) amplification of HPV DNA utilising the universal HPV-targeted primers MY09/11 and GP5/6. 99% of cervical malignancies have been proven to have high-risk HPV DNA using these primers. High-risk HPV-positive squamous intraepithelial lesions (SIL) are more likely to advance than HPV-negative SIL. Furthermore, it found that patients with chronic high-risk HPV infections have a 100–300-fold greater risk of developing cervical intraepithelial neoplasia (CIN) 3 lesions.[46,47] All of these studies make the assumption that the development of CIN is linked to (ongoing) high-risk HPV. However, it has also been demonstrated that high-risk HPVs are eliminated in more than 80% of young women and that many of these infections are temporary.[48,49]

Testing for HPV in Cervical Specimens

Numerous technologies have been developed because HPV detection in cervical specimens has emerged as an effective primary and supplementary test to cytology in the screening for cervical neoplasia.[50] The second-generation Hybrid Capture 2 (HC2) assay, which can identify 13 strains of high-risk HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), was the first test for cervical specimens to receive FDA approval. When cervical cells are killed in a reaction tube, HPV DNA is released, which is then hybridized with corresponding chemical RNA probes. The hybridized product is then seen after being stained on a microtitration plate. The actual advantage of HC2 over PCR was that it was less likely to be contaminated by samples

from different types of samples. Compared to the earlier PCR assays, HC2 is mostly automated but a little less sensitive. Several new commercial assays have been created as of this writing for high-risk HPV testing on cervical samples.[50,51]

Squamous Cell Carcinoma (SCC)

Histology

Any lesion in which epithelial formations invade the underlying stroma by infiltration or destruction is to be classified as invasive carcinoma. Both keratinizing and non-keratinizing squamous cell cancer may originate from the original non-keratinizing stratified ectocervical squamous epithelium or from metaplastic epithelium in the endocervical canal.[52]

Key Features of SCC

Non-keratinizing SCC

• Syncytial aggregates are primarily composed of round-to-oval cells.

• Cyanophilic cytoplasm with a corresponding rise in cytoplasmic volume

• Round to oval nuclei that differ significantly in size and membrane shape

Due to a higher cytoplasmic volume, the nuclear to cytoplasmic ratio is lower than in HSIL.

• Nuclear chromatin that is hyperchromatic and unevenly dispersed.

• There are frequently macronucleoli with atypical shapes.

• A possible connection to unsatisfactory specimens because to haemorrhage and necrosis of the tissue.

Keratinizing SCC

• A mixture of single cells and clusters with highly eosinophilic cytoplasm and odd forms, as well as cells grouped in syncytial aggregates with indistinct cell borders.

• Cytoplasmic strands can be detected.

• The cytoplasm of syncytia cells is cyanophilic.

• Round to oval nuclei with a wide range of sizes

• Nuclei with bizarre shapes and lengths

• The greater volume of cytoplasm results in a lower nuclear to cytoplasmic ratio than in HSIL.

• Densely hyperchromatic, coarsely granular, unevenly dispersed nuclear chromatin

• Cells in syncytia usually include micro- and macronucleoli, although the keratinizing cells are obscured by the obvious hyperchromasia.

PROGNOSTIC BIOMARKERS

Numerous studies have been conducted on the function of immunohistochemistry in the identification of different prognostic biomarkers in cervical cancer.

Cell-cycle and apoptosis-regulatory proteins

Cyclin-dependent kinases [CDK]s and their regulator proteins known as cyclins[52] favorably regulate the cell cycle. Retinoblastoma [Rb] proteins are phosphorylated by cyclin/CDK complexes in G1 phase, releasing E2F transcription factors required for cell entry into S-phase[57]. Two classes of endogenous protein inhibitors, together referred to as the INK4 family, control CDK activity. (including p15 INK4b, p16 INK4a, p18 INK4c, and p19 INK4d) and Cip/Kip family (p21 Waf/Cip1, p27 Kip1, and p57 Kip2).[54,55]

INK4 family proteins, Cip-Kip family proteins, and cyclins

Only a few studies have examined the cervical cancer proteins from the INK4 family and Cip-Kip family.[57-59]. The immunohistochemistry expression patterns of CDK inhibitors vary significantly between investigations, primarily because of methodological variations. For instance, alterations in tissue preparation and antibody selection can affect the staining's

intensity. Variations in the cutoffs for nuclear staining that is considered positive versus negative may also affect the results.

Low expression of p16 INK4a, p21 Waf/Cip1, and p27 Kip1 has been seen in 57.0%, 12.9%, and 58.0% of squamous cell carcinomas[60]. According to an immunohistochemical study, cervical intraepithelial carcinoma and adenocarcinoma exhibit increased expression of TGF-beta ligands and receptors and contractedly decreased expression of p27 when compared to normal endocervix[59]. This suggests that dysregulation of TGF-beta and p27 Kip1 expression may be involved in the pathogenesis of cervical adenocarcinoma. In cervical cancer, Lu et al.[60] discovered substantial positive associations between p16 INK4a and p27 Kip1, cyclin E and p27 Kip1, and cyclin E and p21 Waf/Cip1.

Inhibitors of apoptosis

One of the essential proteins in the cervical cancer apoptotic pathway is BCL-2. According to several writers' findings from univariate or multivariate analyses, BCL-2 expression is related to improved survival.[61]

The role of p16 INK4a as a tumor suppressor

Previous research has shown that the CDK inhibitor p16 INK4a functions as a tumour suppressor. p16 INK4a is a member of the INK4 family including p15 INK4b , p16 INK4a , p18 INK4c , and p19 INK4d . Increased expression of p16 INK4a can bind to and inhibit CDK4 and CDK6, which then results in the active, hypophosphorylated Rb. p16 can also shatter CDK complexes, releasing other sequestered CDK inhibitors like p27 to inhibit other CDK, especially CDK2, and then increase the expression of the active, hypophosphorylated Rb. G1 phase arrest, also known as the S phase entry block, is caused by hypophosphorylated Rb. (**Zhu et al.[62]., 1996; Brookes et al.[63]., 2002; Itahana et al.[64]., 2003).** That is the so-called p16 INK4a /pRb pathway.

Therefore, the expression of p16 INK4a can be controlled by these phosphorylation sites and any pathways that affect the phosphorylation of p16 INK4a. It is well known that the degree of intracellular oxidative stress is directly correlated with the phosphorylation of post-translational proteins. Due to repeated, vigorous cell division, the amount of oxidative stress is noticeably higher in tumor cells. In order to stop tumor cells from turning malignant, oxidative stress may cause the phosphorylation of p16 INK4a, causing them to enter division arrest and premature senescence.

In 2004 **Barbara Tringler et al**.[4] studied 108 cases and concluded that p16INK4A is a sensitive and specific marker for squamous neoplasia of cervical mucosa. p16INK4A expression was consistently observed in cervical intraepithelial neoplasia CIN 2/3 and invasive squamous cell carcinoma of the uterine cervix. They also described the correlation between the expression of p16INK4A and pRb in cervical neoplasia, as p16 is known to play a critical role as a negative regulator of cell cycle progression and differentiation by controlling the activity of the tumor suppressor protein pRb. In 2010 **Gupta R et al**.[1] recognized that in the higher grade CIN, there are disturbances in the expression of several cellular proteins, one of which is p16. They observed a progressive increase in p16 expression with an increase in the grade of cervical malignancy. In 2017 **Vatsala Kishore et al**.[2] where significant overexpression of p16INK4A was observed in carcinoma cervix, and with increasing severity of cervical dysplasia, the p16INK4A expression increased progressively. The essential parameters like age and parity showed a positive expression for p16 and gave high expression scores indicating the association of HPV with cases of carcinoma cervix in the North Karnataka region.

In 2018 **Ashok Sangwaiya et al**.[6] studied **the** p16INK4A marker to distinguish CIN & SCC from non-neoplastic cervical lesions, and as cervical carcinoma is the most common cancer related to mortality and morbidity in the world, it is necessary to identify biomarkers like p16INK4A to distinguish CIN from other non-neoplastic cervical lesions.

Apoptotic Index

In general, single cells are affected by apoptosis. The nuclear chromatin is compacted and segregated, and condensation of the cytoplasm occurs along with the creation of clearly defined, evenly granular masses that border against the nuclear membrane. During this time, the cytoplasm continues to condense, and apoptotic bodies that include nucleus fragments contained in membranes sprout from the cell. [65,66] The adjacent cells immediately consume apoptotic bodies. Their digestion is finished within hours of phagocytosis. In order to determine AI, the H and E sections were studied using oil immersion lenses (x100). Ten fields free of artifacts were chosen from each area. The presence of apoptotic cells and apoptotic bodies were examined in one thousand cells. As a proportion of the whole, the AI was expressed.

There are very few studies from India that have evaluated apoptosis using light microscopy in premalignant & malignant lesions of the uterine cervix, despite the fact that it has been assessed and shown to correlate with tumour grade and subtype in some malignant lesions, including those of the large intestine, endometrium, prostate, and breast. The only technique used in the current study to identify apoptosis is morphology, which has the advantages of being simple, practical, and inexpensive.

Both dysplasia and cancer share significant cell growth traits. A few indices used to measure cell proliferation include the mitotic index, PCNA, Ki-67, and AgNOR count; these have been acknowledged by S. Bhardwaj and F. A. Wani as useful prognostic predictors of malignancy. The interruption of apoptosis, which is recognised as a normal process for controlling cell populations, is thought to contribute to the emergence of cancer. Apoptotic cells on formalin-fixed, H&E-stained slides can be quickly distinguished by their morphology using a light microscope. Apoptosis can also be identified by flow cytometry, electron microscopy,

electrophoresis, in-situ nick translation, and the TUNEL approach (Tdt-mediated dUTP biotin nick labelling).In 2015 **Vidya Viswanathan et al.**[7] evaluated the role and significance of apoptotic indices (AI) and proliferative index (PI) in premalignant and malignant squamous cell lesions of the oral cavity and also found an increase in AI gradually from normal to dysplasia to carcinoma[7]

Sagol, et al. [68](1999) also noted that when compared to pre-neoplastic lesions, the squamous cell carcinoma cervix group displayed considerably greater mitotic and apoptotic cell counts and came to the conclusion that as cervical epithelium progresses toward malignancy, both apoptosis and mitosis are noticeably increased. According to **Vijaya et al. (2008)[67],** the severity of dysplasia in premalignant lesions correlates with an increase in mean AI and MI.

MATERIALS AND METHODS

The present study is a cross-sectional study conducted in the Departmentof Pathology, BLDE Hospital (Deemed to be University) Shri B.M. Patil Medical College, Hospital and Research Centre, Vijayapura. During the period from January 2021 to July 2022. The Ethics Committee granted the study its ethical approval.

A sample of 73 cervical biopsies was examined in total. They were categorized into nonspecific cervicitis which is taken as control, low-grade squamous intraepithelial lesion (CIN 1), high-grade squamous intraepithelial lesion (CIN 2&3), and squamous cell carcinoma(SCC). based on their histomorphological characteristics in hematoxylin and eosin-stained (H&E) sections .

TABLE 2: CERVICAL BIOPSIES CATEGORIZATION IN THE PRESENT STUDY

Category	Number of cases
Non specific cervicitis	20
Low grade squamous intraepithelial lesion (CIN 1)	08
High-grade squamous intraepithelial lesion (CIN 2&3)	07
Squamous cell carcinoma (SCC)	38
TOTAL	**73**

PLACE OF STUDY:

Department of Pathology, BLDE (Deemed to be University) Shri B.M. Patil Medical College, Hospital and Research Centre, Vijayapura.

STUDY PERIOD:

January 2021 to July 2022

INCLUSION CRITERIA

All cervical biopsy specimens were diagnosed with cervical neoplasms & for every two to three neoplastic, one non neoplastic cases of the cervix were included.

EXCLUSION CRITERIA

Patients undergoing radiotherapy and anticancer treatment were excluded from the study.

.

All cervical biopsies received in the department of Pathology, which were diagnosed as cervical intraepithelial neoplasia (CIN) and invasive squamous cell carcinoma (SCC) were studied from January 2021 to July 2022. Tissue was preserved in 10% buffered formalin and processed routinely. Two thin sections were prepared from each tissue block. One section was stained with hematoxylin and eosin (H&E) for histomorphologic diagnosis and for calculating the apoptotic index. The other section was mounted on a poly L lysine-coated slide from paraffin-embedded tissue blocks, which was subjected to p16INK4A immunohistochemical staining.

To confirm the diagnosis, classify the lesions based on histomorphological characteristics, and determine the apoptotic index, H& E-stained microscope slides of the cervical lesions were examined.

Squamous cell carcinoma of the cervix is categorised into three classes using the Modified Broder method.

TABLE 3: MODIFIED BROADER SYSTEM OF GRADING OF SQUAMOUS CELL CARCINOMA OF CERVIX.[69]

Well differentiated(Grade 1)	Tumors have individual cell keratinization characterized by intense eosinophilic cytoplasm. Mitotic figures are often present at the edge of the tumor nests.They show keratin pearls which are deposits of acellular keratin found within the tumor nests.
Moderated differentiated (Grade 2)	Tumor cells are more pleomorphic than Grade 1 tumors, with less cytoplasm and larger irregular nuclei. Cell borders are often indistinct. Although keratin pearls are uncommon individual tumor cells, especially at the center of the tumor nests, show keratinization. Mitotic activity is greater than Grade 1 tumors.
Poorly differentiated (Grade 3)	Tumor cells are primitive, appearing with hyperchromatic oval nuclei and scant indistinct cytoplasm that resembles the cellsof HSIL. Mitosis is common and there often is extensive necrosis. Evidence ofkeratinization is challenging to identify. Occasionally Grade 3 tumors consist of large pleomorphic cells with bizarre nucleiand abnormal mitotic figures seen.

HEMATOXYLIN AND EOSIN (H&E) STAINING PROCEDURE:

1. Deparaffinize wax and bring sections to water.
2. Treat with absolute alcohol- 2min.
3. Immerse in 70% alcohol – 2min
4. Immerse in 50% alcohol – 2min
5. Immerse in water – 2min
6. Immerse in hemotoxylin – 5 to 10min
7. 1% acid alcohol- 3 to 5 dips
8. Immerse in running tap water for bluing- 10 min
9. Immerse in 1% eosin- 1 min
10. Dehydrate, clear in xylene and mount in DPX.

The other section was mounted on poly L lysine-coated slide from paraffin-embedded tissue blocks, which was subjected to p16INK4A immunohistochemical staining. Four-micron thick sections were made and stained with monoclonal antibodies for immunohistochemistry. The procedure for staining as follows.

REAGENTS USED IN IMMUNOHISTOCHEMISTRY

1. Peroxide block
2. Primary antibody- p16INK4a mouse monoclonal antibody (clone E6H4) andki67 rabbit monoclonal antibody (ki67-clone EP5)
3. Power block
4. Chromogen - Diaminobenzidine
5. Liquid DAB substrate
6. Super enhancer
7. Poly HRP reagent
8. Hematoxylin- counterstain
9. Buffer solutions

BUFFERS USED

1. TRIS EDTA : pH- 9.0 TRIS
   buffer salt : 6.05 gm
   Disodium EDTA: 0.744 gm
   Distilled water:1000ml
2. TRIS BUFFER pH - 8 TRIS
   buffer salt: 6.05 gm
   Sodium chloride: 8 gm
   Distilled water: 1000ml
   1N Hydrochloric acid : 3 ml
3. CITRATE BUFFER pH-6
   Trisodium citrate: 2.94 gm
   Distilled water: 1000ml
   1N Hydrochloric acid: 5 ml

IMMUNOHISTOCHEMISTRY PROCEDURE

1. Slides were incubated over night at 600 C in an incubator.
2. For 30 minutes, deparaffinize the tissue slices in xylene.
3. Using two changes of five minutes of pure alcohol washing

4. Wash with tap water for 10 minutes.

5. Rinse for five minutes in distilled water.

6. To retrieve the antigen, the slides were placed in a microwave for 20 minutes with the proper buffers.

7. Allow to cool at room temperature before rinsing with distilled water.

8. For five minutes, wash twice in TBS buffer.

9. For ten minutes, apply a peroxide block.

10. Wash for five minutes with two changes in TBS buffer.

11. For 10 minutes, apply power block to the affected areas.

12. After draining the slide, add the primary antibody, and incubate it for an hour in a moisture chamber at room temperature.

13. Wash for five minutes with two changes in TBS buffer.

14. For thirty minutes, cover the slides with a super enhancer.

15. Wash for five minutes with two changes in TBS buffer

16. After that, use the poly HRP reagent for 30 minutes.

17. For five minutes, wash twice in TBS buffer.

18. For five to eight minutes, apply DAB chromogen.

19. For five minutes, wash twice in TBS buffer.

20. Wash for five minutes with tap water.

21. Counterstain for one minute with Mayers hematoxylin.

22. Wash for five minutes with tap water.

23. Air dry and mount in DPX

Apoptotic index:

For calculating Apoptotic Index (AI), the H&E sections were examined using oil immersion lenses (×100). From each section, 10 fields devoid of artifacts were selected. 1000 cells are evaluated for the presence of apoptotic cells and apoptotic bodies. The AI is expressed as percentage of apoptotic cells relative to the total number of tumour cells counted.

P16INK4a Expression:

The following criteria were used for P16INK4a score. Strong nuclear and cytoplasmic positivity is the p16INK4a staining pattern in tumor cells. Expressions that are both negative and weak are seen negatively. For statistical analysis, only high levels of p16INK4a expression were considered positive.

Scoring criteria for p16INK4A expression:

Score	P16INK4a Expression
0	No positivity or focally scattered positive cells (ie, patchy staining)
1+	low intensity, diffuse positivity restricted to lower 1/3rd of epithelium
2+	continuous positivity in the lower 2/3rd of epithelium
3+	positive cells involving the full thickness of epithelium (diffuse total thickness staining)

STATISTICAL ANALYSIS

The study would require a sample size of 73 (i.e., sample size of 73 assuming 2:1 group sizes of Neoplastic: Nonneoplastic cases), to achieve a power of 80% for detecting a difference in proportions between two groups at a two-sided p-value of 0.05.

Formula used

$$n = \frac{(z_\alpha + z_\beta)^2 \; 2 \; p*q}{MD^2}$$

Where Z= Z statistic at a level of significance

MD= Anticipated difference between two proportions

P=Common Proportion

q= 100-p

Statistical analysis

- The data obtained were entered in a Microsoft Excel sheet, and statistical analysis will be performed using a statistical package for the social sciences (Version 20).
- Results were presented as Mean±SD, counts and percentages and diagrams.
- To compare more than two sub groups, ANOVA/Kruskal Walli\s test was applied.
- Categorical variables between groups were compared using the Chi-square test/Fisher's Exact test.
- $p<0.05$ was considered statistically significant. All statistical tests were performed two-tailed.

OBSERVATION AND RESULTS

TABLE 4: CERVICAL BIOPSY CATEGORIZATION IN THE CURRENT STUDY

CATEGORY	NUMBER OF CASES
Non specific cervicitis	20
Squamous intraepithelial lesion of Low-grade(LSIL)	08
Squamous intraepithelial lesion of High-grade(HSIL)	07
Squamous cell carcinoma(SCC)	38
TOTAL	**73**

TABLE 5: AGE GROUP DISTRIBUTION IN THE STUDY

AGE	CASES	PERCENTAGE
30 – 39	20	27.39%
40 – 49	15	20.54%
50 – 59	16	21.9%
> 60 Yrs	22	30.13%
Total	73	100%

A total of 73 patients, age ranged from 30 to 72 years.

FIGURE 3. DISTRIBUTION OF AGE GROUP IN THE STUDY

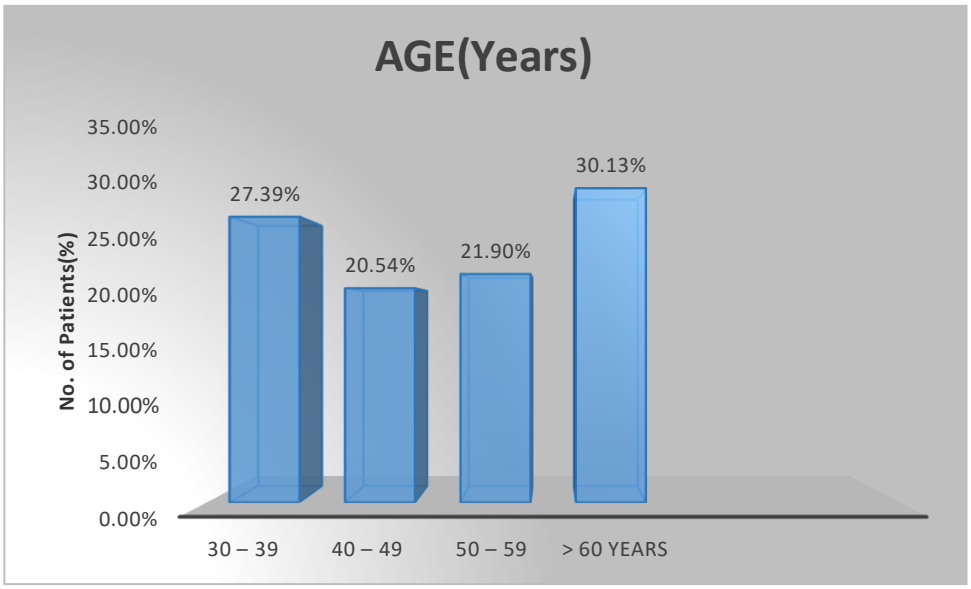


TABLE 6 : PREMALIGNANT CERVIX LESIONS ARE DISTRIBUTED BASED ON DIFFERENT AGE GROUPS.

AGE	CASES	PERCENTAGE
31-40	9	60%
41-50	3	20%
51-60	3	20%
>60 yrs	0	0%

With a mean age of 39 years, the patients' ages range from 30 to 60.

Premalignant lesions were most common in people between the ages of 31 and 40, who made up 60% of all LSIL and HSIL patients.

TABLE 7: DISTRIBUTION OF CARCINOMA CERVIX IN ACCORDANCE WITH DIFFERENT AGE GROUPS

AGE	CASES	PERCENTAGE
30-39	4	10.5%
40-49	7	18.4%
50-59	18	47.3%
>60	9	23.6%
TOTAL	**38**	**100%**

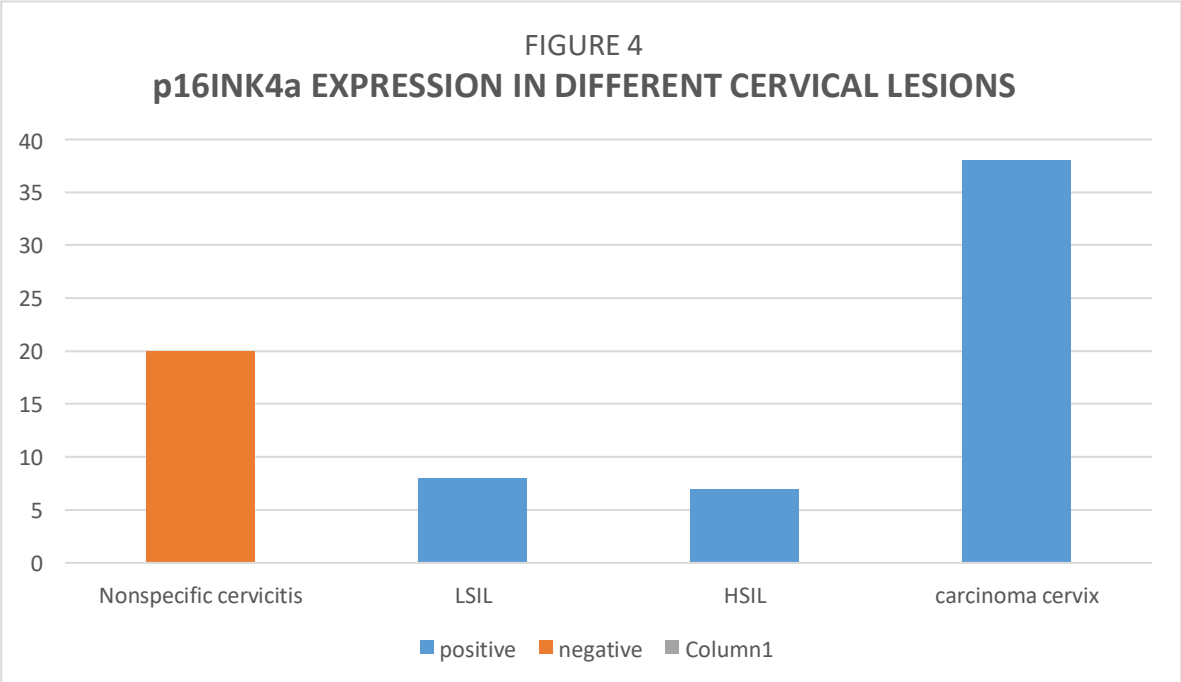
The following findings were made after analyzing a total of 38 cases of cervical cancer. Patients with cervical cancer showing mean age of 56 years. The age range 51–60 years had the highest incidence of cervical carcinoma cases, accounting for 47.3% of all cases of carcinoma cervix.

TABLE 8 : ASSOCIATION OF AGE WITH HISTOPATHOLOGICALDIAGNOSIS

AGE	NON SPECIFIC CERVICITIS	LSIL (CIN 1)	HSIL (CIN 2 & 3)	SCC	TOTAL
30 - 39	15	5	4	4	28
40 - 49	5	2	1	7	15
50 - 59	0	1	2	18	21
> 60 Years	0	0	0	9	9
Total	20	8	7	38	73

TABLE 9: ASSOCIATION OF p16INK4a EXPRESSION INDIFFERENT CERVICAL LESIONS

HISTOLOGICAL DIAGNOSIS	p16INK4a (nuclear and cytoplasmic staining)	
	POSITIVE CASES	NEGATIVE CASES
Nonspecific cervicitis	0	20 (100%)
LSIL	8(100%)	0
HSIL	7 (100%)	0
Carcinoma cervix	38(100%)	0
TOTAL	**47**	**26**



p16INK4a showed positivity was present in 100%(8/8) of LSIL cases, 100%(7/7) of HSIL cases, and 100%(38/38) of carcinoma cervix patients.

TABLE 9: ASSOCIATION OF p16INK4a EXPRESSION INDIFFERENT CERVICAL LESIONS

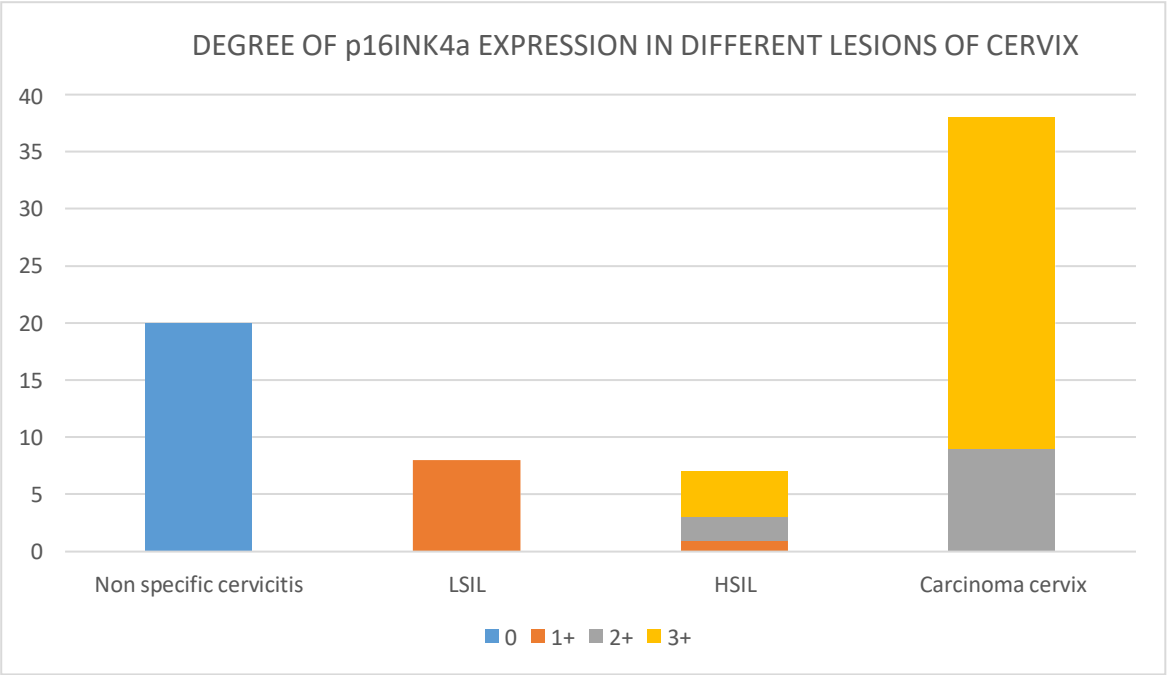
HISTOLOGICAL DIAGNOSIS	p16INK4a SCORE				
	0	1+	2+	3+	TOTAL
	n(%)	n(%)	n(%)	n(%)	N
Non specific cervicitis	20(100)	0	0	0	20
LSIL	0	8(100)	0	0	8
HSIL	0	1(14.2)	2(28.5)	4(57.14)	7
Carcinoma cervix	0	0	9(23.6)	29(76.3)	38

n-number of patients

100% of LSIL, 14.2% HSIL cases show Score 1+ positivity, 28.5% of HSIL, 23.6% of SCC cases show Score 2+ positivity and 57.14% of HSIL and 76.3% SCC cases show Score 3+ positivity.

The scoring of the p16INK4a marker showed difference in expression between non neoplastic and neoplastic lesions and the score gradually increased from premalignant to malignant lesions of cervix.

FIGURE 5 : DEGREE OF p16INK4a EXPRESSION WITH HISTOPATHOLOGICAL DIAGNOSIS



Score 3 p16INK4a positive was seen in 76.3% (29/38) of carcinoma patients and 57.14% (4/6) of HSIL cases. This marker was therefore highly expressed in high-grade cervical lesions.

PREMALIGNANT AND MALIGNANT LESIONS OF CERVIX ON H&E SLIDES

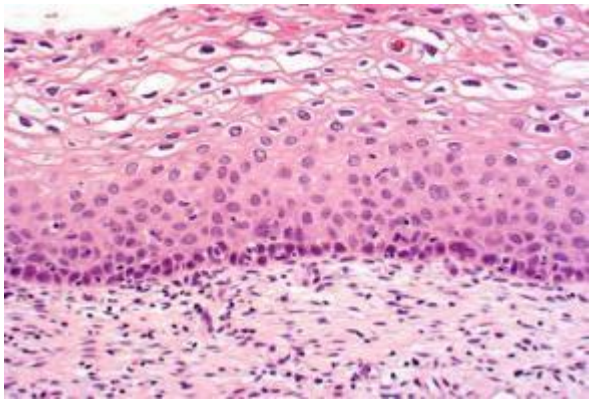


FIG6- Photomicrograph showing LSIL (H& E)

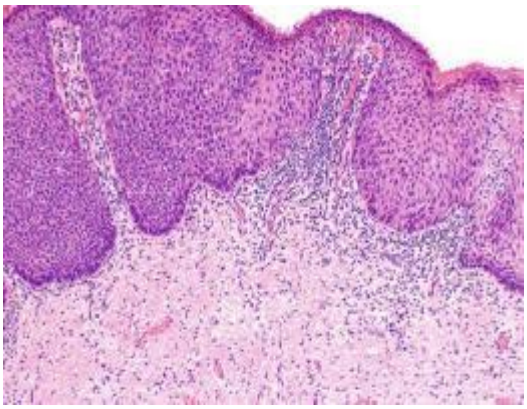


FIG7- Photomicrograph showing HSIL (H&E)

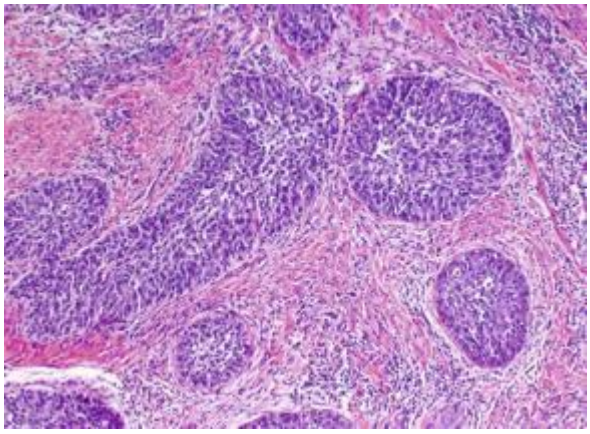


FIG8- Photomicrograph showing SQUAMOUS CELL CARCINOMA (20X) (H&E)

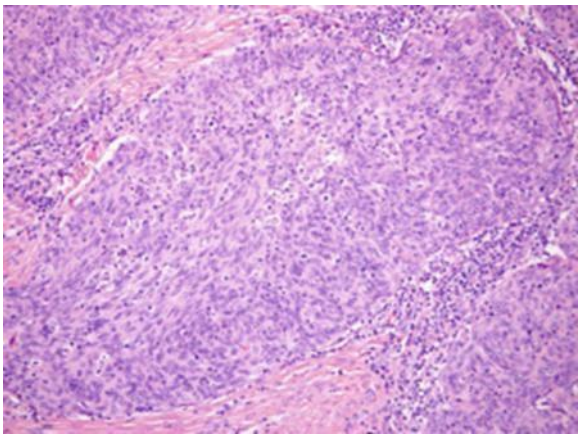


FIG9- Photomicrograph showing SQUAMOUS CELL CARCINOMA (40X) (H&E)

p16INK4a IMMUNOSTAINING EXPRESSION IN DIFFERENT CERVICAL LESIONS

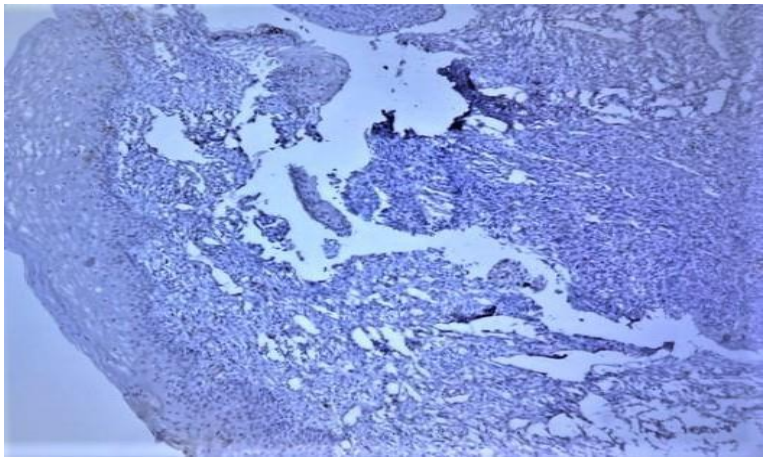


FIG10- P16 NEGATIVE IN NON SPECIFIC CERVICITIS (SCORE 0)

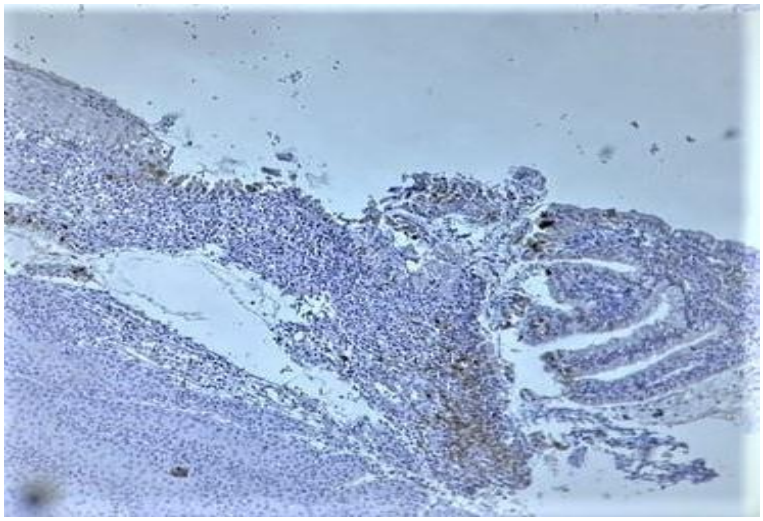


FIG11-P16 POSITIVE IN LSIL (SCORE 1+)

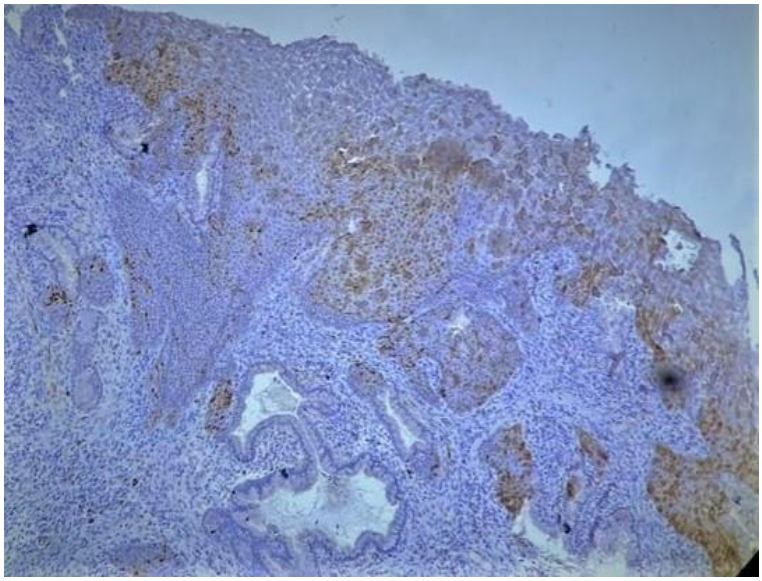


FIG12- P16 POSITIVE IN HSIL (SCORE 2+)

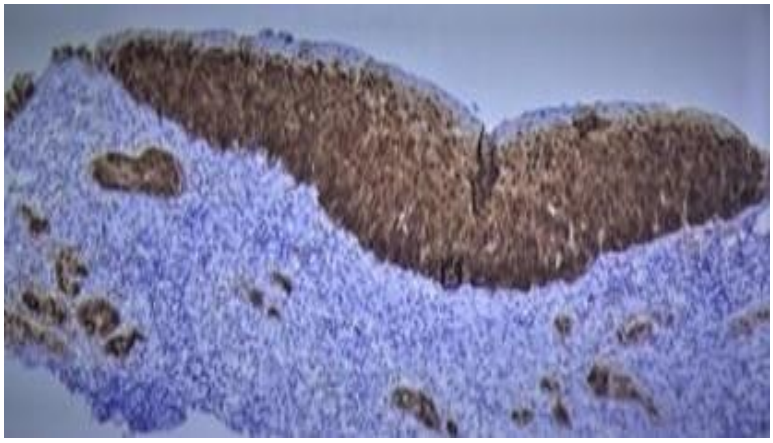


FIG13- P16 POSITIVE IN SQUAMOUS CELL CARCINOMA SHOWING (SCORE 3+)

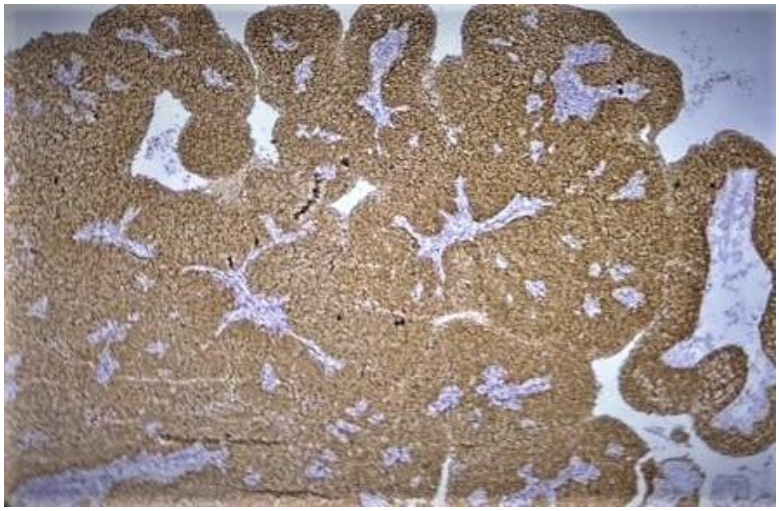


FIG14- TUMOR TISSUE SHOWING HIGH EXPRESSION OF P16INK4A (SCORE 3+)

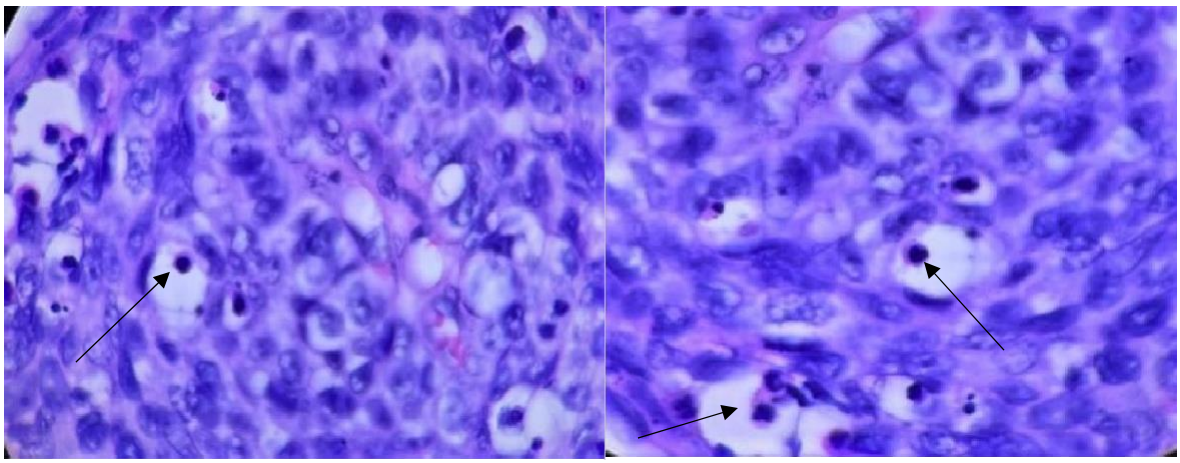


FIG15- H&E, SQUAMOUS CELL CARCINOMA SHOWING APOPTOTIC BODIES

TABLE 11: p16INK4A EXPRESSION IN DIFFERENT GRADES OF CARCINOMA CERVIX

DIAGNOSIS	p16INK4a expression Score				Total
	0	1	2	3	
	n(%)	n(%)	n(%)	n(%)	
Well-differentiated	0	0	1(11.1)	7(24.13)	8
Moderately differentiated	0	0	3(33.3)	16(55.17)	19
Poorly differentiated	0	0	5(55.5)	6(20.68)	11
Total SCC	0	0	9	29	38

n-number of patients

Score 3 positive of p16INK4a was present in 24.13% (7/29) cases of well-differentiated carcinoma, 55.17% (16/29) of moderately differentiated and 20.6% (6/29 poorly differentiated cervical carcinoma.

As a result, there was no significant difference in the expression of p16INK4a between cervical carcinomas of different grades with a p-value > 0.05.

TABLE 12: ASSOCIATION OF APOPTOTIC INDEX (AI) IN DIFFERENT CERVICAL LESIONS

HISTOPATHOLOGICAL DIAGNOSIS	APOPTOTIC INDEX (AI)				Total cases
	0-0.5	0.5-1.0	1-1.5	1.5-2.0	
Non specific cervicitis	20(100%)	0	0	0	20
LSIL	6(75%)	2(25%)	0	0	8
HSIL	0	4	2(28.5%)	1(14.2%)	7
Carcinoma cervix	0	3	16(42.1%)	19(50%)	38
Total	26	7	18	20	**n=73**

- 20/20(100%) cases of nonspecific cervicitis and 6/8 (75%)of LSIL cases showing AI 0-0.5, 2/8(25%) of LSIL cases showing AI 0.5-1.0, 2/7(28.5%) cases of HSIL and 16/38(42.1%) cases of SCC showing AI 1.0-1.5, 1/7(14.2%) of HSIL and 19/38(50%) cases of SCC showing AI 1.5-2.0. Cases of non specific cervicitis showing Mean AI of 0.025±0.05, LSIL cases show Mean AI of 0.413±0.145, HSIL cases showing 1.086±0.29 and carcinoma cervix cases showing AI of 1.788±0.124

Apoptotic index is increased from non neoplastic to neoplastic cervical lesions and from premalignant to malignant conditions.

TABLE 13: APOPTOTIC INDEX (AI) IN DIFFERENT GRADES OF CARCINOMA CERVIX

HISTOLOGICAL DIAGNOSIS	APOPTOTIC INDEX (AI)				Total cases (n)
	0-0.5	0.5-1.0	1.0-1.5	1.5-2.0	
Poorly differentiated	0	8(72.7%)	2	0	11
Moderately differentiated	0	0	13(68.4%)	6(31.5%)	19
Well-differentiated	0	0	0	8(100%)	8
Total cases of Carcinoma cervix	**0**	**8**	**15**	**14**	**38**

- 8/11(72.7%) cases of poorly differentiated SCC showing AI 0-0.5, 13/19(68.4%) cases of moderately differentiated SCC showing AI 1.0-1.5 and 8/8(100%) cases of well-differentiated SCC showing AI 1.5-2.0. cases of poorly differentaied SCC showing Mean AI 1.432±0.124, cases of moderately differentiated SCC showing AI 1.788±0.124 and cases of well differentiated SCC showing 1.936±0.143.

Apoptotic index decreased with the decrease in differentiation of tumor

Apoptotic Index(AI)

TABLE 13: MEAN APOPTOTIC INDEX VALUES IN DIFFERENT CERVICAL LESIONS

Diagnosis	No. of cases	Mean ± SD
chronic cervicitis	20	0.025±0.05
LSIL	7	0.413±0.145
HSIL	8	1.086±0.29
Poorly differentiated squamous cell carcinoma	19	1.432±0.124
moderately differentiated squamous cell carcinoma	11	1.788±0.124
well-differentiated squamous cell carcinoma	8	1.936±0.143
Total	73	0.866±0.649

In our study, it was observed that AI increased gradually from normal through dysplasia to cancer. Squamous cell carcinomas with a significant degree of differentiation showed the highest AI (SCCs).

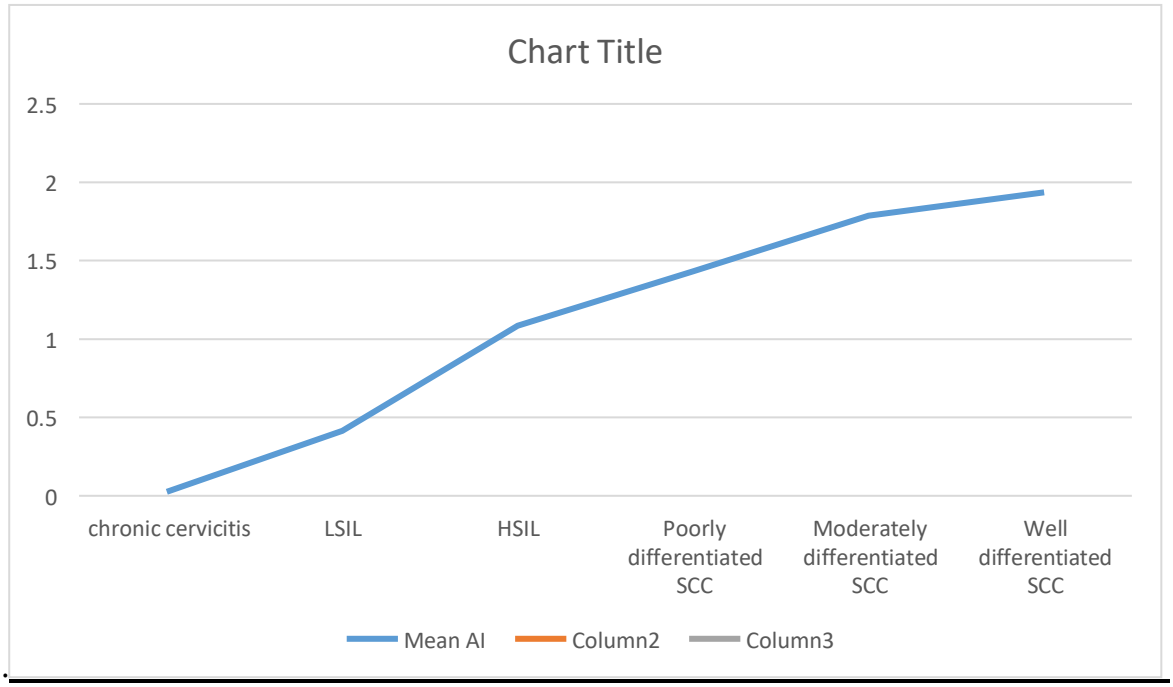


FIGURE 16 : CHART SHOWING INCREASE IN AI WITH INCREASE IN DIFFERENTIATION

In the current study, AI steadily increased from non neoplastic to neoplastic lesions and also increased with increase in tumor differentiation

DISCUSSION

In our study, found no p16INK4a expression in any of the 20 nonspecific cervicitis cases whereas nonspecific cervicitis in the study done by Munhoz et al. also did not show p16INK4a expression.[70]In the present study, the age range between 50 – 60 years has the highest prevalence of cervical cancer (47.3%), whereas the age group between 30 – 40 years has the highest frequency of premalignant lesions (60%) and cervical carcinomas were found to be 56 years old on average.

Table 14:- p16INK4A expression in carcinoma cervix (SCC) in various studies.

STUDY	CASES	POSITIVE	NEGATIVE
Lesnikova Iana et al.,[5]	133	98.5%	1.5%
Srivastava S[7]	15	100%	0%
Agoff et al.[71]	46	92%	8%
Rauschenbach et al[72]	46	100%	0%
In present study	38	100%	0%

All 38 cervical cancer patients 100% displayed nuclear and cytoplasmic positive for p16INK4a. 92% (42 out of 46) of the cervical cancer cases in the **Agoff et al.** study showed p16INK4a expression.[71] **Rauschenbach and colleagues** showed 100% (46 of 46) positive cases for p16INK4a in their study.[72]

In the current study, all 7 HSIL slides showed 100% p16INK4a positivity. In the study conducted **by Fatemah et al.,** 100% HSIL cases showed 100% positivity for the p16INK4a

marker in both the nucleus and cytoplasm.[73] Reuschenbach et alstudy .'s showed that 86% of HSIL cases tested positive for p16INK4a. Out of the 8 LSIL cases in the current study, 8 (100%) were p16INK4a positive with score 1+ involving 25% of epithelial cells whereas in the Agoff et al. study, 16% of patients had p16INK4a positive in fewer than 25% of epithelial cells with score 1 positivity.

In this study SCC and HSIL cases all showed p16INK4a overexpression. The studies conducted by Benevolo et al.,[74] and Volgareva et al.,[75] revealed similar evidence. This shows the significant relationship between the degree of premalignant and malignant lesions and the overexpression of p16INK4a in HSIL and SCC, as well as its crucial involvement in the development of malignancy in the cervix.

Table 15:- Overall pattern of p16INK4A expression in various studies

Study	Chronic cervicitis	CIN 1 (LSIL)	CIN2&3 (HSIL)	Squamous cell carcinoma
Lesnikova Iana et al.,	(0/10)	(180/249)	(178/181)	(131/133)
Srivastava S	(0/15)	(8/10)	(5/5)	(15/15)
In present study	(0/20)	(8/8)	(7/7)	(38/38)

With a p-value of less than 0.0001, we were able to show that p16 INK4a expression increased gradually from non neoplastic to LSIL, followed by HSIL and SCC. As a result, it was shown that an increase in p16INK4a corresponds to the expansion of cells showing dysplasia and subtly indicates the degree of premalignancy and malignancy. In order to diagnose premalignant and malignant lesions, it is necessary to use the expression of the p16INK4a protein in cervical biopsies.

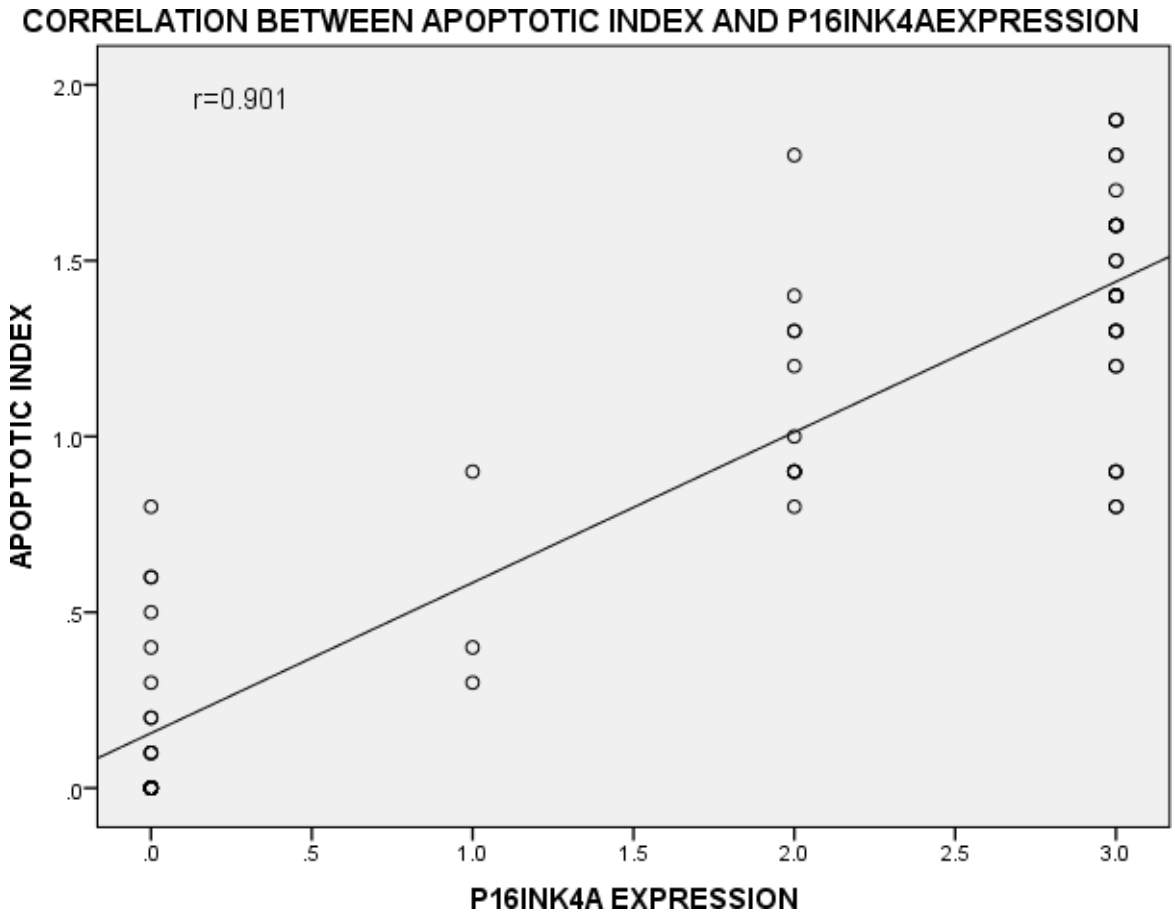
Additionally, our study found no expression of p16INK4a in benign lesions such non-specific cervicitis, in contrast to premalignant (LSIL-100% and HSIL-100%) and malignant (100% positive) lesions. Thus, to improve diagnostic accuracy and to distinguish between benign and malignant tumours, p16INK4a is an useful additional tool.

According to our study, the level of AI continued to increase from normal to dysplasia to cancer. 20/20 (100%) cases of chronic cervicitis, 6/8 (75%) of LSIL cases exhibiting AI 0-0.5, 2/8 (25%) of LSIL cases showing AI 0.5-1.0, 2/7 (28.5%) of HSIL cases, 16/38 (42.1%) of SCC cases showing AI 1.0-1.5, 1/7 (14.2%) of HSIL cases, and 19/38 (50%) of HSIL cases. **Vidya Viswanathan et al.**[7] in their study also found an increase in AI gradually from normal to dysplasia to carcinoma[7]

The Apoptotic Index increased from normal cervical lesions to carcinoma cervix in 19/38 (50%) cases of SCC showing AI 1.5-2.0 Cases of non-specific cervicitis showing Mean AI of 0.025±0.05, LSIL cases showing Mean AI of 0.413±0.145, HSIL cases showing 1.086±0.29, and carcinoma cervix cases showing AI of 1.788±0.124. According to **Vijaya et al. (2008),** the severity of dysplasia in premalignant lesions showed an increase in mean AI[67]. **Sagol, et al.** (1999) also noted that when compared to pre-neoplastic lesions, the squamous cell carcinoma cervix group displayed considerably greater apoptotic cell counts and came to the conclusion that as cervical epithelium progresses toward malignancy apoptosis is noticeably increased.[68]

Squamous cell carcinomas with high degree of differentiation showed the highest AI (SCCs). 8/8 (100%) cases of well-differentiated SCC showed AI 1.5-2.0, while 13/19 (68.4%) cases of moderately differentiated SCC showed AI 1.0-1.5. Cases of poorly differentiated SCC showed Mean AI 1.432±0.124, while Cases of moderately

differentiated SCC showed AI 1.788±0.124, and Cases of well-differentiated SCC showed 1.936±0.143. The apoptotic index decreased with a decrease in the differentiation of the tumor. In study by **Vidya Viswanathan et al.**[7] also found highest AI in well differentiated SCC. In the current study, AI continued to increase from normal to carcinoma but decreased when tumor differentiation decreased.



- The coefficient of correlation between P16INK4A expression and the Apoptotic index is found to be r=0.901, which is statistically significant.

- This study demonstrated that p16INK4a and AI are positively correlated by the Pearsons' coefficient of correlation . r =+0.901and statistically significant p value of 0.001 linked an increase in both of these indicators to an increase in the grades of cervical lesions and cervix carcinoma.

The combination of p16INK4a positive and AI correlation thereby increases the precision of the diagnosis of squamous cell carcinoma, HSIL, and LSIL.

CONCLUSION

In our study we concluded that from non neoplastic to neoplastic lesions and in neoplastic lesions from premalignant to malignant cervical lesions, the The p16INK4a marker shown an increase in scoring showing the degree of histological dysplasia and malignancy, confirming its prognostic and predictive usefulness in the therapy of cervical malignancies.

Apoptotic indices have been demonstrated to be helpful in identifying benign and malignant cervix lesions. In this study, from histological evaluation, it is observed that AI increased with an increase in disease progression, which might act as a good prognostic indicator.

Therefore, integrating p16INK4a expression and AI improves the accuracy of diagnosing premalignant and malignant cervix lesions while also separating them from non-neoplastic cervical lesions to avoid needless surgical operations.

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ANNEXURE – I

B.L.D.E. (DEEMED TO BE UNIVERSITY) IEC/no-09/2021 Date-22/01/2021

(Declared vide Notification No. F.9-13/2007-U.1(A) Dated 29.2.2008 of the Ministry, Government of India under Section 3 of the UGC Act, 1956)

The Constituent College

SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL AND RESEARCH CENTRE

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Institutional ethical committee of this college met on 11-01-2021 at 11-00 am to scrutinize the synopsis of Postgraduate students of this college from Ethical Clearance point of view. After scrutiny the following original/corrected and revised version synopsis of the Thesis has been accorded Ethical Clearance

Title: Significance of apoptotic index and p16INK4A protein expression in cervical intraepithelial neoplasia and invasive squamous cell carcinoma of uterine cervix

Name of PG student: Dr K Elizabeth, Department of Pathology

Name of Guide/Co-investigator: Dr S.B.Hippargi , Professor of Pathology

DR .S.V.PATIL
CHAIRMAN, IEC
Institutional Ethical Committee
B L D E (Deemed to be University)
Shri B.M. Patil Medical College,
VIJAYAPUR-586103 (Karnataka)

Following documents were placed before Ethical Committee for Scrutinization:

1. Copy of Synopsis / Research project
2. Copy of informed consent form
3. Any other relevant documents.

7

ANNEXURE-II

B.L.D.E(DEEMED TO BEUNIVERSITY) SHRI B.M.PATIL MEDICAL COLLEGE, HOSPITAL AND RESEARCH CENTER, VIJAYAPURA-586103

INFORMED CONSENT FOR PARTICIPATION IN DISSERTATION/RESEARCH

I, the undersigned,_____, S/O D/O W/O_____, aged ____years, ordinarily resident of_____do hereby state/declare that Dr_____of _____ Hospital has examined me thoroughly on_____at_____ (place) and it has been explained to me in my own language that I am suffering from _____ disease (condition) and this disease/condition mimic following diseases . Further Doctor informed me that he/she is conducting dissertation/research titled _____under the guidance of Dr_____requesting my participation in the study. Apart from routine treatment procedure, the pre-transfusion, post-transfusion and follow-up observations will be utilized for the study as reference data.

Doctor has also informed me that during conduct of this procedure adverse results may be encountered. Among the above complications most of them are treatable but are not anticipated hence there is chance of aggravation of my condition and in rare circumstances it may prove fatal in spite of anticipated diagnosis and best treatment made available. Further Doctor has informed me that my participation in this study will help in evaluation of the results of the study which is useful reference to treatment of other similar cases in near future, and also I may be benefited in getting relieved of suffering or cure of the disease I am suffering.

The Doctor has also informed me that information given by me, observations made/ photographs/ video graphs taken upon me by the investigator will be kept secret and not assessed by the person other than me or my legal hirer except for academic purposes.

The Doctor did inform me that though my participation is purely voluntary, based on information given by me, I can ask any clarification during the course of treatment / study related to diagnosis, procedure of treatment, result of treatment or prognosis. At the same time I have been informed that I can withdraw from my participation in this study at any time if I want or the investigator can

terminate me from the study at any time from the study but not the procedure of treatment and follow-up unless I request to be discharged.

After understanding the nature of dissertation or research, diagnosis made, mode of treatment, I the undersigned Shri/Smt_____under my full conscious state of mind agree to participate in the said research/dissertation.

Signature of patient:

Signature of doctor:

Witness: 1.

2.

Date:

Place:

ANNEXURE - III

PROFORMA

NAME : OP/IP No.:

AGE :

SEX : D.O.A:

RELIGION : D.O.D:

OCCUPATION :

RESIDENCE :

Presenting Complaints :

Past history :

Personal history :

Family history :

Treatment history :

Per Rectum

Examination finding :

Catheterization : YES/NO

USG (findings) :

VITALS :

PR: RR:

BP: TEMPERATURE:

WEIGHT:

BIOPSY: Cervical biopsy -

HPR Finding:

Benign OR Malignant:

If malignant:

POSITIVE-

Score 0 –

Score 1 –

Score 2 -

Score 3 -

IHC show: - p16INK4A-

Diagnosis:

APOPOTOTIC INDEX:

KEY TO MASTER CHART

LSIL – Low grade squamous intra epithelial lesion

HSIL – High grade squamous intra epithelial lesion

SCC- Squamous cell carcinoma

CIN – Cervical intra epithelial neoplasia

AI – Apoptotic index

MASTER CHART

NAME	AGE	HPR NO.	DIAGNOSIS	P16INK4A EXPRESSION	APOPTOTIC INDEX
Padmavati bhimappa	70	6749/19	well differentiated squamous cell carcinoma	3	1.9
Padmavati . k	50	7443/19	moderately differentiated squamous cell carcinoma	3	1.6
Neelawwa	70	8040/19	moderately differentiated squamous cell carcinoma	3	1.4
Shakuntala	54	411/20	moderately differentiated squamous cell carcinoma	3	1.5
Shantabai	63	847/20	moderately differentiated squamous cell carcinoma	3	1.6
Iramma	80	1803/20	Poorly differentiated squamous cell carcinoma	3	1.2
Gorakka	54	2138/20	Poorly differentiated squamous cell carcinoma	2	0.9
Husanbi	75	2321/20	moderately differentiated squamous cell carcinoma	3	1.4
Ningamma	60	2445/20	moderately differentiated squamous cell carcinoma	3	1.4
Ayesha patil	40	2935/20	LSIL (CIN-1)	0	0.6
Neelabai	66	2975/20	well differentiated squamous cell carcinoma	2	1.8
Basamma	42	3081/20	LSIL (CIN-1)	0	0.4
Kamala	36	121/20	HSIL (CIN-3)	1	0.9
Aneesa	37	3563/20	LSIL (CIN-1)	1	0.4
Boramma	38	3919/20	HSIL (CIN-2)	2	0.9
Bharati	32	965/21	HSIL (CIN-3)	3	0.8
Deepa.B	34	1325/21	LSIL (CIN-1)	0	0.3
Bhimawwa	80	2494/20	Poorly differentiated squamous cell carcinoma	2	1.2
Padmavati . C	49	3621/21	LSIL (CIN-1)	0	0.2
Sunita	52	292/21	well differentiated squamous cell carcinoma	3	1.8
Gwalamma	65	135/21	moderately differentiated squamous cell carcinoma	3	1.7
Shivamma pujari	56	993/21	moderately differentiated squamous cell carcinoma	2	1.4
Neelamma	51	1143/21	well differentiated squamous cell carcinoma	3	1.6
Sumitra	58	1098/21	moderately differentiated squamous cell carcinoma	3	1.5
Laxmibai	35	1914/21	HSIL (CIN-2)	2	0.9
Vimala	52	1954/21	moderately differentiated squamous cell carcinoma	3	1.3
Kashibai	55	2130/21	moderately differentiated squamous cell carcinoma	3	1.6
Roopa hiremath	53	2246/21	moderately differentiated squamous cell carcinoma	2	1.3
Shankaramma	82	2555/21	well differentiated squamous cell carcinoma	3	1.9
Savitha	55	3937/21	HSIL (CIN-3)	3	1.6
Savitri	56	4658/21	moderately differentiated squamous cell carcinoma	3	1.4
Basamma	53	4902/21	moderately differentiated squamous cell carcinoma	3	1.3
Shobha	37	5892/21	Poorly differentiated squamous cell carcinoma	2	0.9
sivamma	58	237/22	well differentiated squamous cell carcinoma	3	1.9
gurudevi	57	3570/22	well differentiated squamous cell carcinoma	3	1.8

shantamma	53	3274/22	Poorly differentiated squamous cell carcinoma	2	0.8
yallawa	55	3319/22	moderately differentiated squamous cell carcinoma	3	1.4
shantamma n	53	3197/22	moderately differentiated squamous cell carcinoma	3	1.4
girija	58	2363/22	moderately differentiated squamous cell carcinoma	3	1.3
Neelamma	50	2322/22	Poorly differentiated squamous cell carcinoma	2	1
sangamma	59	2074/22	HSIL (CIN-3)	3	1.3
Borama	60	590/22	moderately differentiated squamous cell carcinoma	3	1.4
meenakshi	38	1866/22	LSIL (CIN-1)	1	0.3
madasai	50	1535/22	HSIL (CIN-3)	3	1.2
nagamma	32	8136/19	LSIL (CIN-1)	0	0.5
mariyamma	45	88/22	well differentiated squamous cell carcinoma	3	1.6
haleema	38	1572/21	LSIL (CIN-1)	0	0.6
shamima bhanu	37	2438/21	chronic cervicitis	0	0.1
kaveri	36	2973/21	chronic cervicitis	0	0
bheemavva	42	3729/21	chronic cervicitis	0	0
gurulingamma	46	3837/21	chronic cervicitis	0	0.1
vidhyaindi	34	4715/21	chronic cervicitis	0	0.1
pravathi	31	4909/21	chronic cervicitis	0	0.2
sheetal	39	4958/21	chronic cervicitis	0	0
nandini	32	5019/21	chronic cervicitis	0	0
gangamma	36	3489/22	chronic cervicitis	0	0
vinutha	31	2625/22	chronic cervicitis	0	0
geerishma	31	1098/22	chronic cervicitis	0	0
jayammma	33	832/22	chronic cervicitis	0	0
padmavathi	31	1674/22	chronic cervicitis	0	0
nandini	36	3261/22	chronic cervicitis	0	0
gousbee	40	2325/22	chronic cervicitis	0	0
ranjana kamble	40	1804/22	chronic cervicitis	0	0
nandini	31	5019/21	chronic cervicitis	0	0
Rukmini	42	2265/21	moderately differentiated squamous cell carcinoma	2	1.3
Shantadevi	49	3328/21	Poorly differentiated squamous cell carcinoma	3	0.9
Mumtaz	43	4321/22	Poorly differentiated squamous cell carcinoma	3	0.9
Savitri	31	4815/22	Poorly differentiated squamous cell carcinoma	3	0.8
Beejanbi	48	2683/22	Poorly differentiated squamous cell carcinoma	3	0.9
Parvathi malli	43	1948/22	Poorly differentiated squamous cell carcinoma	0	0.8
Ningamma halli	36	485/22	chronic cervicitis	0	0
Zubeda begum	31	1648/22	chronic cervicitis	0	0
Shantamma p	48	1926/22	chronic cervicitis	0	0

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