"IMMUNOHISTOCHEMICAL EXPRESSION OF P16^{ink4a} IN PATIENTS WITH SQUAMOUS CELL CARCINOMA OF THE CERVIX- A ONE YEAR PROSPECTIVE AND RETROSPECTIVE STUDY IN A TERTIARY CARE HOSPITAL"

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LIST OF ABBREVIATIONS USED

WHO	-	World Health Organisation
HPV	-	Human papilloma virus
PCNA	-	Proliferation cell nuclear antigen
SCC	-	Squamous cell carcinoma
RB	-	Retinoblastoma
IHC	-	Immunohistochemistry
H & E	-	Haematoxylin and eosin
EGFR	-	Epidermal growth factor receptor
DNA	-	Deoxyribonucleic acid
NCRP	-	National Cancer Registry Program
VIA	-	Visual inspection with acetic acid
HLA	-	Human leucocyte antigen
LSIL	-	Low grade squamous intraepithelial lesion
HSIL	-	High grade squamous intraepithelial lesion
CIN	-	Cervical intraepithelial neoplasia
FIGO	-	Federation International of Gynaecology and Obstetrics
CDK	-	Cyclin dependant kinases
CDKI	-	Cyclin dependent kinase inhibitors
FDA	-	Food and Drug Administration
VEGF	-	Vascular endothelial growth factor

ABSTRACT

"IMMUNOHISTOCHEMICAL EXPRESSION OF P16^{ink4a} IN PATIENTS WITH SQUAMOUS CELL CARCINOMA OF THE CERVIX- A ONE YEAR PROSPECTIVE AND RETROSPECTIVE STUDY IN A TERTIARY CARE HOSPITAL."

BACKGROUND: Worldwide, carcinoma of uterine cervix is the fourth common cancer in women and becomes the prevalent cause of mortality in women. The P16^{ink4a} is a tumour suppressor protein located on chromosome 9p21. It acts by preventing the progression of cell cycle from G1 to S phase. Its overexpression is demonstrated by immunohistochemical staining and it is known to have an effect on treatment response and survival of patients. It is not known to show positivity in normal cervical epithelium. This research was undertaken to study the expression of P16ink4a in cervical squamous cell carcinoma cases. Its role as a possible marker by correlating its positive expression with clinicopathological parameters was also studied.

OBJECTIVES: To study the expression of P16^{INK4A} in histologically diagnosed cases of cervical squamous cell carcinoma and to compare the histological grading with intensity of P16^{INK4A} expression.

METHODOLOGY: Thirty-five paraffin embedded blocks of histopathologically diagnosed squamous cell carcinoma cervix were retrieved from the Department of Pathology, J. N. Medical College, Belagavi, Karnataka and were stained with Haematoxylin and Eosin (H and E) and immunohistochemical staining with P16^{ink4a} antibody in KAHER's basic research laboratory and were evaluated for staining intensity and percentage of tumour cells showing p16 positivity. Final IHC score was

given as their product and various clinic-pathological parameters were correlated with the score.

RESULTS: All the thirty-five cases of SCC cervix showed P16 positivity. The correlation between parity and P16 score was moderately significant with a p value of 0.03. Also, correlation between keratinising status of the tumour with P16 score was significant statistically with p value of 0.02. The P16 expression increased with increased age and in postmenopausal women with no statistical significance.

CONCLUSION: Overexpression of $P16^{ink4a}$ intensity increases with increasing histological grade.

KEY WORDS: Squamous cell carcinoma cervix, Keratinizing, Non-keratinizing, $P16^{ink4a}$, IHC, clinic-pathological parameters

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INTRODUCTION

Carcinoma of uterine cervix is considered to be the second most common cancer in India and fourth most common cancer worldwide.[1] According to WHO, developing countries for example India, contribute for about 80% of total cervical cancer cases and hence, becomes the leading cause of mortality.[2,3,4]

Approximately 1 in 53 women in India experience cervical cancer in their lifetime and is the main cause of mortality.[5] This is because most of the cases present at a fairly advanced stages.[3,6,7]

Almost all cervical cancers and most of its precursor lesions are caused by persistent cervical infections by the 12 to 15 high-risk human papillomavirus (HPV) genotypes.[8] There are several other risk factors associated with etiology and pathogenesis of carcinoma of the cervix like

i. Women with early age of sexual activity.
ii. Women having multiple sex partners.
iii. Role of high-risk male partner such as promiscuous male having previous multiple sex partners, having history of penile condyloma or male who had previous spouse with cervical cancer.[9]

The presence of immature metaplastic squamous cells at squamo-columnar junction and the immature basal cells in cases of epithelial ulcers are mainly infected by HPV. The ectocervix lined by mature superficial squamous cells is not infected by these organisms.[10]

The viral proteins E7 and E6, which interrupt the activity of tumor suppressor proteins which help in regulating cell survival and growth, are required for HPV to act as a carcinogen.[10] It has been documented that morphologic abnormalities in cervical lesions have good correlation with cellular events by use of techniques such as proliferation cell nuclear antigen (PCNA), P16, P53 and P63 expression.[9]

Almost 80% of cervical cancer cases account for morphologic subtype squamous cell carcinoma (SCC).[10] They could be:

- Keratinizing: morphologic variant with nests (masses) of keratin producing squamous cells.
- Non-keratinizing: morphologic variant with nests (masses) of squamous cells without keratin.

Hysterectomy and cervical cone excision are the procedures mostly done for early invasive lesions and for advanced lesions, radiation and chemotherapy may also be necessary. Cervical carcinoma can be prevented with adequate screening and appropriate management of women with histopathologically diagnosed precancerous conditions of cervix.[10]

Stage of cervical cancer and also the histologic subtype during diagnosis, play a major role in prognosis and survival rate.[10]

In normal cell cycle, the progression of cells to S phase is inhibited by the hypophosphorylated retinoblastoma (RB) protein which is also regulated by cyclin D1. Its growth suppressive activity and inactivation can be achieved by lengthened phosphorylation of the retinoblastoma protein. The release of E2f-like transcription

factors from this protein mediate this inactivation, leading to the stimulation of cyclin-dependent kinases (CDK) and activation of transcriptional target promoter.[11]

$P16^{INK4A}$, a tumour suppressor protein is present on short arm of chromosome 9 and locus 21, It acts by phosphorylating RB protein by inhibiting CDK4 and 6, and therefore $P16^{INK4A}$ can decelerate the cell cycle. Therefore, the overexpression of $P16^{INK4A}$ in proliferating cells can indicate increasing oncogenic alterations.[11]

Study by Lesnikova et al suggests that there exists considerable significant association between the grades of cervical lesions and intensity of $P16^{INK4A}$ immunohistochemical staining pattern[11]. The project by ASCCP (American society for colposcopy and cervical cytology) defines $P16^{INK4A}$ as a strong nuclear or nuclear with cytoplasmic stain. Only cytoplasmic positivity, either patchy or focal should be considered negative.[12] Its expression is not seen in the normal cervical epithelium.[11]

This research study was undertaken to study the expression of $p16^{INK4a}$ by immunohistochemistry (IHC) in histopathologically diagnosed cases of squamous cell carcinoma (SCC) cervix. Role of $P16^{INK4A}$ as a possible biomarker for cervical cancer screening by correlating its expression with histological grading was also studied.

AIMS AND OBJECTIVES

Primary objective:

- To assess the expression of $P16^{INK4A}$ in histologically diagnosed cases of squamous cell carcinoma of cervix.

Secondary objective:

- To compare the histological grading of squamous cell carcinoma of cervix to intensity of $P16^{INK4A}$ expression.

REVIEW OF LITERATURE

Embryogenesis:

Around sixth week of embryogenesis, the cervix is derived from the two paramesonephric ducts[13] (also called Mullerian ducts). As the development progresses, a single urogenital canal is formed by the fusion of these ducts which later go on to become the vagina, cervix and uterus.

The relative size of the cervix decreases with time, as it grows at lesser rate compared to the body of uterus.

The paramesonephric duct gives rise to the columnar epithelium and the urogenital sinus gives rise to the squamous epithelium lining the cervix during its development. The junction between the two epithelia is called the squamo-columnar junction.

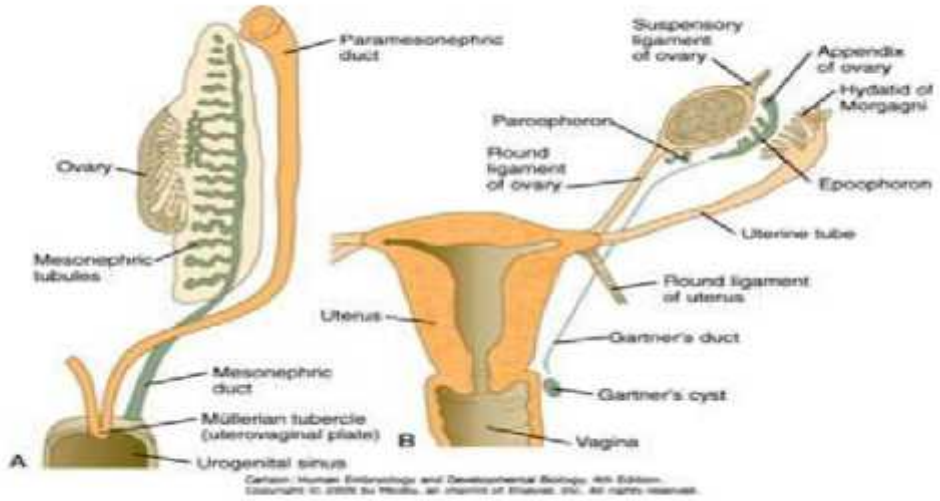


Figure 1: Development of cervix[13]

Anatomy:

Anterior to the rectum and recto-uterine pouch and posterior to the bladder, lies uterus which is thick walled and muscular. Distention of rectum and bladder may cause a change in its position.

Uterus has two parts: Upper two-third which forms the body of the uterus (corpus uteri) and lower one-third which forms uterine cervix (cervix uteri).

In nulliparous adults, anteversion means the cervix tilting forwards relative to the axis of the vagina. If relative to the cervix, the body of the uterus tilts forwards it means anteflexion.[14]

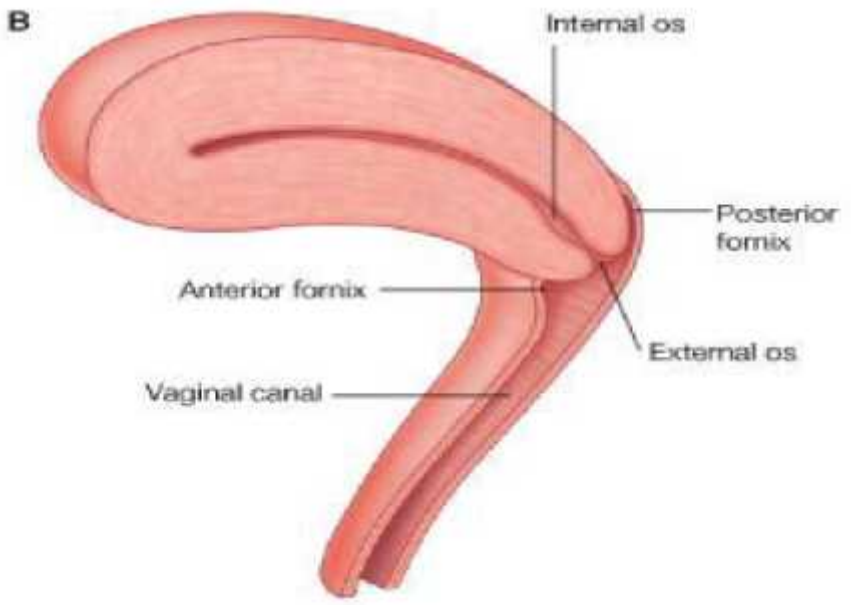


Figure 2: parts of cervix with uterus and vagina[14]

From fundus superior to cervix, inferiorly extends the body which is pear in shape. The bilateral fallopian tubes enter the body of uterus on both sides near its upper end. The round ligament is inferoanterior to each cornua and the ovarian

ligament is inferoposterior. Superior to the entry of fundus lies the dome shaped fundus.

The uterine cavity is flat in the anteroposterior plane and measures 6cms from external os to the fundus. On coronal section, it is narrow below (at internal os) and broad above (joining of fallopian tubes).[14]

The cervix is lower portion of uterus connecting uterus to vagina through endocervical canal.[15] The cervix in a non-pregnant adult women is 2.5 cm long and is cylindrical and more narrower than the body of the uterus.[14] Cervix has two parts one that protrudes into vagina is portio vaginalis and the other that lies above the vaginal vault is supravaginal portion.[15] Anteriorly cellular connective tissue, the parametrium separates the supravaginal part from the bladder , which also passes between layers of broad ligament laterally and to the sides. Posteriorly supravaginal part is related to rectouterine pouch which contains coils of intestine and rectum. On each side supravaginal part of cervix is related to ureter and uterine artery.[16] The spaces between vagina and vaginal part of cervix are called vaginal fornices. The cavity of cervix is called cervical canal and it is fusiform in shape. The cervical canal is connected with the body of uterus via internal os above and the lower end at the external os opens into the vagina.[16] External os is a circular aperture in nulliparous women whereas it becomes a transverse slit during the postpartum period. The cervical canal is flattened from before backwards thus have anterior and posterior walls.[16] Anterior and posterior walls have longitudinal ridges which give small oblique folds that ascend laterally like the tree and its branches. These folds in the walls of cervix interdigitate to close the canal.[14]

Blood supply:

The uterine artery, one of the branches of internal iliac artery reaches supravaginal part of cervix and divides. The smaller vaginal artery supplies lower cervix and main branch supplies upper part of the cervix.[17]

Venous blood is drained into ureteric veins via small plexus in the broad ligament.

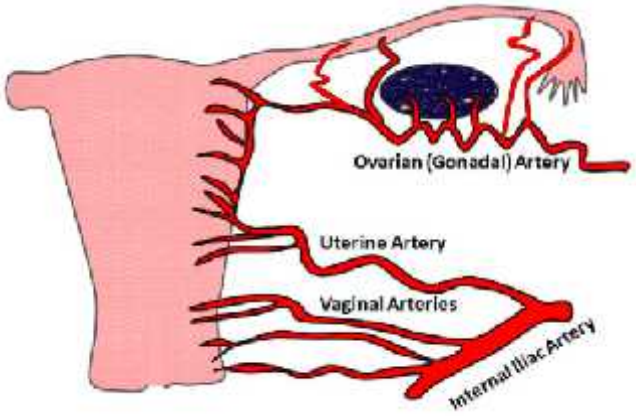


Figure 3: Blood supply of cervix[17]

Lymphatic drainage:

Lymphatic drainage is by multiple groups of lymph nodes.[18] Cervix drains

- Posteriorly to sacral lymph nodes
- Laterally to iliac (external) lymph nodes
- Postero-laterally to iliac (internal) lymph nodes
- Inguinal lymph nodes

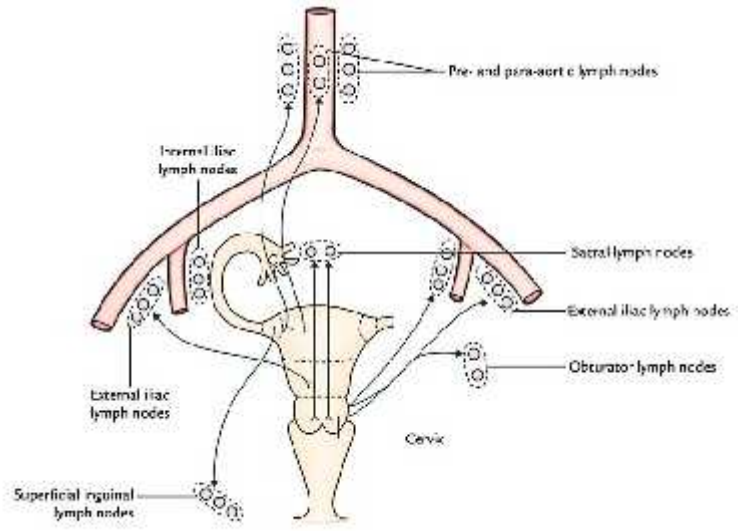


Figure 4: Lymphatic drainage of cervix[18]

Histology:

The uterine cavity opens into vagina by containing the endocervical canal as uterine cervix protrudes into upper vagina. This canal is lined by mucus secreting endovervical epithelium containing a layer of tall columnar ciliated cells.[19]

Stratified squamous epithelium lines the ectocervix. This lining helps in withstanding the hostile vaginal environment. These ectocervical cells have abundant glycogen and so have a clear cytoplasm. At the external os, lies the junction of ectocervix and endocervix which is abrupt and is located near the external os. The main bulk of the cervix is formed by abundant collagenous tissue with compact smooth muscle component.[19]

Squamo-columnar junction or transformation zone is the most common site which undergoes malignant change.[19] At this junction, the stroma is often infiltrated by polymorphonuclear leucocytes which act as defense against the microorganisms.

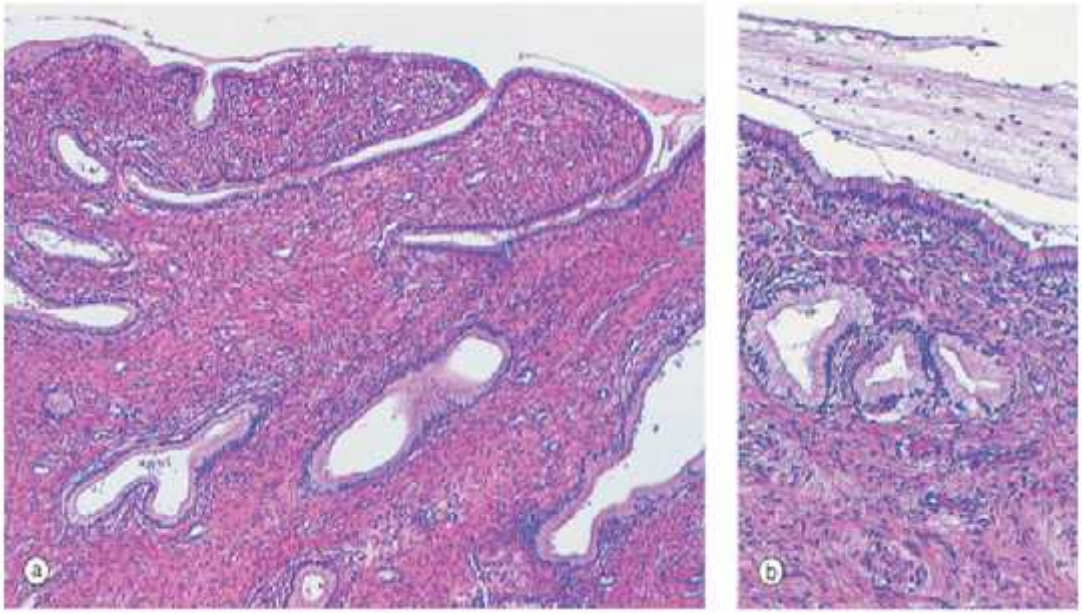


Figure 5: a) showing normal endocervical glands b) fibro-collagenous stroma[19]

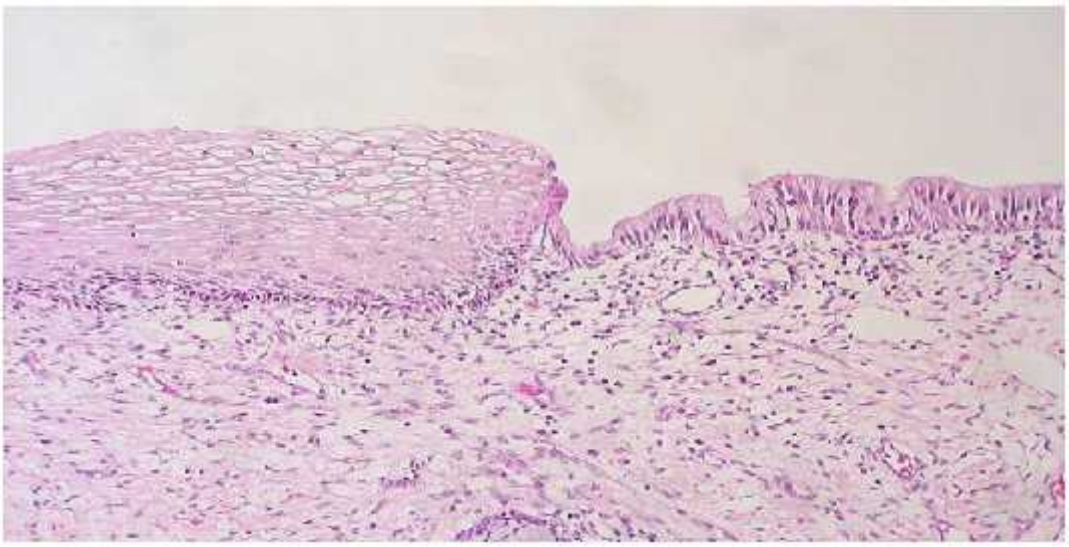


Figure 6: Transformation Zone[19]

Physiology:

The cervix connects the uterus to the vagina and contains glands that produce mucus. During pregnancy and most of the menstrual cycle, cervical glands secrete

thick mucus which prevents entry of sperm into the uterus and also helps in protecting the uterus and bilateral adnexae from infectious pathogenic organisms.[20]

During ovulation, secretions in cervix become thin, thus allowing sperms to pass through it into the uterus.[20]

History:

As early as 400 BC, the Greek physician and scientist Hippocrates identified warts and also wrote about cervical carcinoma.[21]

Distinct types of warts have been established by Aulus Celsus C in 25 AD.[21] They being:

- Skin tag as Acrochordon
- Genital wart as Thymion
- Non-genital wart as Myremecia

One of the ancient Greek physicians Aretaeus, in 2nd or 3rd century BC, had described that uterine cancer most likely presents in two different forms: as an ulcer, which would later be seen infiltrating the uterus or as a growth in the uterus. He distinguished between the two lesions and identified that the symptoms and prognosis of cancers presenting clinically as ulcers were worse.[21,22]

In the middle of 19th century, a surgeon named Rigoni-stern, who had immense interest in epidemiology has studied the death certificates of women who died of cervical carcinoma. He is pioneer in creating awareness about the pathogenesis leading to carcinoma cervix. He observed that uterine cancer was rare in celibate nuns.[22]

In early 20th century, it was observed by epidemiologists that cervical carcinomas is more common in female sex workers and also in women with their spouses having multiple number of sexual partners and it was less common in Jews.[23,24,25]

In precursor cancerous lesions, HPV was initally identified by Zur hausen and his colleagues in the year 1983. HPV DNA was demonstrated in cancer cells of cervix in the year 1985.[22,26] All of these interpretations by leading scientists formed a ground for further research leading to development of vaccinations against these genital infections. Two vaccinations have been approved by FDA in 2006.[22] They are:

- Cervarix- Against HPV 18 and 16.
- Gardasil- Against HPV 6, 11, 18 and 16.
- Gardasil 9- Against nine HPV subtypes

The physiological and cytological features of the female reproductive unit was elucidated by a renowned scientist named George Nicholas Papanicolaou. He is the mastermind behind the creation of Papanicolaou test, commonly called Pap smear test. The book named " Diagnosis of Uterine Cancer by the Vaginal Smear " was published in the year 1943, as a part of his research along with Dr. Herbert T, a gynaeco-pathologist, at the New York hospital.[27]

In 1988, Bethesda system was developed for standardizing the results of screening test with further improvisation in 2001.[22]

Burden of carcinoma cervix in the community:

Cervical cancer becomes second most commonly diagnosed cancer in women with an estimated 5,70,000 cases worldwide and also has become the leading cause of cancer related deaths with about 3,11,000 deaths in 2018.[28]

It becomes a major concern of public health in India, so much so that it holds for about one-fourth of the cervical cancer cases throughout world.[29]

In developed countries, it is estimated to occur in about 1 in 100 women in their entire lifetime as compared to India, where it is almost seen in 1 in 53 women during the entire lifetime.[30]

It is observed that women between 30 to 69 years of age account for 17% of cancer related deaths making it the leading cause of cancer mortality in this age group.

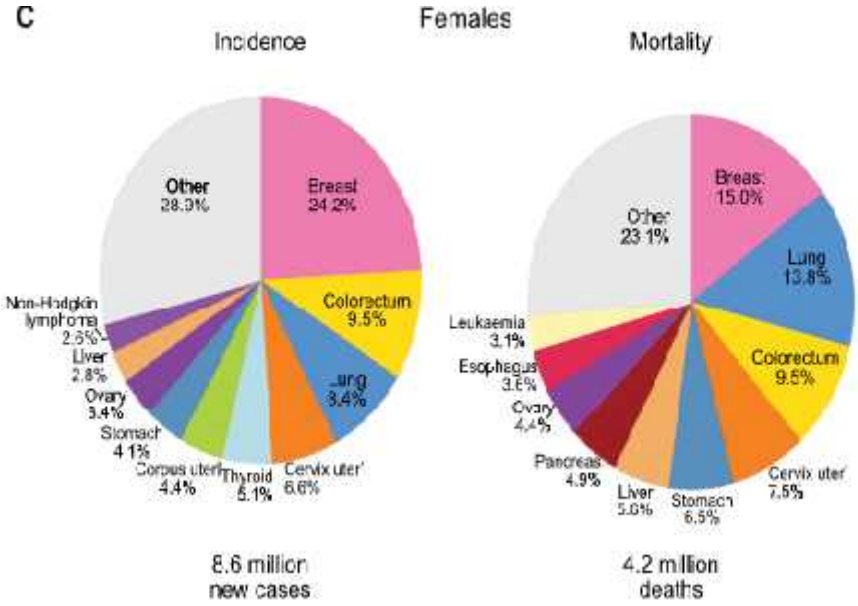


Figure 7: Pie chart depicting both incidence and mortality of carcinoma cervix globally[28]

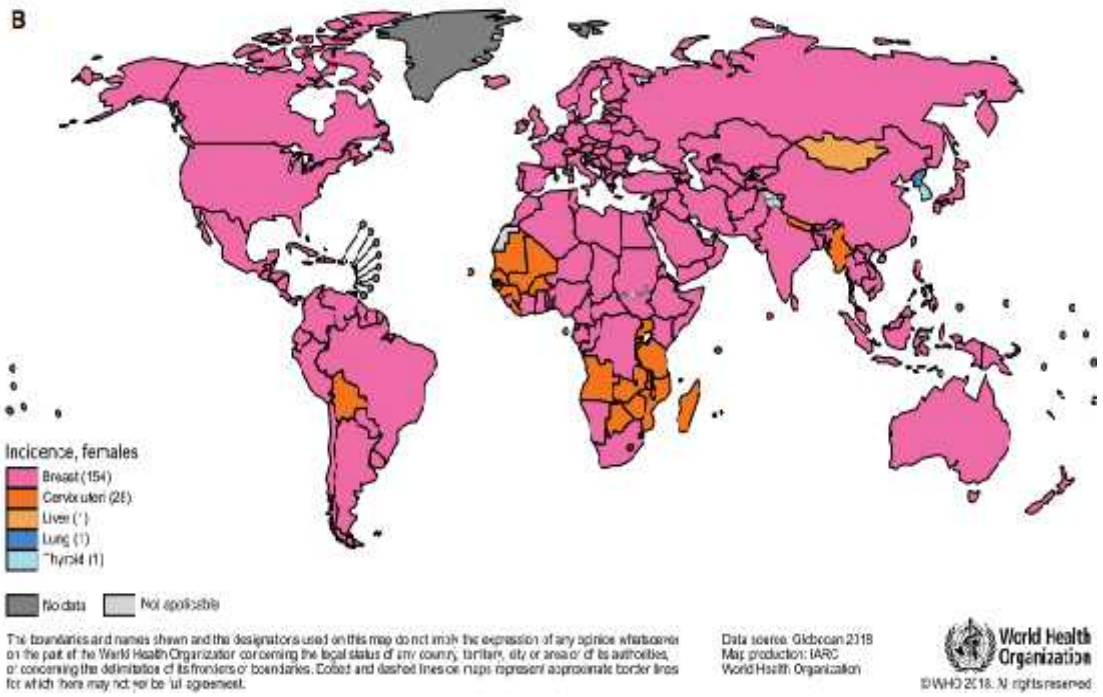


Figure 8: Global map depicting the incidence of most common cancers[28]

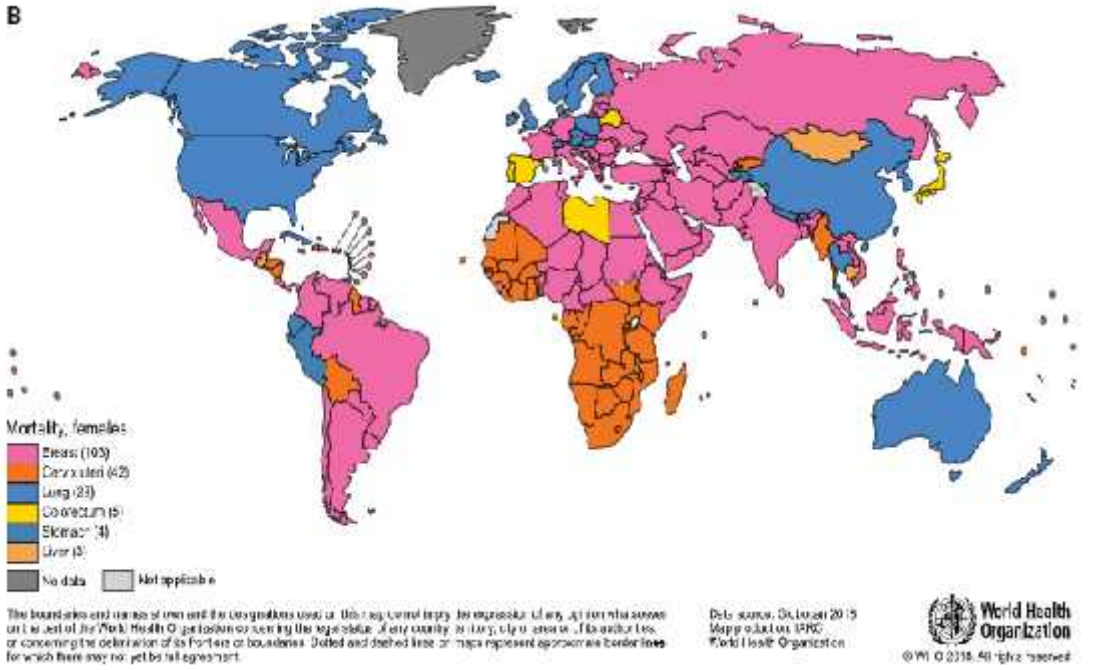


Figure 9: Global map depicting the mortality rates of most common cancers[28]

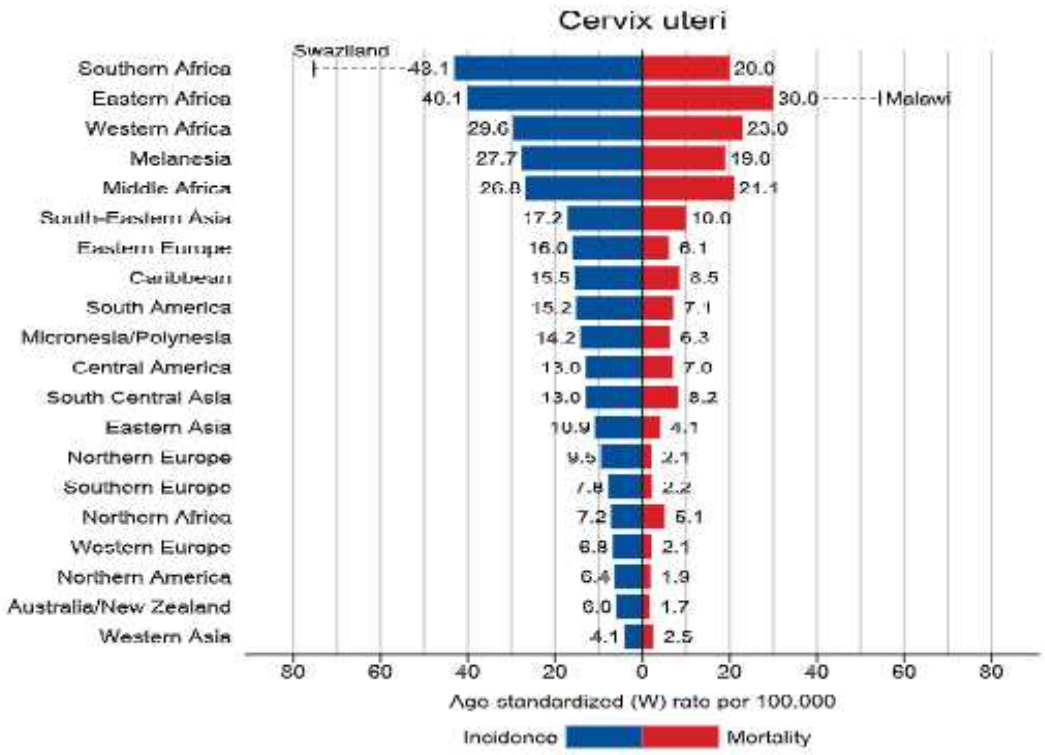


Figure 10: Region specific mortality and incidence rates[28]

The above chart depicts that there are higher incidence rates in Africa and South east Asia.[28]

In India, according to the recent National Cancer Registry Program (NCRP), cervical cancer contributes to about 6-29% of all cancers in females.[31] Papumpare District (30.2), Aizawl District (28.0), Mizoram state (23.1) and Pasighat PBCR (22.5) occupied the top four places in a population-based cancer registry in a three year report from 2012-2014.[32]

The incidence rate which is age adjusted of cervical carcinomas was found to vary widely among registries.[32]

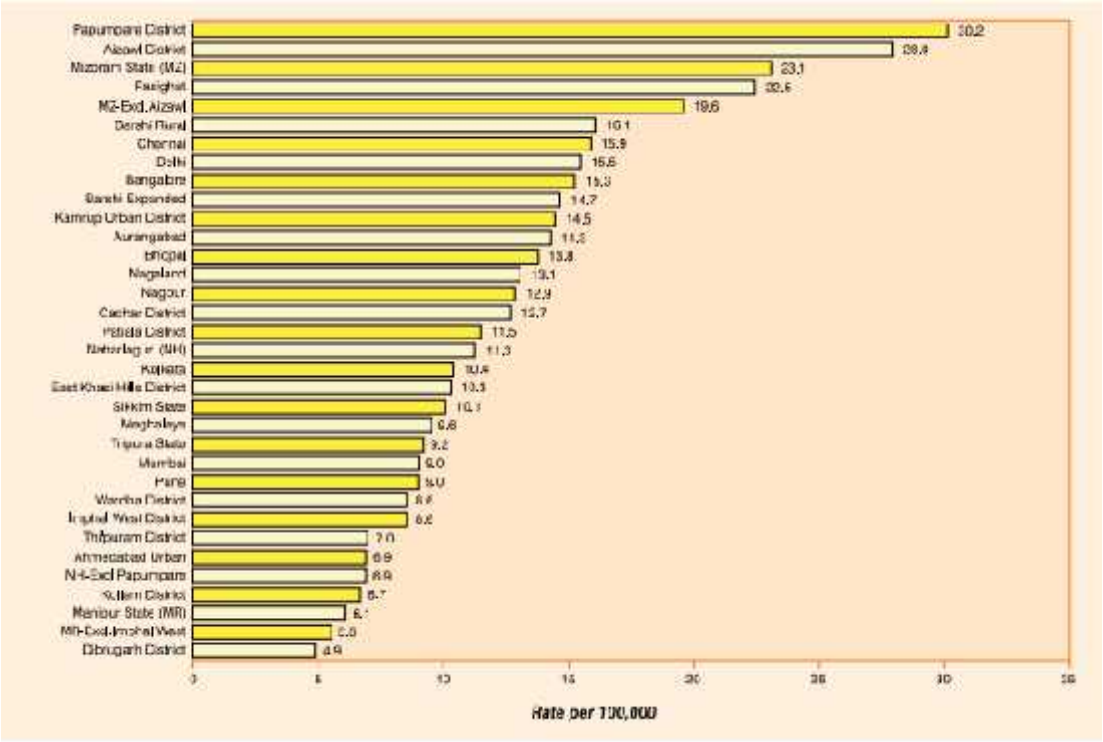


Figure 11: Age adjusted incidence rates by NCRP[32]

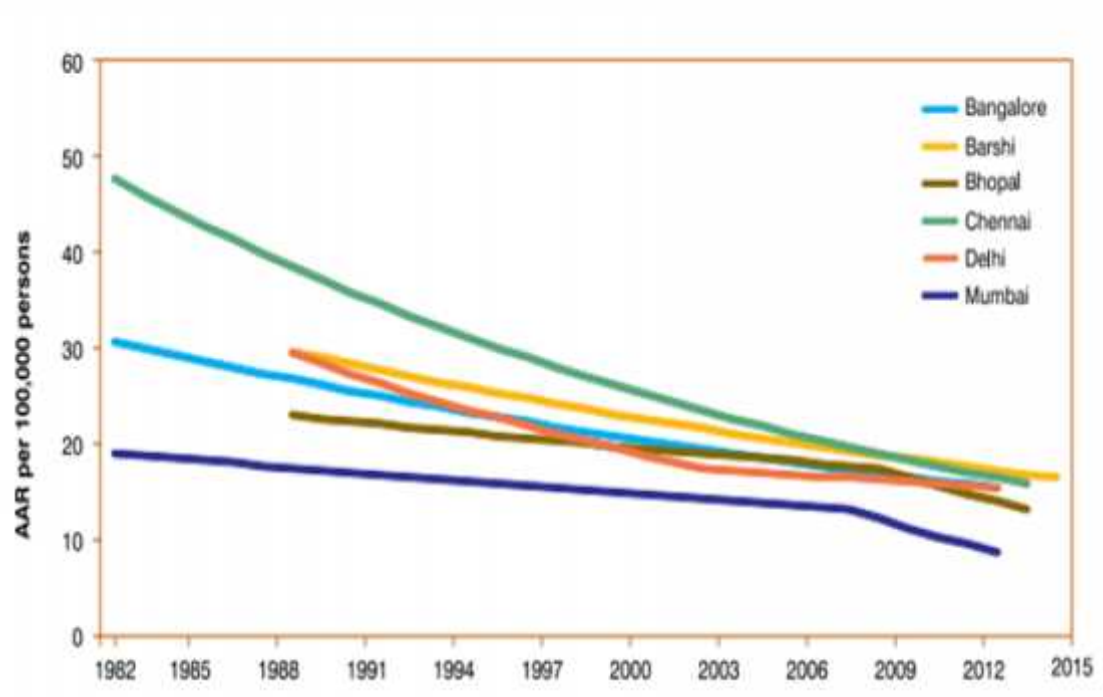


Figure 12: Trend of cervical cancer over time in India[33]

Though the above graph shows a decreasing trend for the incidence rates over a period of time for cancer cervix, it still continues to be the leading cause of mortality in Indian women. This is because of the ineffective screening programs for detection of precancerous lesions in the developing countries like India.[34]

There are two prerequisites for screening programs to be effective. Firstly, screening must be done much advance to the time of diagnosis of cancers. And secondly, early treatment should have some advantage over treatment at the time of clinical presentation.[35,36]

WHO ideally recommends the screening of women between the age group 30 to 49 years every 3 to 5 years either by VIA (visual inspection with 3-5% acetic acid) or by Pap smear tests, or every 5 years through HPV testing.[37]

Etiology:

The cervical cancer has a pre-invasive state which is long and many cytological screening tests are currently being used which help in effective treatment of pre-invasive lesions and therefore carcinoma cervix is considered a preventable disease.[38]

Cancer of uterine cervix arises mostly at the junction between and the squamous epithelium lining the ectocervix and columnar epithelium lining the endocervix as this site undergoes metaplastic change continuously.[39]

Various risk factors in carcinogenesis of cervix are as under:

- Age – two peaks have been observed, one at 35 to 39 years and another at 55 to 60 years.[38,34]

- Race
- Low socioeconomic status
- More than one sexual partner
- First early intercourse (< 16yrs of age)
- A spouse having more than one sexual partner or whose first wife died of cancer cervix
- Increased parity
- Persistent high-risk HPV infection
- Immunocompromised
- Few HLA subtypes
- Excessive use of oral contraceptives or high estrogenic states
- Cigarette smoking

Classification:

Cervical intraepithelial Neoplasia: These are the pre-invasive counter parts of the invasive cervical squamous cell carcinoma. They have significant amount of malignant potential but they may also regress or remain stable or progress.[40]

Various systems have been established for classifying premalignant cervical findings either cytologic or histologic. The Bethesda system divides it into two groups[39]:

- HSIL-High- grade squamous intraepithelial lesions
- LSIL- Low- grade squamous intraepithelial lesions

LSIL: It represents the morphological manifestation of production of HPV on the host squamous cells. Low-grade signifies low risk of developing into invasive cancer. These are usually related to low-risk HPV subtypes.[39]

Histopathology shows about one-third of the epithelium showing proliferation of parabasal or basal cells. Mitotic activity is confined to this zone and it could be normal. The upper two-third of the epithelial cells differentiate and gain cytoplasm, however enlarged hyperchromatic nuclei are seen. There is development of a well-defined halo around the nucleus. These changes together are termed as koilocytotic atypia or HPV cytopathic effect.[41]

HSIL: It represents the cervical intraepithelial lesion that has serious risk of progressing into invasive cancerous lesion. They are usually related to high-risk HPV subtypes.[39]

Histopathology shows proliferation of squamous cells mostly near the squamocolumnar junction. The proliferation is seen extending up to the entire middle third. These cells have atypical nuclear features like irregular nuclear membrane, increased size, and nucleo-cytoplasmic ratio is increased. Mitotic figures are increased which are abnormal.[41] The lower layers or layers near the epithelial surface exhibit foci of mucinous differentiation with scattered mucin secreting cells

Cervical intraepithelial neoplasia is divided into three categories:

CIN 1 or Mild dysplasia: The epithelium's lower one third area in these lesions shows presence of neoplastic basaloid cells and mitotic figures. Irregularities of nuclear membrane, multinucleation, perinuclear halos, and hyperchromatic nucleus are the HPV related cytopathic effects that are frequently seen in these lesions.[42] It is also called flat condyloma.

CIN 2 or Moderate dysplasia: The epithelium's lower two third area in these lesions shows presence of neoplastic basaloid cells and mitotic figures.

CIN 3 or Severe dysplasia: The entire epithelium in these lesions shows presence of neoplastic basaloid cells and mitotic figures. These cells have irregular nuclear outlines with enlarged dense, clumped chromatin and hyperchromatic nucleus with very scant cytoplasm.[42]

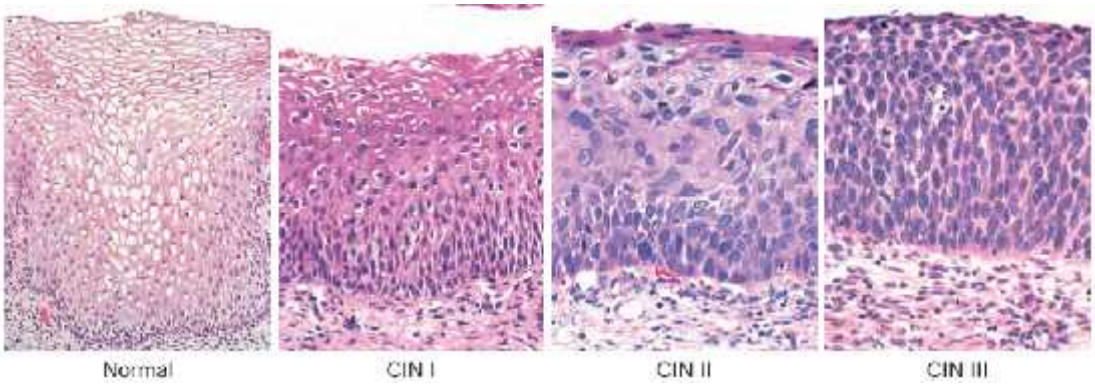


Figure 13: Cervical dysplasia[43]

Table 1: WHO classification of cervical carcinoma[44]

WHO CLASSIFICATION			
Epithelial Tumours			
Squamous cell			
	Intraepithelial lesions		
		HSIL	
		LSIL	
	Carcinomatous Lesions		
		Papillary	
		Keratinizing	
		Basaloid	
		Non keratinizing	
		Verrucous	
		Lymphoepithelioma like	
		Warty	
		Squamotransitional	
	Benign lesions		
		Metaplasia	
		Papilloma	
		Condyloma acuminatum	
		Transitional metaplasia	
Glandular tumours			
	Adenocarcinoma-in-situ		
	Adenocarcinoma		
		Endocervical	
		Mucinous	
			Intestinal type
			Gastric type
			Clear cell
			Mesonephric
			Endometrioid
			Serous
			Signet ring cell type
			Villoglandular
			Adeno with neuroendocrine
	Benign		
		Endocervical polyp	
		Nabothian cyst	

		Mullerian papilloma	
		Microglandular hyperplasia	
		Tunnel clusters	
		Ectopic prostate	
		Lobular laminar endocervical hyperplasia	
		Tuboendometriod metaplasia	
		Mesonephric hyperplasia	
		Endometriosis	
		Endocervicosis	
		Diffuse laminar endocervical hyperplasia	
		Arias stella reaction	
Other Epithelial tumours			
	Adenoid basal carcinoma		
	Adenosquamous		
	Adenoid cystic carcinoma		
	Glassy cell carcinoma		
	Neuroendocrine		
		High grade	
			Large cell
			Small cell
		Low grade	
			Atypical carcinoid
			Carcinoid
	Undifferentiated		
Mesenchymal tumours			
	Benign		
		Rhabdomyoma	
		Leiomyomas	
	Malignant		
		Rhabdomyosarcoma	
		Leiomyosarcoma	
		Angiosarcoma	
		Malignant peripheral nerve sheath tumour	
		Alveolar soft part	

		sarcoma	
		Other sarcomas	
			Undifferentiated endocervical
			Liposarcoma
			Ewings sarcoma
	Tumour-like		
		Lymphoma like lesion	
		Post-operative spindle cell nodule	
Mixed mesenchymal and epithelial tumours			
	Adenosarcoma		
	Malignant Mullerian mixed tumour		
	Adenomyoma		
Melanocytic tumours			
	Blue nevus		
	Malignant melanoma		
Germ cell tumours			
	Yolk sac tumour		
Haematologic tumours			
	Lymphoid and myeloid neoplasms		

The major histological type (around 80%) are squamous cell carcinomas, followed by adenocarcinomas which constitutes about 10-12% of cervical cancer cases.[10]

SCC: It is a tumor comprising of neoplastic squamous cells showing varied differentiation. They differ in types of cells, their differentiation and pattern of their arrangement.

The common type are non-keratinizing tumors associated with high risk HPV.[41,42] Invasive SCC of cervix has three pathological variants. They are:

- Large cell Keratinizing carcinoma
- Small-cell carcinoma
- Large-cell non keratinizing carcinoma

But, histopathological tumor grade (poorly, well or moderately differentiated) and type do not have much importance in predicting patient's outcome as compared to the depth of invasion and lympho-vascular tumor embolization.[42]

Cervical SCC is divided by Wentz and Reagan in 1958 into three types:

- Large cell non-keratinizing
- Small cell carcinoma
- Large cell keratinizing .[45,46]

Non-Keratinizing tumors: Polygonal squamous cells with intercellular bridges are arranged in sheets or nests. No any keratin pearl formation is seen. Numerous mitotic figures with nuclear pleomorphism is evident. The cells show course granular chromatin with large nuclei and prominent nucleoli.[45,46]

Keratinizing tumors: Cells with abundant keratohyaline granules and dense cytoplasmic keratinization are seen. keratin pearl formation may be present. They may be of any grade. The chromatin is coarse with enlarged hyperchromatic nuclei with or without prominent nucleoli.[45,46]

Small cell carcinoma: Uniform, monomorphic, small basophilic cells with increased nuclear-cellular ratio. The cell or nuclear size, both are uniform, the nuclei are coarsely granular. Isolated keratinized cells or keratin pearl formation is not seen. The mitotic activity is high.[45,46]

Small cell carcinomas are now known as heterogenous group of tumours with the help of immunohistochemistry and electron microscopy. These include:

- Small cell squamous carcinoma
- Small cell neuroendocrine carcinoma
- Small cell anaplastic carcinoma

By demonstrating neuroendocrine differentiation by IHC markers like synaptophysin and chromogranin, newer classification has extended the neuroendocrine tumours including even poorly differentiated large cell carcinomas.[47]

Modified Broder's grade: Based on the amount of keratin, nuclear pleomorphism, and the mitotic activity, histological grading was given.[38,40]

- **Well differentiated tumours:** These tumours show presence of mature squamous cells with abundant keratinisation with pearl formation, few cells with intercellular bridges with mild pleomorphism and less mitotic activity.
- **Moderately differentiated tumours:** These tumours show neoplastic cells with less distinct cell borders and cytoplasm compared to well differentiated tumours but increased nuclear pleomorphism and increase in mitotic activity
- **Poorly differentiated tumours:** These tumours show small primitive cells with scant cytoplasm, enlarged hyperchromatic nucleus and increased mitotic activity. Keratinisation is occasional or absent. It simulates HSIL

Revised 2018 **FIGO** staging of Carcinoma of cervix :[48]

Stage	Description
I	The carcinoma is strictly confined to the cervix (extension to the uterine corpus should be disregarded)
IA	Invasive carcinoma that can be diagnosed only by microscopy, with maximum depth of invasion <5mm[a]
IA1	Measured stromal invasion <3mm in depth
IA2	Measured stromal invasion ≥3mm and <5mm in depth
IB	Invasive carcinoma with measured deepest invasion ≥5 mm (greater than Stage IA), lesion limited to the cervix uteri[b]
IB1	Invasive carcinoma ≥ 5mm depth of stromal invasion, and < 2cm in greatest dimension
IB2	Invasive carcinoma ≥ 2cm and < 4cm in greatest dimension
IB3	Invasive carcinoma ≥ 4cm in greatest dimension
II	The carcinoma invades beyond the uterus, but has not extended onto the lower third of the vagina or to the pelvic wall
IIA	Involvement limited to the upper two-thirds of the vagina without parametrial involvement
IIA1	Invasive carcinoma < 4cm in greatest dimension
IIA2	Invasive carcinoma ≥ 4cm in greatest dimension
IIB	With parametrial involvement but not to the pelvic wall
III	The carcinoma involves the lower third of the vagina and/or extends to the pelvic wall and/or causes hydronephrosis or nonfunctioning kidney and/or involves pelvic and/or para-aortic lymph nodes[c]
IIIA	The carcinoma involves the lower third of the vagina, with no extension to the pelvic wall
IIIB	Extension to the pelvic wall and/or hydronephrosis or nonfunctioning kidney (unless known to be due to another cause)
IIIC	Involvement of pelvic and/or para-aortic lymph nodes, irrespective of tumor size and extent (with r and p notations)[c]
IIIC1	Pelvic lymph node metastasis only
IIIC2	Para-aortic lymph node metastasis
IV	The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. (A bullous edema, as such, does not permit a case to be allotted to Stage IV)
IVA	Spread to adjacent pelvic organs
IVB	Spread to distant organs

The association between cervical premalignant and malignant epithelial neoplasms has been well established.[49]

The major risk factor or causative organism for cervical neoplastic lesions or invasive tumors is known to be Human papilloma viruses (HPV) infections. More than 200 HPV sub types have been recognised[50] and are subdivided into two categories based on their association with invasive cervical carcinoma as high or low-risk.[51]

The most common low-risk types are HPV 6, 11 and high risk types are HPV 45, 16, 31, and 18 which are known to be causative organisms in about 90% of cervical carcinoma cases.[49,52] Cervical intraepithelial lesions and carcinomas either squamous or adenocarcinomas are known to be caused by subtypes HPV 16 and 18.[53]

Most of the HPV infections are known to cause LSIL, of which about 90% heal spontaneously and only about 10% transform to infections causing several molecular changes.[54]

Many studies have indicated the importance of immune responses in HPV infections. The protein necessary for completion of viral cell cycle is HPV L1 capsid protein. This protein is expressed in the active phase of the viral infection. Hence, the immunochemical expression of the capsid protein gives the clue of active infection in the examined tissue.[55] And so, this capsid protein is the target for cellular immune response.[56]

$P16^{INK4A}$:

$P16^{INK4A}$, a tumour suppressor protein, is located on short arm of chromosome 9 and locus 21. It also codes for other tumor suppressor genes like p14, p15and p19.[57,58] Many animal studies have shown that P14 plays an important role in tumor genesis, however, $P16^{ink4a}$ and its role in carcinogenesis in humans is well recognized.

The cell cycle normally is a coordinated sequence of events which result in DNA replication and cell duplication, as a response to mitogenic stimuli. The human cell cycle is divided into four phases:

- Growth phase (G1)
- DNA synthesis phase (S)
- Growth phase (G2)
- Mitotic phase (M).

A coordinated cellular response may be due to wide variety of mitogenic and quiescence-inducing stimuli.[59] The cell cycle is regulated through activating and inactivating phosphorylation by cyclins, cyclin dependent kinases (CDKs) and their inhibitors (CDKIs).[60] Cell differentiation and proliferation occurs in the G1 phase of cell cycle as a result of the major regulatory events.

In the middle of G1 phase, cyclin D which is at its maximum level of expression, combines with CDK4 or CDK6 forming functional kinases whereas near the G1-S boundary, cyclin-E is associated and expressed with CDK2 in an active complex.[60,61,62]

Cell cycle is slowed down by preventing advancement from G1 to S phase by a cyclin-dependent kinase inhibitor (CDKI) $P16^{ink4a}$.

Active CDK complexes are controlled as and when they start binding to CDKI ($p16^{INK4A}$, $p21^{waf1}$ and $p27^{KIP1}$) as they act by inhibition of progression of cell cycle. The two oncoproteins E7 and E6 impairs the function of these proteins in carcinoma cervix associated with HPV.[60,63,64]

Normally, retinoblastoma protein (RB) is phosphorylated by the formation of an active protein complex when CDK4 or CDK6 binds to cyclin D. Once this protein is phosphorylated, transcription factor E2F1 dissociates from it. Thus, E2F1 enters the nucleus after it is freed from its bound state in cytoplasm. E2F1 promotes the target genes transcription required for transition from G1 to S phase after entering the nucleus.[65,66]

Cell is protected from various stress factors and various hyperproliferative signals by a growth arrest mechanism called as cellular senescence.[67] This mechanism is activated in response to stress stimuli like oxidative stress DNA damage, and exposure to drugs (premature senescence). It is also activated while ageing (replicative senescence).[68] However, non-senescent cells also undergo same mechanisms which involve the p16ink4a/rb pathway for senescence and cell cycle arrest.[69]

The usefulness of this tumour suppressor protein in senescence and ageing is demonstrated by the expression of $P16^{ink4a}$ in mouse tissues, human skin and kidney tissues. Its expression markedly increases with age.[70]

$P16^{Ink4a}$ protein also plays an important role in various other processes like angiogenesis, apoptosis and cell invasion.

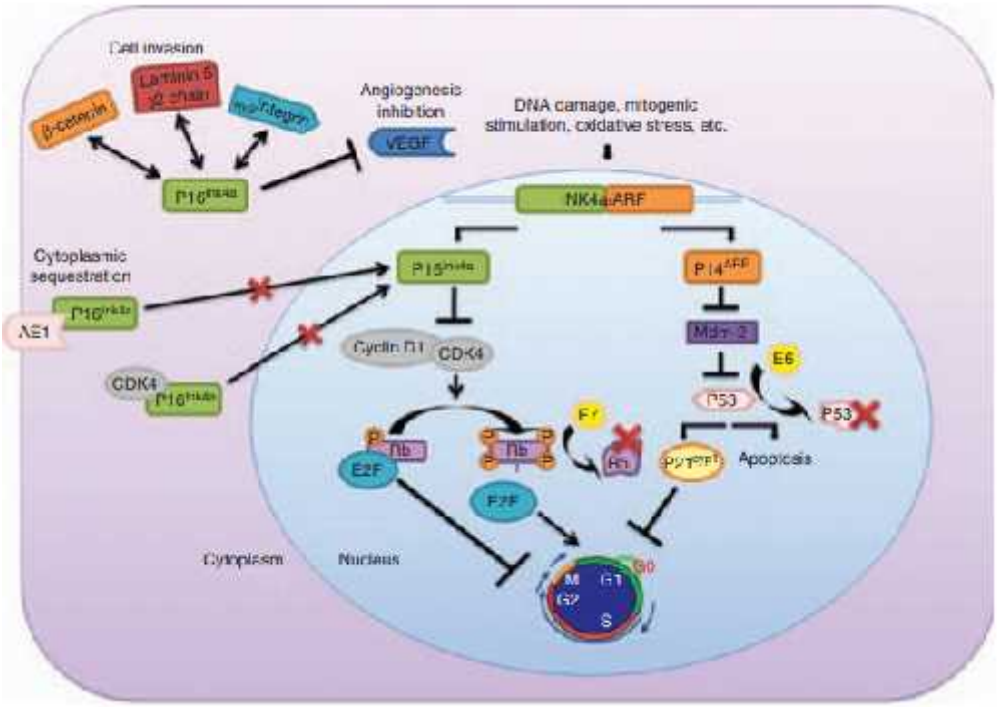


Figure 15: Depicts $P16^{INK4A}$, a cell cycle inhibitor[71]

$P16^{INK4A}$ molecular pathways in different aspects is explained in the above figure. They are:

- Phosphorylation of retinoblastoma (Rb) is blocked as INK4 family binds and inactivates CDK4/6 thus inducing arrest of cell cycle.
- The presence of viral oncoproteins E7 and E6 explain the molecular mechanism involved in overexpression of $P16^{Ink4a}$ in HPV-related neoplasms. The interaction between high-risk HPV oncoprotein E7 and E6 inactivates the retinoblastoma protein which bring about the degradation of the tumor suppressor gene p53.
- Sequestration by proteins like CDK4 is associated with $p16^{Ink4a}$ overexpression in cytoplasm of cells.
- Proteins like beta-catenin, G2 chain of laminin 5 or vascular endothelial growth factor (VEGF) interact with $P16^{Ink4a}$ protein and help in inhibition of invasion and angiogenesis of the cell.[56]

Studies by Natarajan et al, Jung et al, Horree et al, Svensson et al observed that there is an overexpression of p16 in cases of basal cell carcinoma, colorectal and endometrial, carcinoma.[72,73,74,75] Overexpression of $p16^{ink4a}$ in tumor cells is also been associated with many other molecules that are in association with invasive status which supports the hypothesis of relationship between invasion and $p16^{ink4a}$.[72,73,76] Also, few of the in-vitro studies have recognised that expression of $p16^{ink4a}$ is also implicated for the regulating cell migration which is matrix dependant[77]. P16 is also implicated for glioma invasion[78] and also breast cancer migration inhibition[79].

One study by Harada et al reveals association of $p16^{ink4a}$ in angiogenesis and apoptosis. It revealed that the restoration of $p16^{Ink4a}$ results in inhibition of

angiogenesis in case of malignant gliomas and in downregulation of VEGF (vascular endothelial growth factor) in cell lines.[80]

Also, P16 promotes differentiation of erythroid series of cells by regulating bcl-x functions and hence plays a role in haematopoiesis.[81]

Many normal human tissues including gastric antral cells, proliferative endometrium, breast ducts, thymus, esophageal squamous epithelium, some neuroendocrine cells and salivary glands also express P16.[82]

While evaluating cervical squamous and glandular lesions, several studies observed $P16^{INK4a}$ overexpression could be a useful marker in predicting prognosis. In order to differentiate between immature squamous metaplasia and cervical intraepithelial lesions, immunochemical staining of cervical punch biopsies with $P16^{INK4a}$ has been observed to considerably improve the decision-making process. Immature squamous metaplasia is observed to be $p16^{INK4a}$ negative, whereas high-grade CIN lesions have shown a diffuse and strong $P16^{INK4a}$ immunostaining.[83,84] Normal cervical epithelium is also reported to be P16 negative.

Study by Gupta et al revealed that cervical cancers caused by HPV showing $p16^{INK4a}$ overexpression are radio-sensitive with better prognosis than those not related to HPV. So, response to treatment and expected survival rate in patients of cervical cancer are likely to be impacted by $P16^{ink4a}$ overexpression.[85]

This research work was undertaken to study the expression of $p16^{INK4a}$ by immunohistochemistry (IHC) in histopathologically diagnosed cases of squamous cell carcinoma (SCC) of cervix. Role of $P16^{ink4a}$ as a potential biomarker by correlating expression of P16 with histological grades is also assessed.

METHODOLOGY

The study was carried out at the Department of Pathology, KAHER's Jawaharlal Nehru Medical College, and Dr. Prabhakar Kore charitable Hospital and medical research centre, Belagavi. Patient identities were concealed and approval from the KAHER University's Institutional Research Ethical Committee was obtained prior to conducting this study (ref no. MDC/DOME/35)

Study Design: Cross Sectional study

Study Period: January 2018 **–** December 2019

- One-year prospective 2019
- One-year retrospective 2018

Study population: Old (2018) and new (2019) specimens of cervical biopsy received in the histopathology laboratory (diagnosed as squamous cell carcinoma of cervix on histology) at Dept of Pathology, JNMC, Belagavi.

Inclusion criteria: Histopathologically diagnosed cases of SCC

Exclusion criteria:

- Metastatic lesions of cervix
- Inflammatory lesions of cervix
- Adenocarcinoma of cervix
- Cervical intraepithelial neoplasia (Dysplastic Lesions)

Sample Size: 35 (Universal sample)

Data Collection: For the retrospective cases, requisitions were retrieved from the records of the Department of Pathology, JNMC. Detailed history of the prospective cases was taken. Total of 35 paraffin embedded tissue blocks were collected.

Requisitions collected for the retrospective cases were used to collect patient's age and clinical complains, while paraffin embedded blocks of all cases were subjected for processing in the KLE's basic research laboratory.

Sampling Procedure:

35 Formalin fixed paraffin embedded blocks which were diagnosed as squamous cell carcinoma of cervix was collected from the departmental records.

Two ribbons from each block of 3micron thick are obtained using rotatory microtome (Leica RM2245).

Each ribbon is floated on tissue floatation bath maintained at 50-55-degree Celsius.

The first ribbon was taken on regular glass slide coated with Egg albumin as adhesive which was stained with Hematoxylin and Eosin. The second ribbon was taken on commercially available Poly-L-lysine coated glass slides for immunohistochemistry with $P16^{ink4a}$.

After staining with Haematoxylin and Eosin, the slides were then studied under light microscopy for histopathological features and were graded according to Modified Broder's Grade into poorly differentiated, moderately differentiated or well differentiated.

After the immunohistochemical staining, the slides were evaluated for the brown reaction product under light microscope.

Immunohistochemical Analysis:

Only nuclear or nuclear with cytoplasmic staining was considered positive. Only cytoplasmic, either patchy or focal staining was considered negative.[11] Scoring and grading of P16 was done as under:[11]

Grading of intensity of P16 stain:

Staining pattern	Grade
Mild	1+
Moderate	2+
Severe	3+

Scoring of percentage positive tumour cells:

Percentage of tumour cells showing positivity	Grade
0%	0
0-5%	1
5-25%	2
>25%	3

Immunohistochemistry score (P16 score) was obtained as product of percentage positive tumor cells and intensity of the staining. Thus, maximum will be taken as 9.

Analysis plan:

Data collected was analysed using appropriate statistical test. The Statistical softwares namely MedCalc 19.4.1 and Microsoft excel Analysis Toolpak were used for the analysis of the data and to generate tables and graphs.

The frequency of expression of P16 in carcinoma cervix was analysed as

Significance	P-value
Suggestive	$0.05 < P < 0.10$
Moderately	$0.01 < P < 0.05$
Strongly	$P < 0.01$

RESULTS

In the present study conducted in the Department of pathology, Jawaharlal Nehru Medical College, Belagavi, 35 cases of cervical biopsy specimens which were histopathologically diagnosed as SCC cervix during time period January 2018 to December 2019 were evaluated. All the cases were evaluated for $P16^{ink4a}$ expression.

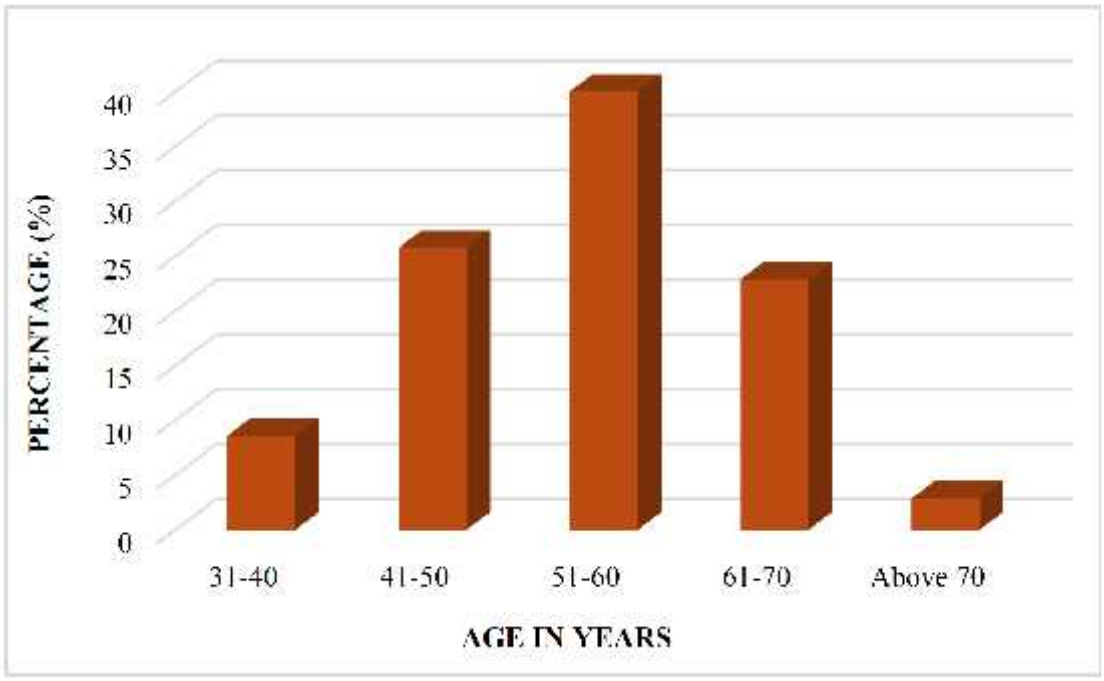
In the present study, all the 35 cases were punch biopsy specimens.

Table 2: Age distribution

Age	No. of Cases	%
31-40	3	9%
41-50	9	26%
51-60	14	40%
61-70	8	23%
Above 70	1	3%
Total	35	100%

In the present study, age of the patients ranged between 35-72 years youngest being 35 years and oldest being 72 years. The mean age of patients was about 54.17 ± 9.1years. The peak incidence was seen between 51-60 years of age. (Table 2, Graph 1).

Graph 1: Age Distribution

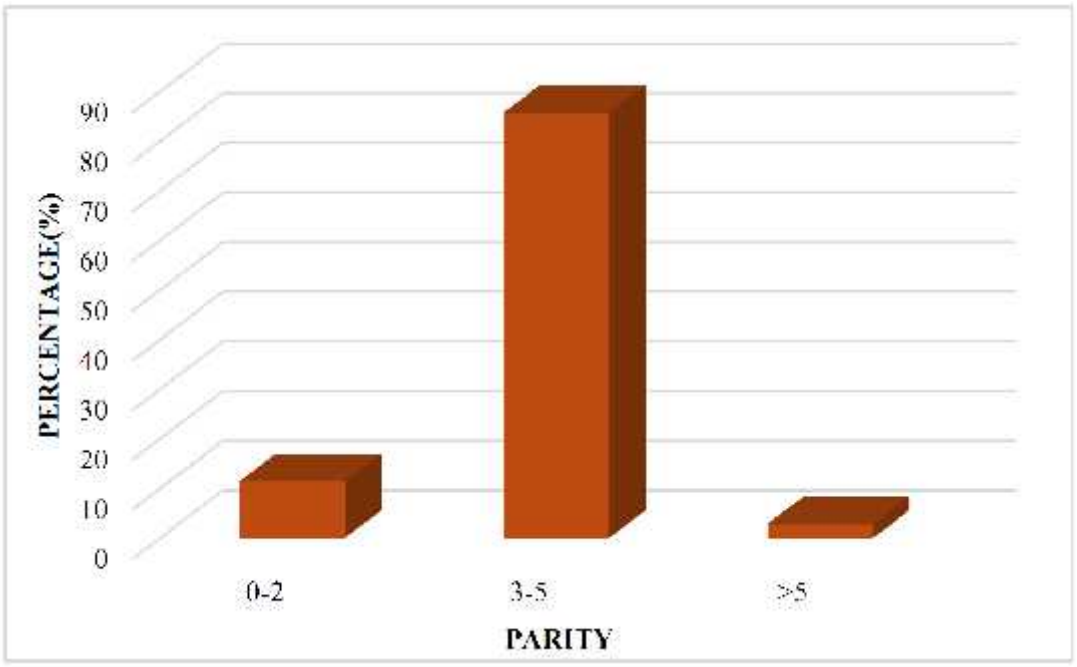


In the present study, all the patients were married. Of all 35 cases, 4 patients (11%) were of parity 0 to 2, 30 patients (86%) were of parity 3 to 5 and 1patient (3%) was of parity more than 5. Maximum number of cases had parity of 3 to 5 (80%). (Table 3, Graph 2).

Table 3: Parity distribution

Parity	No of Cases	%
0-2	4	11%
3-5	30	86%
>5	1	3%
Total	35	100%

Graph 2: Parity distribution



In the present study, premenopausal women were 6 (17%) and postmenopausal were 29 (83%). It is observed that cervical cancer is most commonly seen in postmenopausal women. (Table 4, Graph 3)

Table 4: Menopausal status

Menopausal status	No of Cases	%
Pre-menopausal	6	17%
Post-menopausal	29	83%
Total	35	100%

Graph 3: Menopausal distribution

In the present study, out of 35 cases of carcinoma cervix, about 30 cases (64%) presented with bleeding per vagina (BPV), 6 (13%) cases presented with white discharge per vagina (WDPV), 5 cases (11%) with cervical growth, 3 cases(6%) with pain in the abdomen, 2 (4%) cases with back pain and 1(2%) case with abdominal growth.

The most common clinical presentation in post-menopausal women is bleeding per vagina. (Table 5)

Of the 5 cases who presented with cervical growth, 4 cases (80%) had presented with a cauliflower like growth and one case (20%) had an ulcerative lesion.

Table 5: Distribution of clinical symptoms

Clinical presentation	Premenopausal cases	Postmenopausal cases	%
BPV	6	24	64%
WDPV	0	6	13%
Cervical growth	0	5	11%
Pain abdomen	1	2	6%
Back pain	1	1	4%
Abdominal mass	1	0	2%
Total			100%

Family and past history:

All the cases did not have any significant past history.

Histopathological examination:

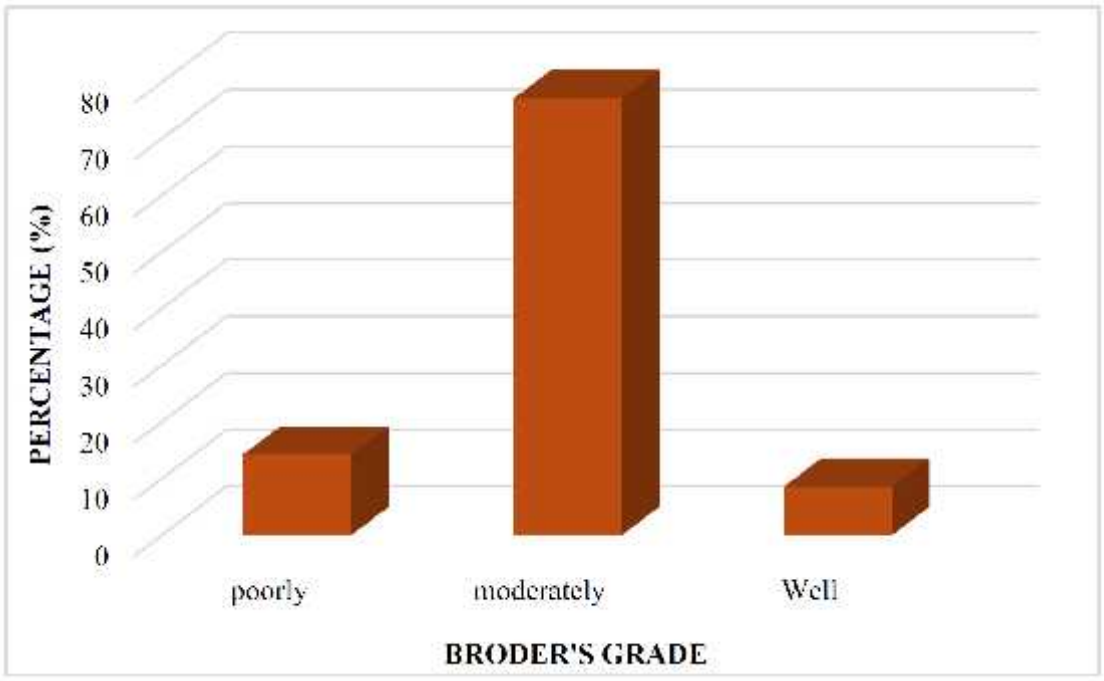
The SCC of cervix were typed according to WHO classification system, the modified Broder's grading was given and histological subtypes according to Wentz and Reagan.[39,40]

Among the 35 cases of SSC carcinoma of cervix studied, 3 cases (9%) were well differentiated, 27 cases (77%) were moderately differentiated and 5 cases (14%) were poorly differentiated carcinoma. (Table 6, Graph 4)

Table 6: Distribution of SCC according to Broder's grade

Grade	No of Cases	%
Well	3	9%
Moderately	27	77%
Poorly	5	14%
Total	35	100%

Graph 4: Distribution of SCC according to Broder's grade

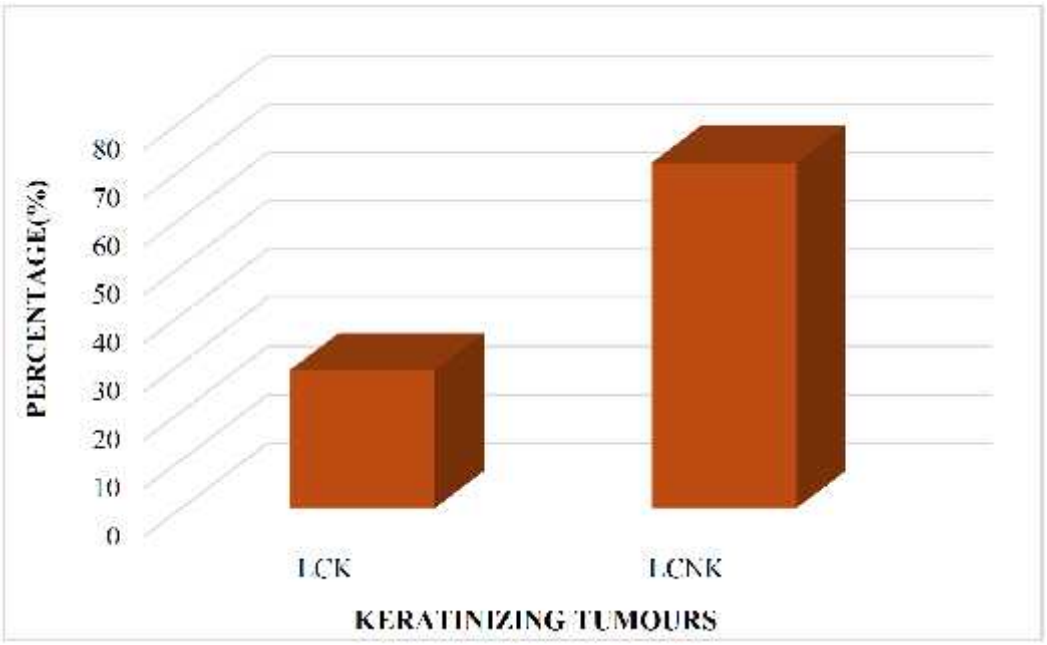


The cases of SCC were further subtyped into large cell non-keratinizing (LCNK), small cell type and large cell keratinizing (LCK). Out of 35 cases, 25 cases (71%) were LCNK and 10 cases (29%) were LCK. No case of small cell type was found. (Table 7, Graph 5)

Table 7: Distribution of histologic subtypes

Histologic subtype	No of cases	%
LCNK	25	71%
LCK	10	29%
Total	35	100%

Graph 5: Distribution of histologic subtypes



P16^{ink4a} Immunohistochemistry:

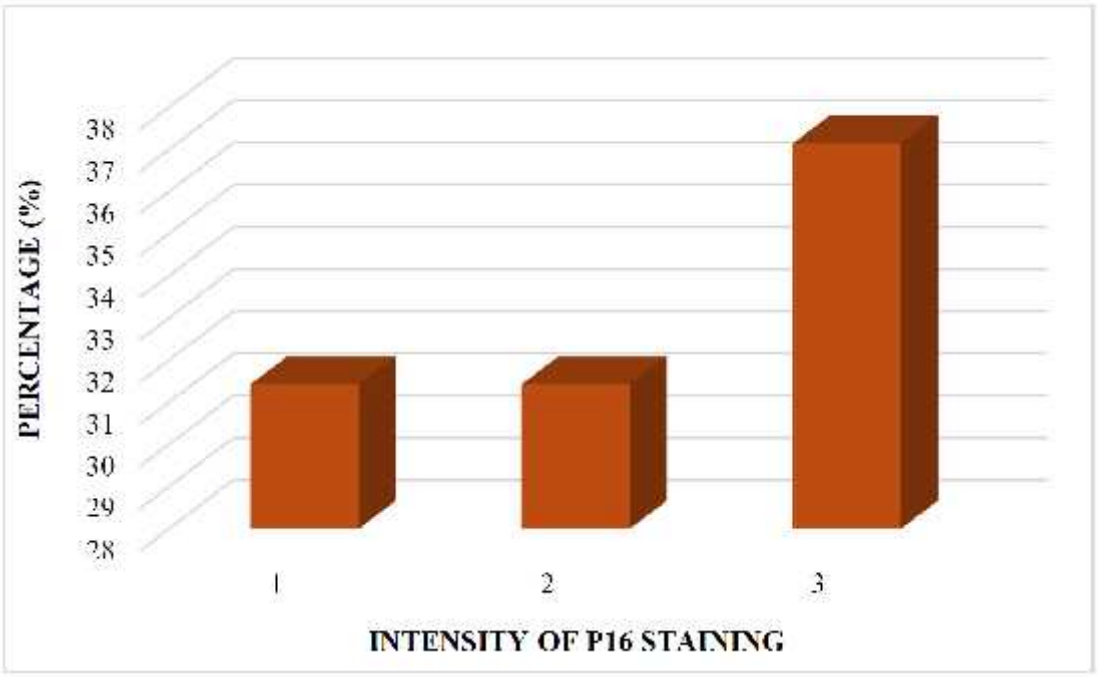
All the 35 cases of SCC cervix were subjected to immunohistochemistry staining with P16, intensity and grade of P16 expression was studied. Final IHC score was given by multiplying both the intensity and grade of P16 expression.

All the 35 cases showed positiveP16 expression. The intensity of P16 expression was studied, out of 35cases,mildpositivity was seen in 11 cases (31%), moderate positivity in 11 cases (31%) and severe intensity in 13 cases (37%).(Table 8, Graph 6)

Table 8: Distribution of P16 intensity

Intensity staining	No of Cases	%
1+(Mild)	11	31%
2+(Moderate)	11	31%
3+(Severe)	13	37%
Total	35	100%

Graph 6: Distribution of P16 intensity

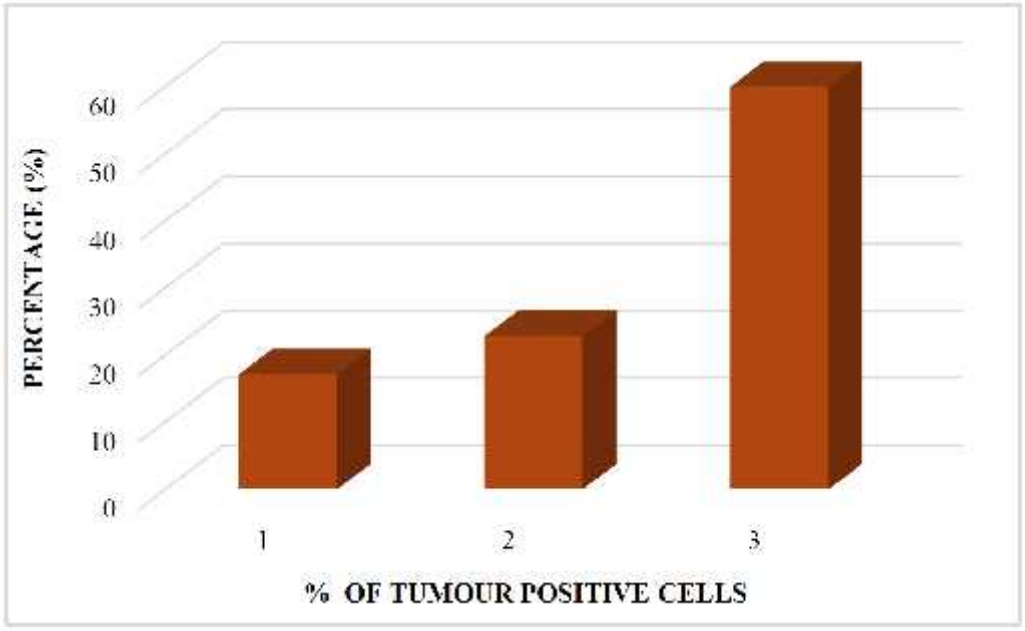


The percentage of tumour cells showing positivity of P16 expression was studied. (Table 9, Graph 7)

Table 9: Distribution of percentage of tumour positive cells

Percentage tumour positive cells	No of Cases	%
0%	0	0%
0-5%	6	17%
5-25%	8	23%
>25%	21	60%
Total	35	100%

Graph 7: Distribution of percentage of tumour positive cells



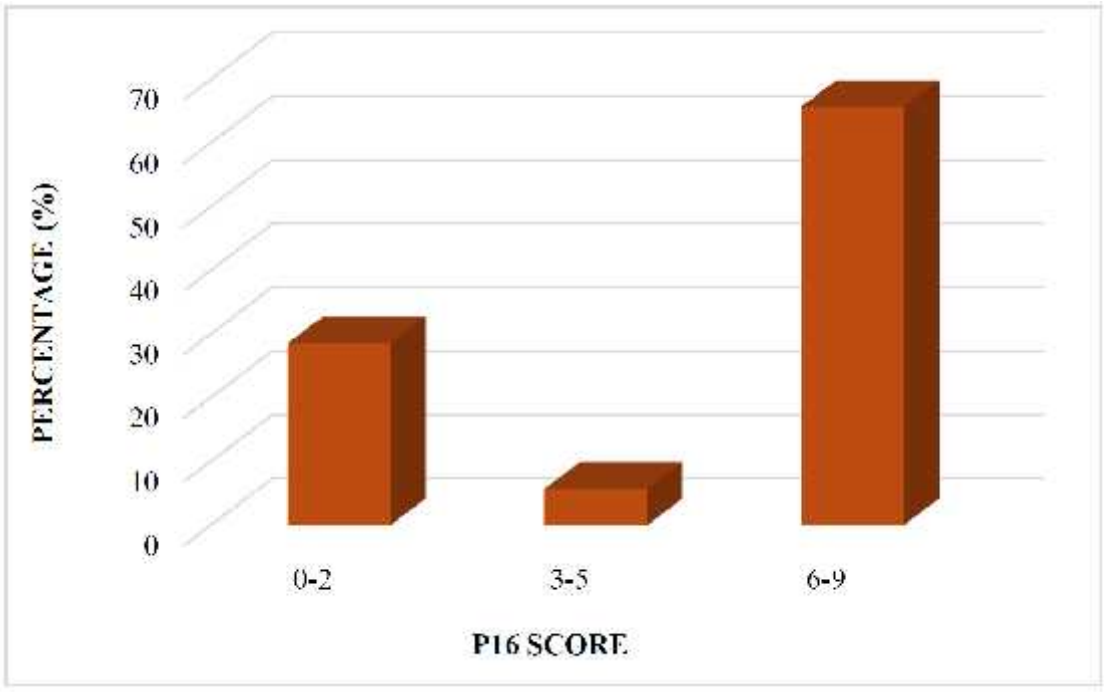
The final IHC score was given as a product of intensity grade and percentage positivity grade with maximum being 9.

A low score of 0-2 was seen in 10 cases (29%), moderate score of 3-5 was in 2 cases (6%) and a high score of 6-9 in 23 cases (66%). (Table 10, Graph 8)

Table 10: Distribution of final IHC (P16) score:

IHC Score	No of Cases	%
0-2	10	29%
3-5	2	6%
6-9	23	66%
Total	35	100%

Graph 8: Distribution of final IHC (P16) score:



The expression of P16 was studied in 35 cases of SCC cervix. While correlating P16 score with age, among the 10 cases with a low P16 score, 2 patients (20%) in 3rd decade, 1 patient (10%) in 4th decade, 5 patients (50%) in 5thdecade and 2 (20%) patients in 6th decade.

Among the 2 cases with moderate P16 score, 2 patients (100%) were in 5th decade. And among the 23 cases with high P16 score, 1 patient(4%) were in 3rd decade, 8 patients (35%) in 4th decade,7 patients (30%) in 5th decade, 6 patients (26%) in 6th decade and 1 (4%) patient in 7th decade. However, the p value was 0.148 which was not statistically significant. (Table 11, Graph 9)

While correlating parity with P16 score, of the 10 patients with low P16 score (0-2); parity of 0-2 was seen in 1 patient (10%) and parity of 3-5 in 8 patients (80%). Among the 2 patients with moderate P16 score (3-5); parity of 0-2 was seen in 1 patient (50%) and parity of 3-5 in 1 patient (50%). Among the 23 patients with high P16 score (6-9); parity of 0-2 was seen in 2 patients (9%) and parity of 3-5 in 21 patients (91%). This finding was statistically significant (p value=0.03). (Table 12, Graph10)

Table 11: Correlation of P16 score with age

Age	0-2	3-5	6-9	Total
31-40	2(20%)	0	1(4%)	3(9%)
41-50	1(10%)	0	8(35%)	9(26%)
51-60	5(50%)	2(100%)	7(30%)	14(40%)
61-70	2(20%)	0	6(26%)	8(23%)
70+	0	0	1(4%)	1(3%)
Total	10(100%)	2(100%)	23(100%)	35(100%)

Graph 9: Correlation of P16 score with age

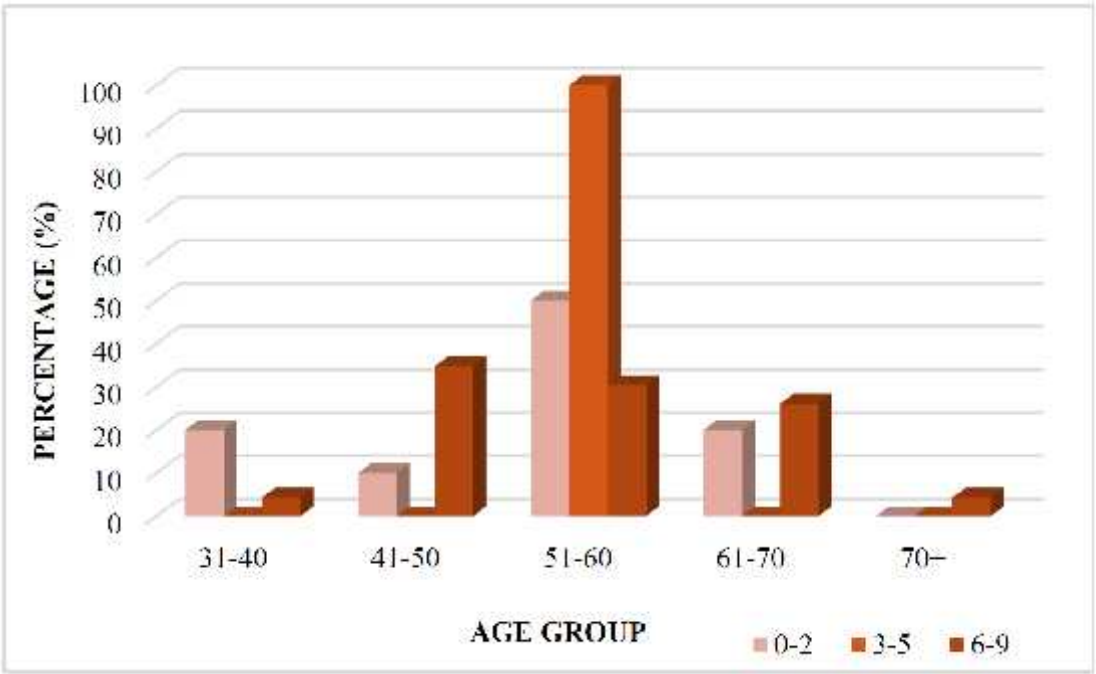
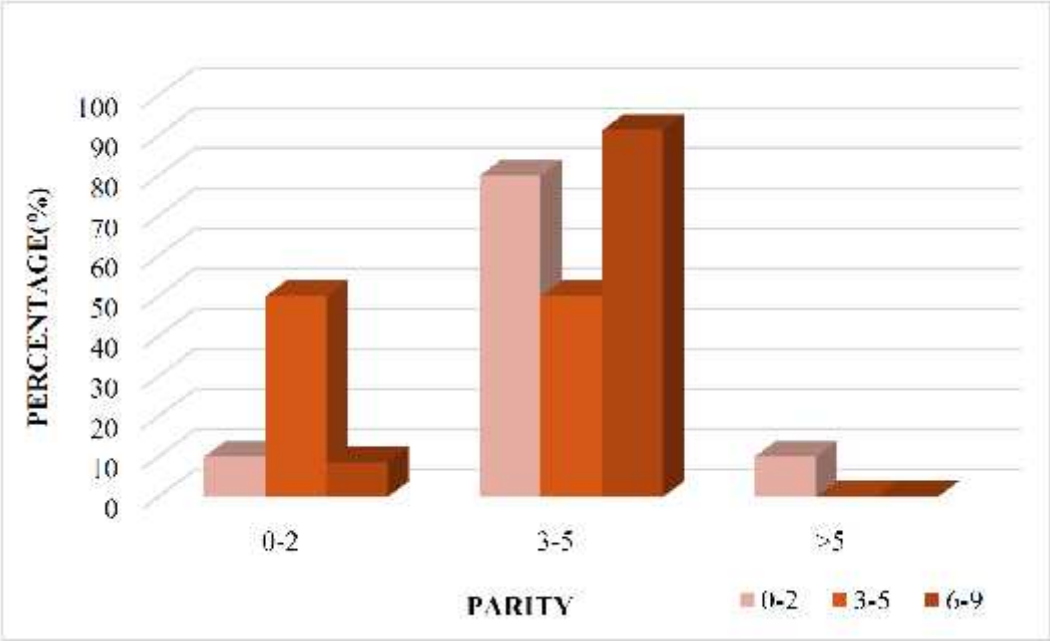


Table 12: Correlating parity with IHC score

Parity	0-2	3-5	6-9	Total
0-2	1(10%)	1(50%)	2(9%)	4(11%)
3-5	8(80%)	1(50%)	21(91%)	30(86%)
>5	1(10%)	0	0	1(3%)
Total	10(100%)	2(100%)	23(100%)	35(100%)

Graph 10: Correlating parity with IHC score

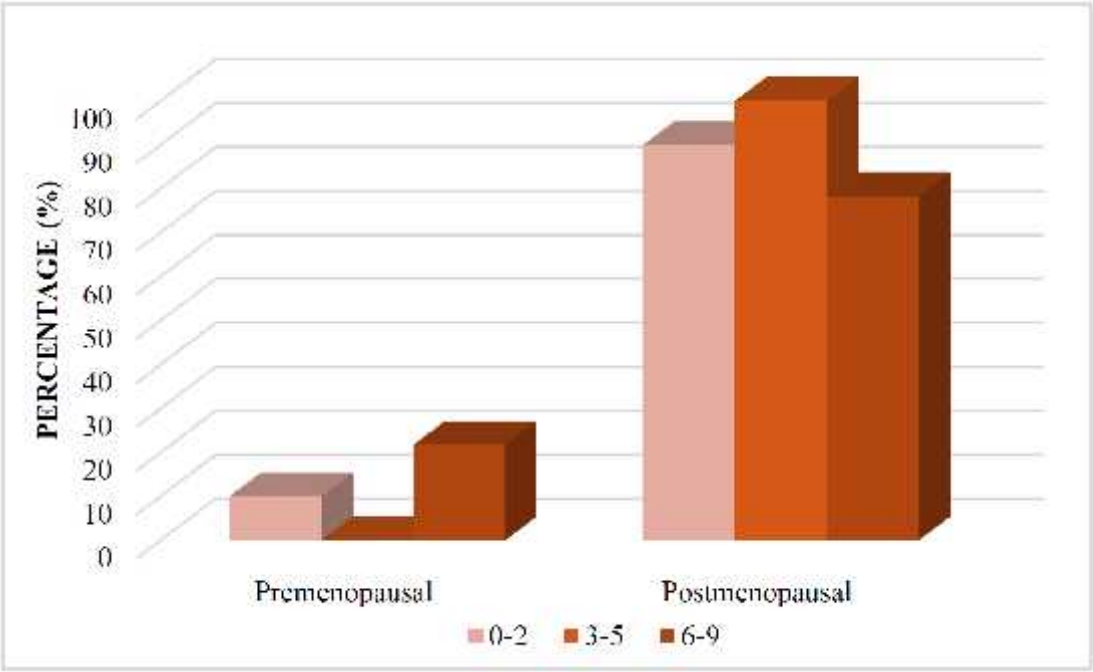


While correlating P16 score with menopausal status, among the 10 cases with a low P16 score, 1 patient (10%) was PM and 9 patients (90%) were POSM. Among the 2 cases with a moderate P16 score, 2 (100%) were POSM. Among the 23 cases with high P16 score, 5 patients (17%) were PM and 18 patients (83%) were POSM. However, this was not significant statistically with a p value of 0.814. (Table 13, Graph 11)

Table 13: Correlating menstrual status with P16 score

Menstrual status	0-2	3-5	6-9	Total
Premenopausal (PM)	1(10%)	0	5(17%)	6(17%)
Postmenopausal (POSM)	9(90%)	2(100%)	18(83%)	29(83%)
Total	10(100%)	2(100%)	23(100%)	35(100%)

Graph 11: Correlating menstrual status with P16 score

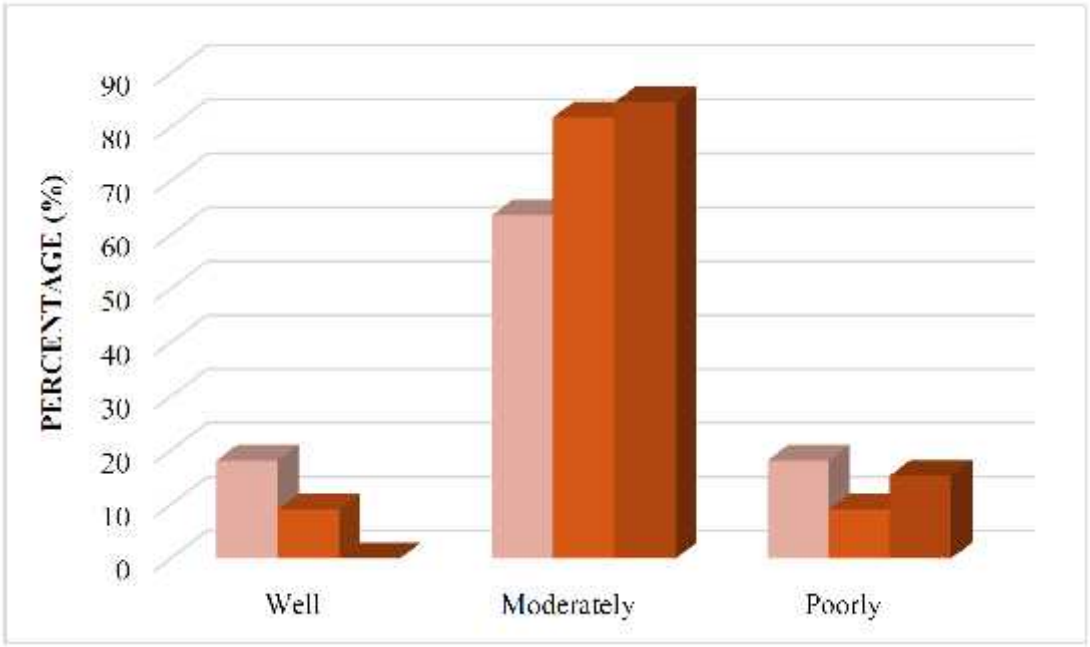


Intensity of P16 expression was correlated with Broder's Grade. Moderately differentiated carcinoma showed high intensity of P16 expression. Among the cases showing 3+ intensity of P16, 11 cases (85%) were of moderately differentiated carcinomas. However, this correlation was not significant (p value = 0.431). (Table 14, Graph12)

Table 14: Correlating intensity of P16 with Broder's grade

Broder's grade	1+	2+	3+	Total
Well	2	1	0	3(9%)
Moderately	7	9	11	27(77%)
Poorly	2	1	2	5(14%)
Total	11	11	13	35(100%)

Graph 12: Correlating intensity with Broder's grade

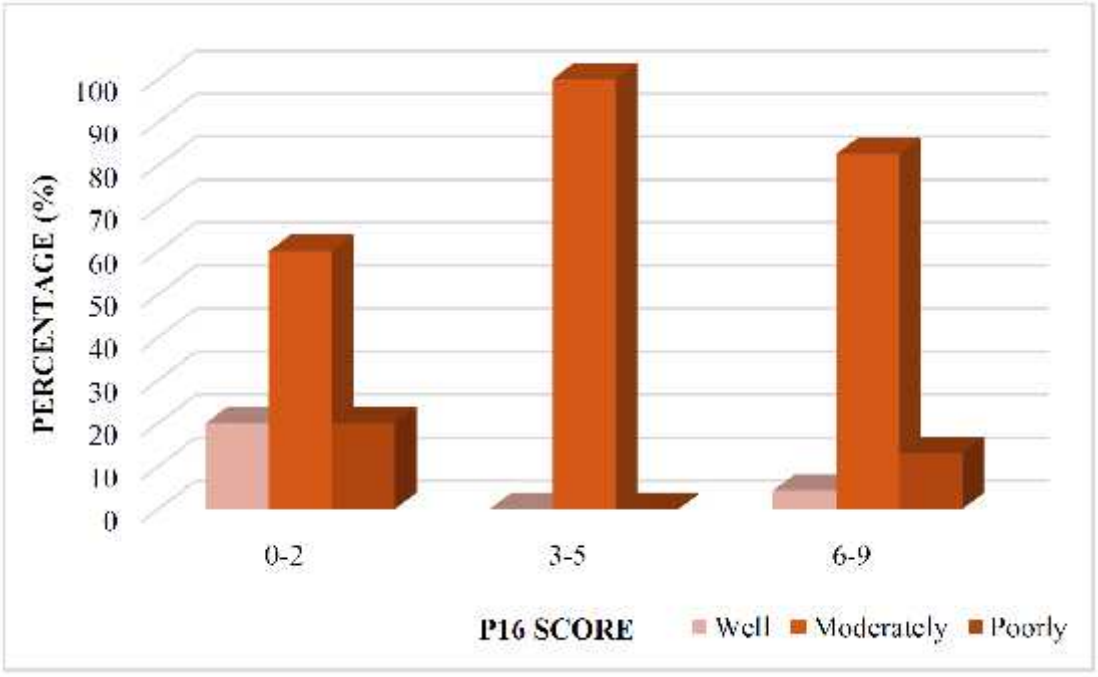


The P16 score was correlated with Broder's grade. Among the 23 cases showing high P16 score, 19 cases (83%) were of moderately differentiated type, 3 cases (13%) were of poorly differentiated type and 1 case (4%) but the correlation was not significant statistically with a p value of 0.709.(Table 15, Graph 13)

Table 15: Correlating P16 score with Broder's grade

Broder's grade	0-2	3-5	6-9	Total
Well	2	0	1	3(9%)
Moderately	6	2	19	27(77%)
Poorly	2	0	3	5(14%)
Total	10	2	23	35(100%)

Graph 13: Correlating P16 score with Broder's grade

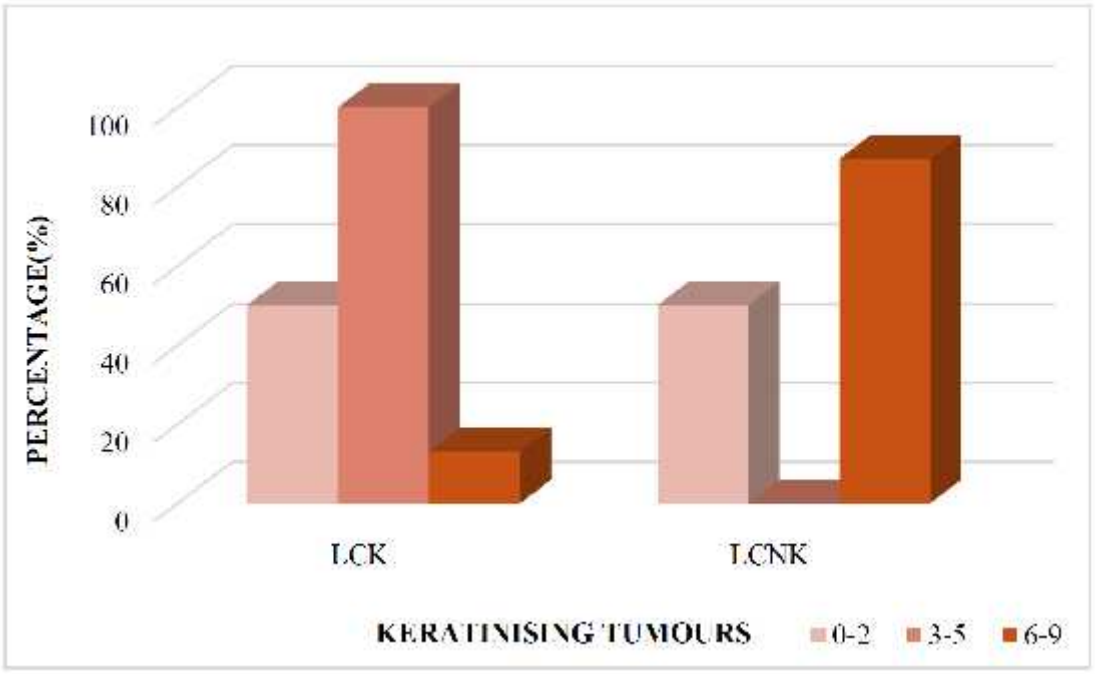


Histological subtypes of SCC also were correlated with P16 score. Among 25 cases of LCNK tumours, a low P16 score of 0-2 was seen in 5 cases and a high score of 6-9 was seen in 20 cases. Among the 10 cases of LCK tumours, a low score of 0-2 was seen in 5 cases, moderate score of 3-5 was in 2 cases and ahigh score of 6-9 in 3 cases. This correlation was significant statistically with p value being 0.02 (Table 16, Graph14)

Table 16: Correlating P16 score with histologic subtypes

Subtype	0-2	3-5	6-9	Total
LCK	5	2	3	10(29%)
LCNK	5	0	20	25(71%)
Total	10	2	23	35(100%)

Graph 14: Correlating P16 score with histologic subtypes



DISCUSSION

Squamous cell carcinoma of cervix is a leading public health issue among the developing world and India accounts for about one quarter cervical cancer cases worldwide.[28] Human papilloma virus is considered to be the primary risk factor.

Immunohistochemical expression of $P16^{ink4a}$ was seen only in dysplastic or neoplastic cells of cervical epithelium.[86] its positive expression is never seen in normal cervix. It stains both nucleus and cytoplasm of neoplastic cells. However, it is been observed that the intensity of staining pattern in different epithelial layers are similar to each other.[86]

Recent studies conducted by klaes et al[87] and Gurrola et al[88], highlight the role of P16 as an extremely sensitive marker for high-risk HPV related neoplasia and cervical epithelial dysplasia.

The study's purpose is to assess the expression of P16 in cases of cervical squamous cell carcinoma as it is now considered to be the most potential biomarker in the diagnosis of cervical neoplasms and can also be used in cytology specimens.[89]

In this study, 35 histologically diagnosed cases of cervical squamous cell carcinoma were studied, all of which are punch biopsy specimens. These were correlated to various clinicopathological variables.

In the present study, mean age of patients was 54.17 ± 9.1years and the peak incidence was observed between 51-60 years of age. These findings correlate with the studies conducted by Tan G.C. et al [90] (50.3 years), Geok Chin Tan et al [91] (51.1

years), Rajaram S. et al [92] (52.1 ± 12.46 years) and Lokesh et al[93]. One study by Chaloob et al [94] has shown mean age about 44.13±1.4 years.

This study has shown that most of the patients of carcinoma cervix were of parity 3 to 5 (80%). One study done by Rajaram S. et al [92](5.23 ± 2.34) also had shown similar results. However, one Swedish study revealed most of the carcinoma cervix patients were of parity 2.7.[11]

Current study shows that cervical carcinoma was more frequently observed in post-menopausal women (83%) which correlates to that of Madhumati et al [95] study.

In this study, highly common clinical feature observed was bleeding per vagina (64%) followed by white discharge per vagina (13%). Most of the postmenopausal women presented with bleeding per vagina. A recent study by JK Sandhu et al showed similar results.[96]

All the cases histologically diagnosed as SCC, were graded as poorly differentiated, moderately differentiated and well differentiated based on the morphology of squamous cells, nuclear pleomorphism and mitotic activity.[97]

Our study has shown that most of the cases were moderately differentiated carcinomas (77%), which correlates with the studies by JK Sandhu et al[96] (86.2%), florina et al[98] and Chaloob et al.[94]

This research work was done to assess the expression of $P16^{ink4a}$ in 35 cases of histologically diagnosed cases of SCC.

IHC scoring was done based on percentage positivity of tumour cells and intensity of staining. Final IHC score is taken as their product, maximum being 9.

All the IHC stained slides (100% of cases) have shown either focal or diffuse positivity, either in nucleus or both in cytoplasm and nucleus.

These observations correlate to multiple studies as mentioned below in the table.

Table 17: Studies showing P16 positivity

Study	Percentage of SCC cases with p16 positivity
Focchi et al[99]	100% (47/47)
Benevolo et al[100]	100% (8/8)
Agoff et al[101]	92% (42/46)
Tringler et al[102]	100% (19/19)
Ruchi gupta et al[103]	95% (19/20)
Lokesh et al[93]	90% (18/20)
Klaes et al[87]	98% (52/53)
Srivastava S[104]	100% (15/15)
Umar et al[105]	88.6% (47/63)
Tsoumpou et al[106]	86% (31/35)
Present study	100% (35/35)

The varying range in different studies may be because of the fixation, antigen retrieval methods, antibody selection and different scoring system used in various studies having different cut off levels of positive P16 expression.

In the present study, most of the cases have shown strong positivity (3+ intensity, 13/35, 37%) for P16 with maximum number of cases showing positivity in more than 25% of tumour cells (21/35,60%).

The present study reveals that majority of the cases have shown diffuse and strong positivity of P16 expression (37%) which is in accordance to the studies done by Lokesh et al[93](69.2%), Ruchi et al[103] (37.5%) and Chaloob et al[94] (28.3%). It is observed that as the cervical lesion progresses from mild dysplasia to invasive cancer, the intensity of expression of P16 increases.[107]

For correlating intensity of P16 positivity, a semi quantitative method was used. In our study about 60% cases showed P16 positivity in more than 25% of tumour cells. Studies conducted by Lokesh et al[93], Ruchi et al[103] and Focchi et al[99] have shown similar results.

While correlating P16 expression with age of the cervical carcinoma patients, P16 score was high in the patients of 5^{th} decade and low in patients of 3^{rd} and 7^{th} decade. This finding is in concordance with studies done by Chaloob et al[94] and Shin A et al[108]

The P16 score was correlated with parity of patients with carcinoma cervix, a high P16 expression was seen in women with parity 3-5. This finding suggests that expression of P16 increased in women with high parity. This is statistically significant in current study.

High P16 score was seen in postmenopausal women in this study than premenopausal women with carcinoma cervix.

Our study has shown that majority of cases (27/35,77%) were of moderately differentiated SCC. Most of these cases have shown intensity of 3+ (11/27) and an IHC score of 6-9 (19/27). However, in the present study, these findings are not statistically significant with a p value of 0.431. These findings are in concordance to the study conducted by Chaloob et al[94] where 92.9% of moderately differentiated carcinomas have shown positivity for P16 and also to studies done by Shin A et al[108] and Wu H et al[109].

A study conducted by Hellman K et al[110] has shown that about 85% cases of moderately differentiated carcinomas have shown strong positivity for P16 with a statistically significant p value of 0.004.

P16 score was also correlated to the histologic subtypes of SCC in this study. Most of the cases are large cell non keratinising type with a high score of P16 (20/35 cases). A p value of 0.02 indicated that this was statistically significant.

Fuste et al study reports that HPV-positive PCV is more frequently of warty type, basaloid and non-keratinising than HPV-negative PCV.[111] In carcinogenesis of SCC cervix, HPV is known to be the major risk factor. Hence, this correlation of non-keratinising tumours with high P16 score could be ascertained to the presence of HPV.

CONCLUSION

Our study evaluated the expression of $P16^{ink4a}$ by immunohistochemistry in 35 cases of squamous cell carcinoma cervix. Its expression was correlated with various clinical and histopathological parameters.

Bleeding per vagina in postmenopausal women was the common presentation clinically. The peak incidence was seen in 5^{th} decade.

Semi quantitative method was used for analysis of $P16^{ink4a}$ positivity. The percentage of tumour cells with positive stain and staining intensity of P16 expression were studied and a final IHC score was given.

All the cases (100%) have shown positivity for $P16^{ink4a}$ expression. Most of the tumours were showing 3+ intensity (37%) and most (60%) have shown P16 expression in >25% of tumour cells. Almost 66% cases have shown high P16 score (6-9).

All the clinical parameters like age, menstrual status, parity and keratinisation status were correlated with P16 score. Of these, correlation of P16 score with parity and non-keratinising status was statistically significant

SUMMARY

- In the present study, 35 histopathologically diagnosed cases of cervical squamous cell carcinomas (punch biopsies) were studied from the time period January 2018 to December 2019 at Dept of Pathology, JNMC, Belagavi.

- The gross morphology, microscopic findings and various appropriate clinical parameters for example menopausal status, sexual history, parity and clinical presentation were studied.

- Paraffin embedded blocks of all 35 cases were subjected to immunohistochemical staining for $P16^{ink4a}$ and its result was correlated with clinicopathological parameters.

- The peak incidence of cervical cancer was observed between the age group 51-60 years with the mean of 54 years.

- Most of the women were of parity 3 to 5 (86%).

- SCC was most commonly seen in postmenopausal women (83%).

- The most common clinical presentation was bleeding per vagina (64%) followed by vaginal white discharge.

- Most of the patients (77%) were moderately differentiated carcinomas according to Broder's grading system and most common subtype was large cell non-keratinizing type.

- All the cases of SCC cervix in this study have shown positivity for $P16^{ink4a}$ immunostaining. However, cases varied in intensity of staining and percentage positive tumor cells.

- Maximum cases have shown 3+ intensity of staining and also was positive in more than 25% of the tumor positive cells in most cases.

- The patients in 5^{th} decade showed high P16 score. Though, the correlation between age and p16 score was not statistically significant (p value =0.15).

- The P16 score was high in patient's parity between 3-5 and this was statistically significant in this present study (p value=0.03).

- The P16 score was also high in post-menopausal women, however was not statistically significant (p value=0.81).

- High P16 score was seen in moderately differentiated SCC, with no statistical significance (p value= 0.43).

- High P16 score was seen most commonly in non-keratinising tumours with a p value of 0.02 which is significant statistically.

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ANNEXURE I

INFORMED CONSENT FORM

IMMUNOHISTOCHEMICAL EXPRESSION OF P16 IN PATIENTS WITH SQUAMOUS CELL CARCINOMA OF THE CERVIX - A ONE YEAR RETROSPECTIVE AND PROSPECTIVE STUDY IN A TERTIARY CARE HOSPITAL

Purpose of the study: The purpose of this study is to determine the efficacy of p16 in squamous cell carcinoma of cervix.

Procedure: During this study, you will be asked questions regarding history and background and you are supposed to answer to the best of your knowledge.

If you agree to enroll yourself in this study, you will be interviewed regarding your present, past and family history and your clinical manifestations.

Risks and benefits: There are no risks involved in taking part in this study and benefit is we will be able to know a better way to diagnose invasive cancers which is essential for providing appropriate treatment.

Alternatives: Taking part in this study is voluntary. You may choose not to take part in this study or if you decide to take part now, you can later change your mind and withdraw from the study. The study doctor or sponsor may terminate your participation in this study anytime.

Privacy and confidentiality: All information collected about you during the course of this study will be kept confidential to the extent permitted by law. The code numbers will identify you in this research record. Information from this study will be published but your identity will be confidential in any publication. No information about you or information provided by you during research will be disclosed to other without your written permission except:

1. In emergency to protect your rights and welfare.
2. If required by law.

Financial incentives for participation: You will not be paid / offered any gift /incentives for participating in this study.

Authorization to publish results: The results of this study would be forwarded to the KAHER University, Belagavi as a part of requirement towards the completion of MD degree, review and publishing.

CONSENT STATEMENT

I voluntarily agree to take part in this study by signing below. I may withdraw at any time. I am not giving up any legal rights by signing this form. My signature below indicates that I have read, or it has been read to me, this entire consent form and have had all my questions answered.

In case of the queries during the study or in future you may contact following person.

Principal Investigator: _____

Guide : _____

If you have any queries about your rights as a study subject, you may call Dr. Roopa Bellad, Professor, Department of Paediatrics, Chairman of J.N. Medical College Institutional Ethical Committee of Human Subjects Research, Ph No- 9448113403, at J.N. Medical College, Belagavi

Name of the participant:

(signature/thumbprint)

Name of the witness : (signature)

Name of the investigator: (signature)

Date:

Address:

Phone no:

ANNEXURE-II-ETHICAL CLEARANCE LETTER

K.L.E. ACADEMY OF HIGHER EDUCATION AND RESEARCH
(Deemed – to- be- University)
Accredited 'A' Grade by NAAC (2nd Cycle) Placed in Category 'A' by MHRD (GoI)
JAWAHARLAL NEHRU MEDICAL COLLEGE,
NEHRU NAGAR, BELAGAVI-590010 (KARNATAKA-INDIA)
Website: http//www.jnmc.edu
E-Mail : domc@jnmc.edu
Phone: (+ 91-(0)831 Office : 2472550
Principal: 2471701
Fax No. +91 (0)831 – 2470759

Ref: MDC/DOME/35 **Date: 24/11/2018**

To,

REG. NO: BN0118003

Sub: Institutional Ethical Clearance for the study.

With reference to the above, we wish to inform you that your proposed research project titled **"IMMUNOHISTOCHEMICAL EXPRESSION OF P16^{INK4a} IN PATIENTS WITH SQUAMOUS CELL – A ONE YEAR RETROSPECTIVE AND PROSPECTIVE STUDY IN A TERTIARY CARE HOSPITAL",** is ethical and justifiable. The proposed research project has been cleared by the JNMC Institutional Ethics Committee on Human Subjects Research.

(Dr. Arathi Darshan)
Member Secretary
JNMC Institutional Ethics Committee
on Human Subjects Research,
J.N.Medical College, Belagavi.

(Dr. Roopa M Bellad)
Chairman,
JNMC Institutional Ethics Committee
on Human Subjects Research,
J.N.Medical College, Belagavi.

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ANNEXURE-III

PROFORMA

NAME:

AGE:

SEX:

OCCUPATION:

ADDRESS:

PRESENT HISTORY:

PAST HISTORY:

History of similar complaints in past:

Any history of hormonal medications:

Any history of infections in past:

GYNAECOLOGICAL HISTORY:

Menstrual History:

Age of menarche:

Length of cycle:

Age of menopause:

OBSTETRIC HISTORY:

Number of living children

Age at the time of first baby:

History of use of oral contraceptives:

FAMILY HISTORY:

Any history of similar complaints in family

Any history of any cancerous lesion in family

Any history of sexually transmitted infections in husband:

PERSONAL HISTORY:

Sexual history:

History of weight loss:

Habits:

CLINICAL EXAMINATION:

Per-vaginal examination:

Colposcopy findings:

Pap smear:

CLINICAL DIAGNOSIS:

HISTOPATHOLOGICAL REPORT:

GRADE:

IHC DIAGNOSIS:

INTENSITY:

ANNEXURE IV

HAEMATOXYLIN AND EOSIN STAINING PROTOCOL

- Deparaffinize in Xylene I and II and III changes. [III change use warmedxylene] (5 minutes in each)
- Rehydrate using:
    - Absolute Ethanol 100% (5 minutes)
    - Absolute Ethanol 100% (5 minutes)
- Rinse in distilled water (5 minutes)
- Rinse in running tap water (5 minutes)
- Stain in Harris's hematoxylin by progressive method (2 minutes) Fresh and filtered
- Rinse in running tap water (20 minutes)
- Decolorize in 1% acid alcohol (1 second)
- Rinse well in tap water (5 minutes)
- Immerse in hot water bath, 550 C for bluing (3 Seconds)
- Rinse in tap water (5 minutes)
- Counterstain in Eosin (15 seconds)
- Dehydrate absolute alcohol 100 % (2-4 dips)
- Clear in Xylene I and II (5 minutes)
- Mount with DPX.

Stock Solutions – EOSIN:

Stock – 1% aqueous Eosin-Y

Stock – 1% aqueous Phloxin B

Working Solutions – Eosin:

100ml stock Eosin

10 ml stock Phloxin B

780 ml 95% Ethanol

4 ml glacial Acetic Acid

Working Solution: - Hematoxylin

Harris Hematoxylin, 1 Liter

Working Solution: - 0.25% Acid Alcohol

95% Ethanol, 2578 ml

dH2O, 950ml

HCL, 9ml

Result: Nuclei – Blue, Cytoplasm – Pink, RBCs – Red.

Reference: Bancroft D, Laylon C. The haematoxylin and eosin, In: Kim SS Ed, Bancroft's Theory and practice of histopathological techniques. 7th Ed., China, Churchill

Livingstone; 2013: p173-87

ANNEXURE V

IMMUNOHISTOCHEMICAL STAINING PROTOCOL

- 2-4µ thick sections to be taken on Poly l lysine coated slides.
- Bake slides for 30minutes at 80 degrees Celsius.
- Deparaffinize and rehydrate the tissue in series of xylene (3 changes) and graded alcohol (100%, 90%,70% ethyl alcohol) 5minutes each and wash in running tap water for 5minutes.
- Soak the slides in PBS* buffer for 2minutes
- Antigen retrieval to be done using Heat induced epitope retrieval (HIER) method using BIOGENX EZ- RETRIEVER System V.3 microwave. Slides to be kept in retrieval jar containing TRIS EDTA**/ citrate buffer.
- Antigen retrieval done in 2 cycles -

1st cycle (Preheat cycle) - 85 degrees Celsius for 5minutes.

2nd cycle (Retrieval cycle) - 98 degree Celsius for 15minutes. Take precaution to note evaporation of the buffer during this heat cycle.

- The slides are then allowed to cool at room temperature for 15 minutes.
- Humid chamber to be prepared using a wooden box, moist cotton and glass rods.
- Wash the slides with PBS buffer 3 times. The area on the slide containing the tissue to be marked using paraffin wax.
- Peroxidase block to be added to the tissue for 10minutes.
- Wash slides with PBS 3times.
- Add power block for 5minutes. Excess to be drained out after 5minutes.
- Add primary antibody for p16 and keep for 1.5 hours.

- Wash slides with PBS 3 times. Add super enhancer and keep it for 20minutes.Slides to be washed with PBS 4 times.
- Prepare DAB working solution using the chromogen and substrate15minutes prior use.
- Add DAB substrate and allow for 7-10minutes.
- Wash with PBS 4 times.
- Wash with distilled water 4 times.
- Counterstain with Gill's/Meyer's commercial haematoxylin (1-3minutes)following which wash in running tap water for 1minute.
- Dehydrate the slides in graded alcohol and clear in xylene.
- Mount the slides using DPX

Preparation of phosphate buffer [PBS] (pH 7.1-7.4)

Solution A – 2.1g of sodium monobasic phosphate + 100ml distilled water.

Solution B – 2.9g of sodium dibasic phosphate + 100ml of distilled water.

Solution C – 0.9g sodium chloride + 100 distilled water.

Working solution is prepared using 68ml solution B + 32ml Solution A + 100ml ofsolution C

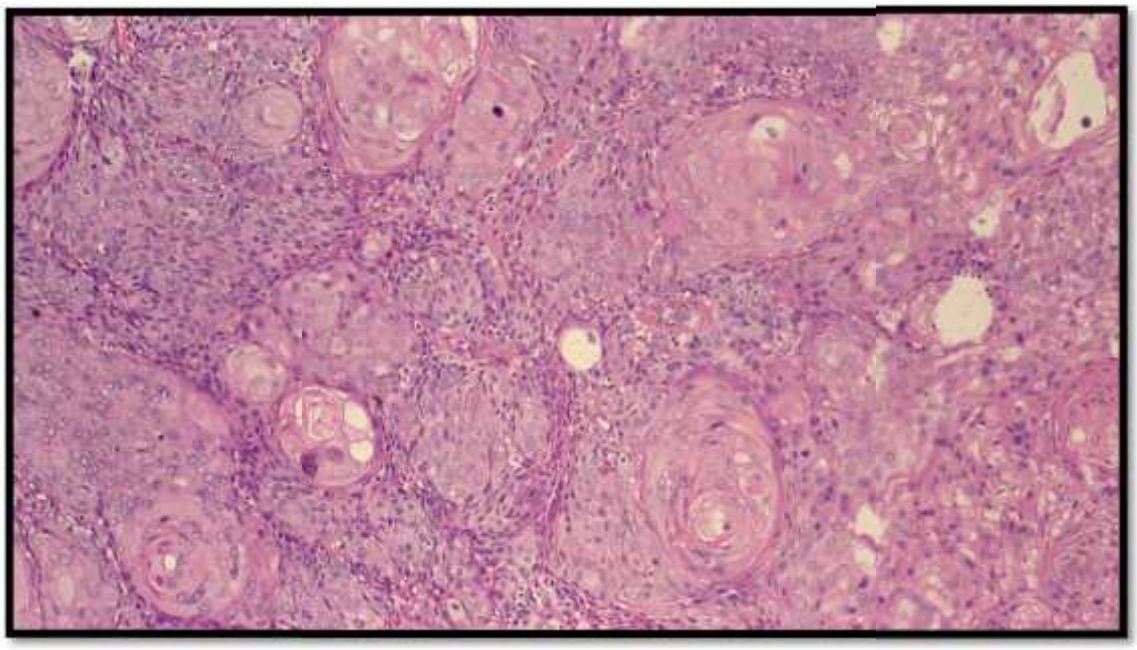
Preparation of TRIS EDTA antigen retrieval buffer (pH – 9)

- Take 1.21g of TRIS base and 0.37g of EDTA.
- To this add 1000ml of distilled water.

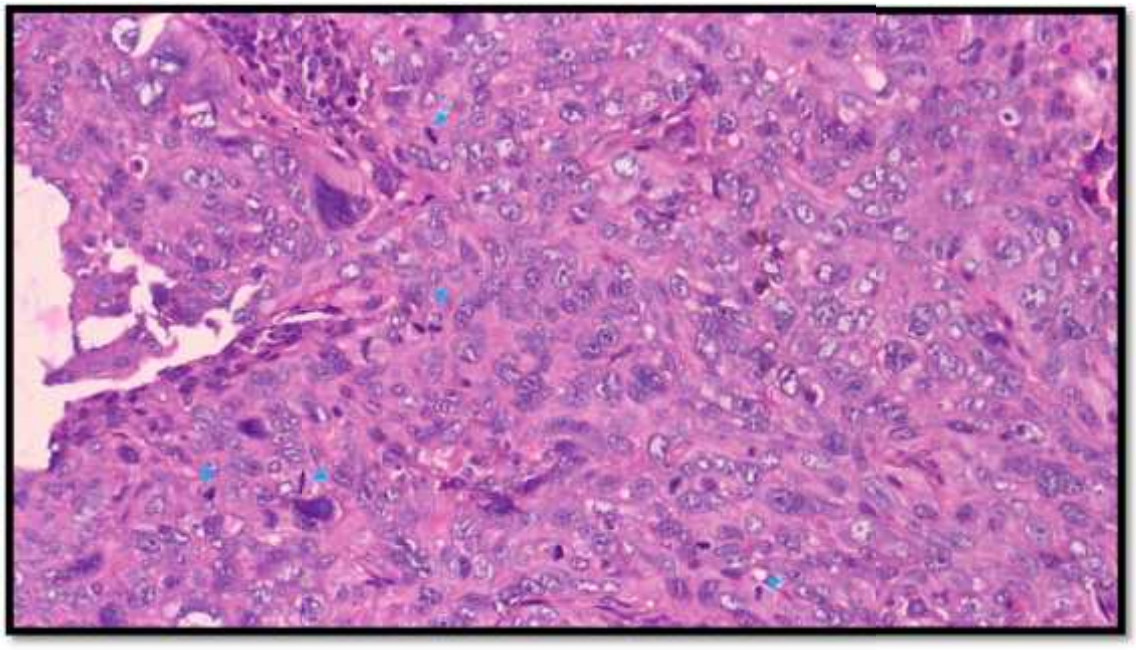
Preparation of DAB [3,3 –Diaminobenzidine substrate] working solution

- 1ml DAB substrate + 1-2 drops of DAB chromogen.
- To be prepared fresh 15minutes prior to use

ANNEXURE VI–PHOTOMICROGRAPHS

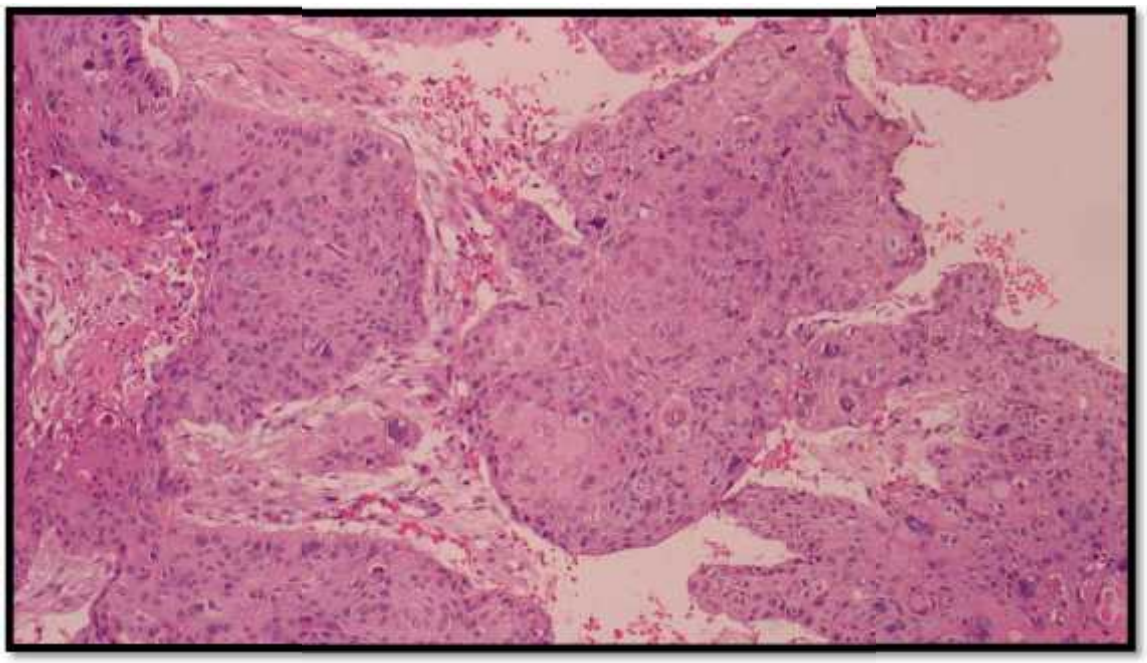


Photomicrograph 1: Well differentiated SCC with keratin pearls (H&E, 4x)

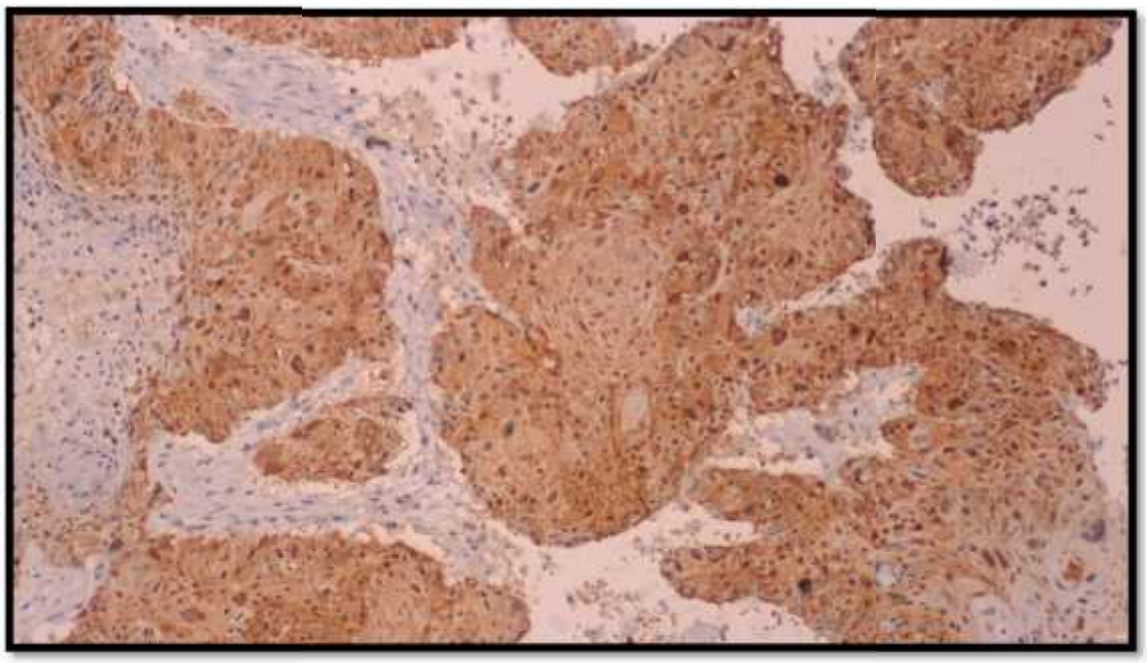


Photomicrograph 2: Poorly differentiated SCC with mitotic activity (blue arrows)

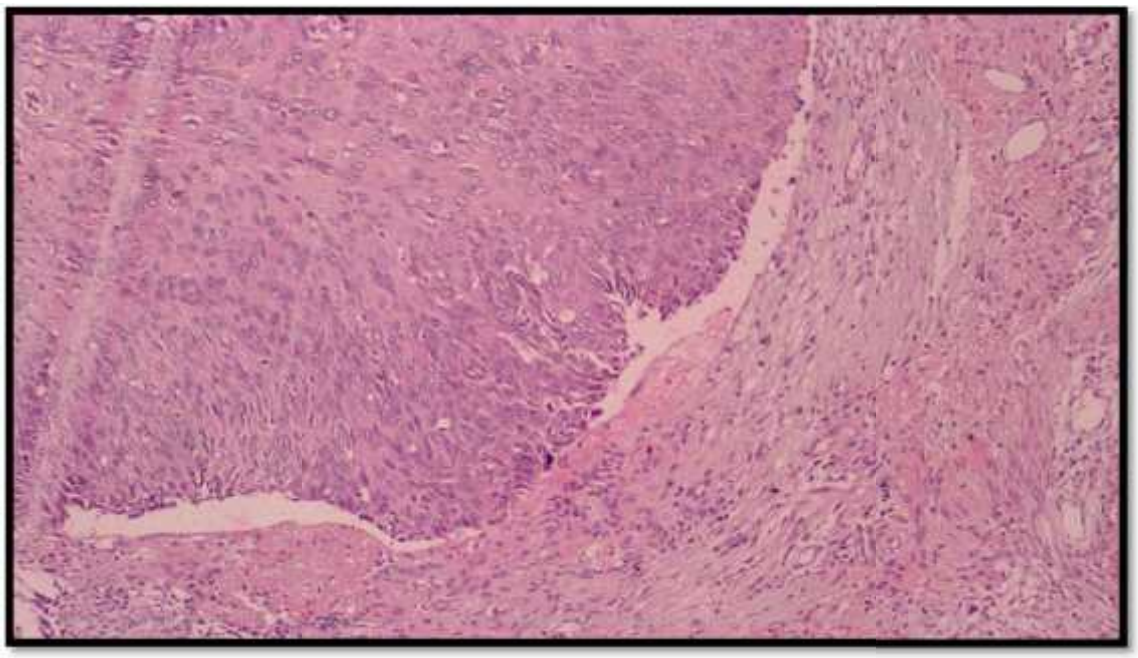
(H&E, 20x)



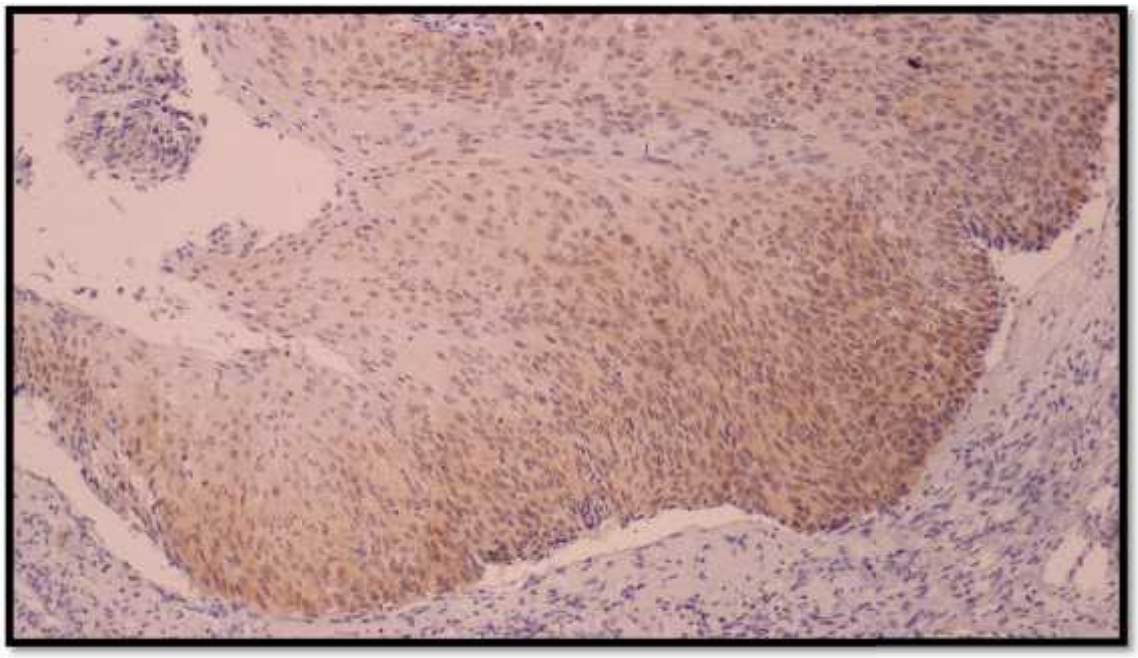
Photomicrograph 3: Shows well differentiated SCC (H&E, 20x)



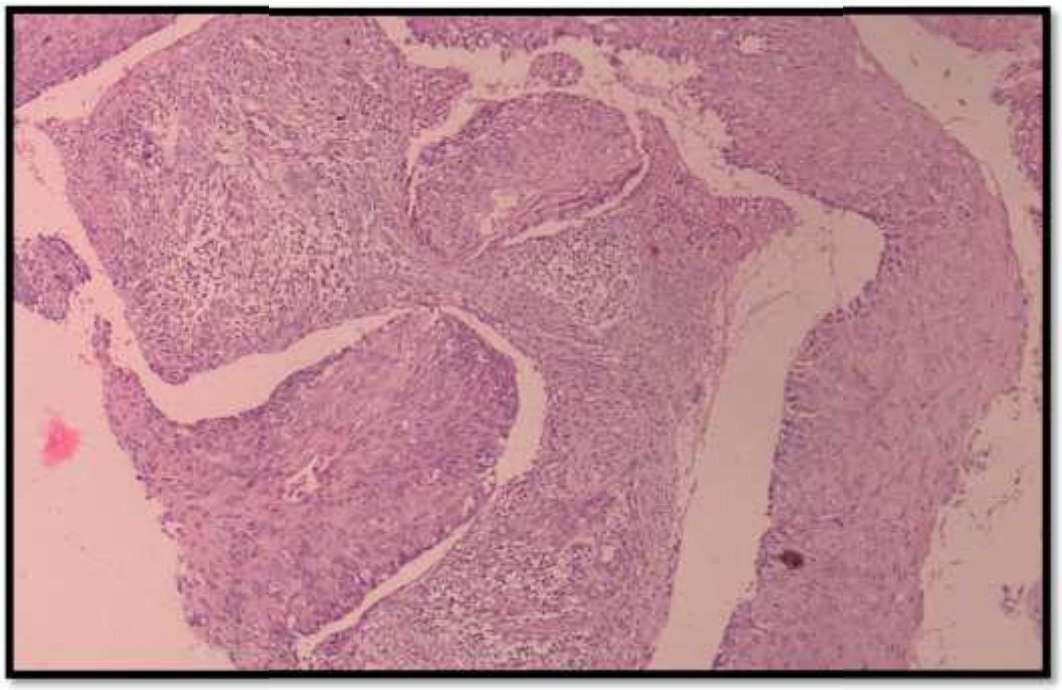
Photomicrograph 4: Shows well differentiated SCC with P16 scores 6 (IHC, 20x)



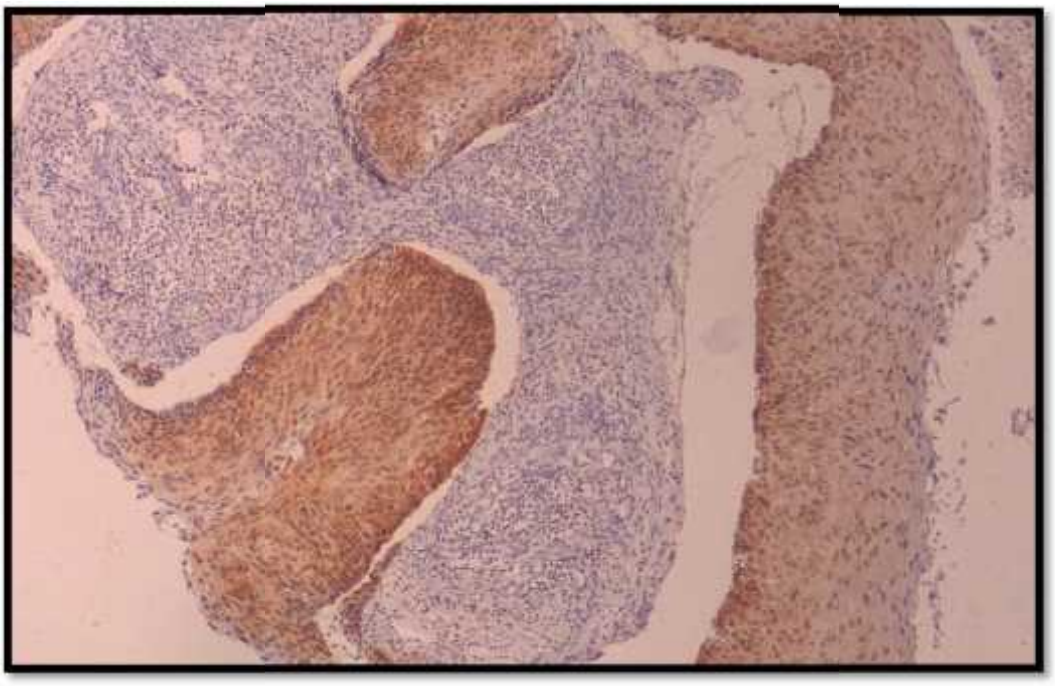
Photomicrograph 5: Shows moderately differentiated SCC (H&E, 20x)



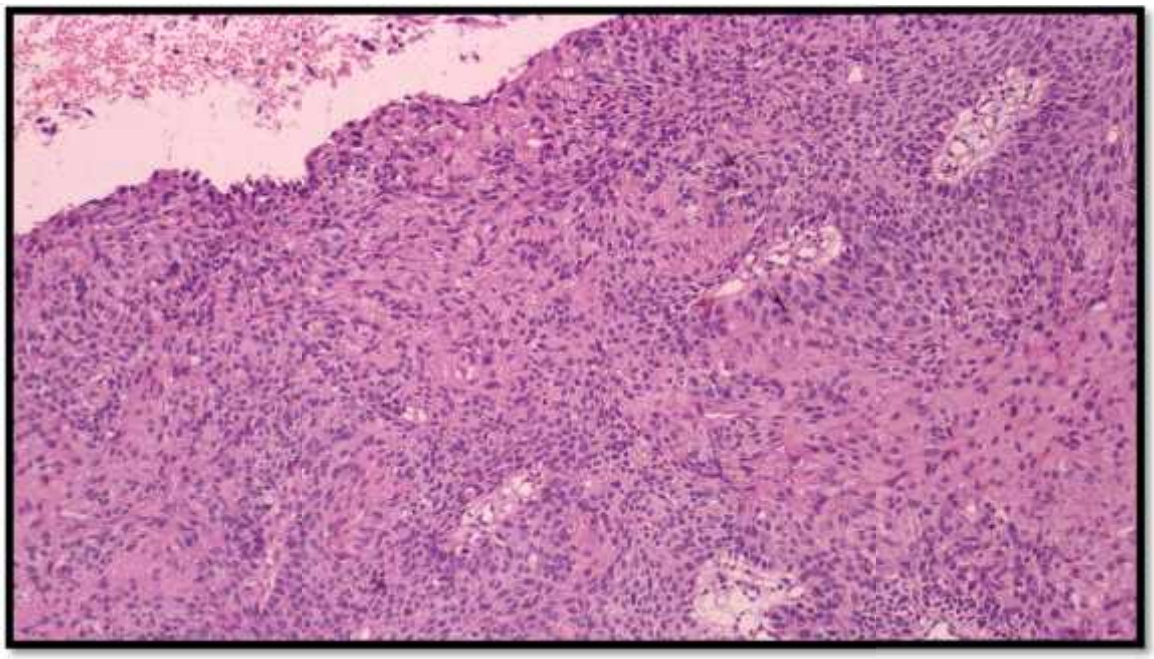
Photomicrograph 6: Moderately differentiated SCC with P16 score of 3 (IHC, 20x)



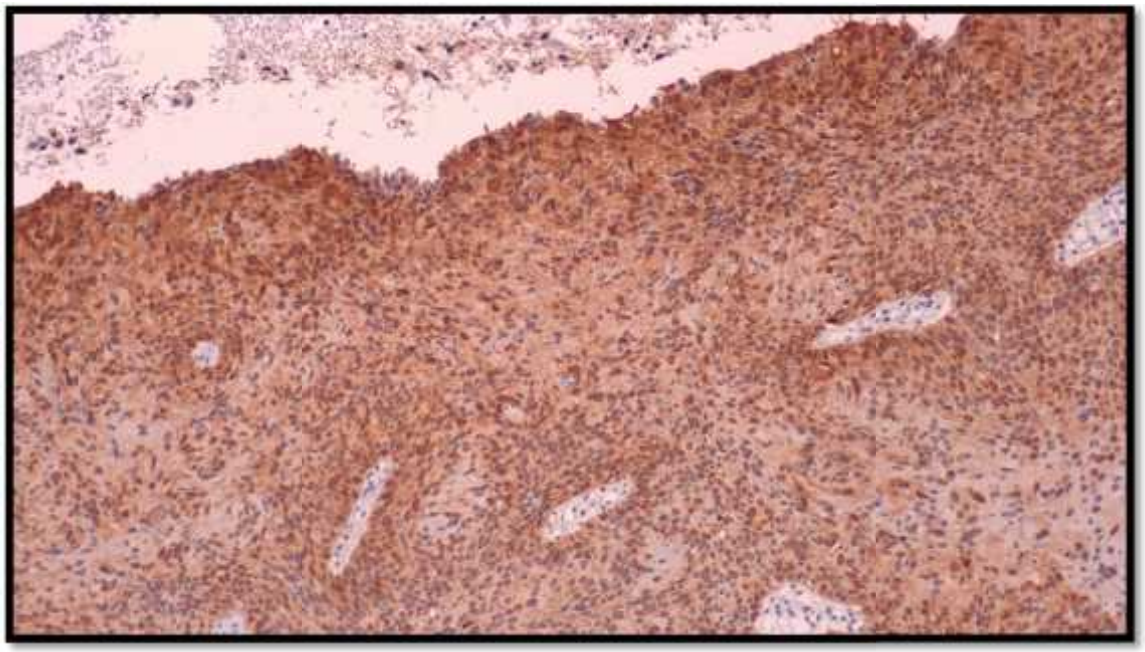
Photomicrograph 7: Shows Moderately differentiated SCC (H&E, 20x)



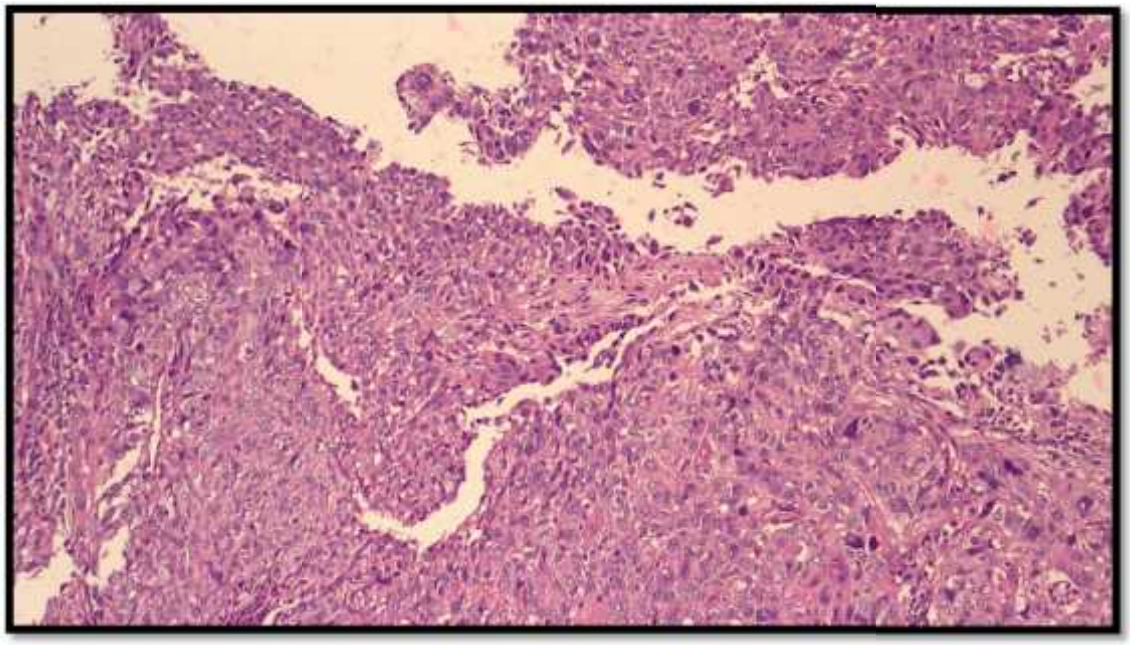
Photomicrograph 8: Moderately differentiated SCC with P16 score of 6 (IHC, 20x)



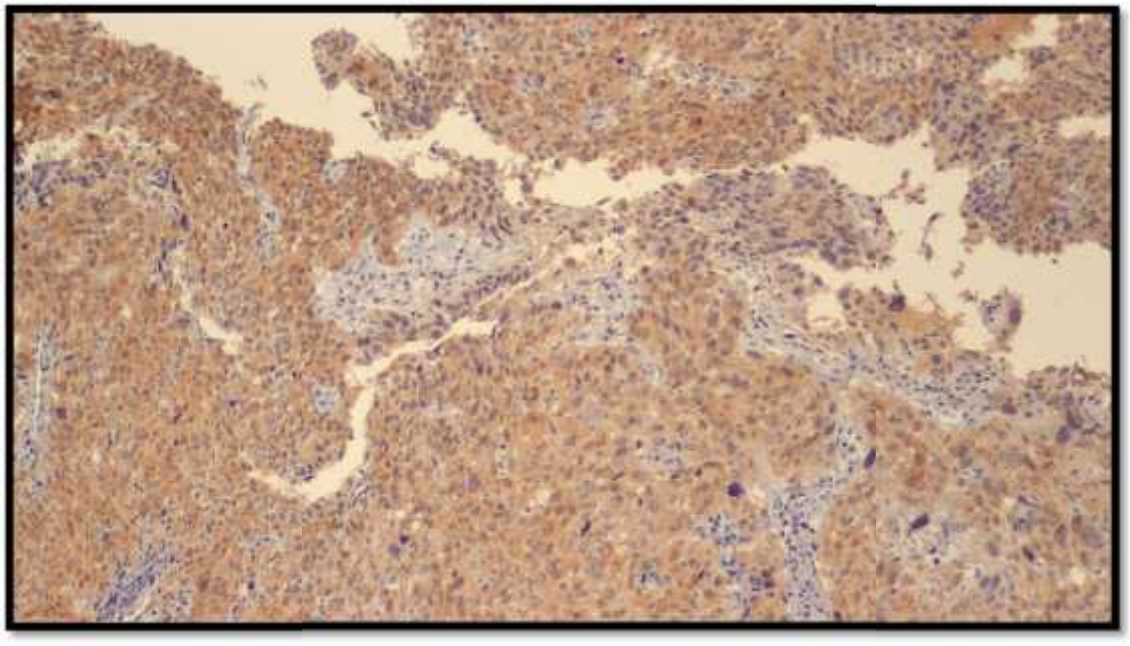
Photomicrograph 9: Moderately differentiated SCC (H&E, 10x)



Photomicrograph 10: Moderately differentiated SCC with P16 score of 9 (IHC, 10x)



Photomicrograph 11: Poorly differentiated SCC (H&E, 10x)



Photomicrograph 12: Poorly differentiated SCC with P16 score of 6 (IHC, 10x)

ANNEXURES VII - MASTER CHART

Case Number	Age	Clinical features (CF)	Parity	Menstruation status	Broder's grade (BG)	Keratinizing status	P16 intensity	% of tumour positive	Final P16 score
1	63	BPV, CG	5	POSM	M	Y	1	2	2
2	55	WDPV	3	POSM	M	N	3	3	9
3	45	BPV, BP	5	PM	P	N	2	3	6
4	57	BPV	3	POSM	M	Y	1	2	2
5	46	WDPV, BP	3	POSM	P	N	1	1	1
6	38	BPV	2	POSM	M	N	1	1	1
7	55	WDPV, PA	3	POSM	M	N	2	3	6
8	65	BPV	5	POSM	M	N	3	3	9
9	50	BPV, WDPV	3	POSM	P	N	3	2	6
10	42	BPV	3	PM	M	Y	2	3	6
11	60	BPV	7	POSM	M	N	1	1	1
12	55	BPV	3	POSM	M	N	3	3	9
13	61	BPV	3	POSM	M	N	3	3	9
14	56	BPV, WDPV	2	POSM	W	Y	2	3	6
15	63	BPV	5	POSM	M	N	2	3	6
16	50	BPV	3	POSM	M	N	3	2	6
17	42	BPV	2	PM	M	N	2	3	6
18	55	BPV	3	POSM	W	Y	1	2	2
19	52	BPV, CG	3	POSM	M	Y	1	1	1
20	61	CG, PA	3	POSM	M	N	1	2	2
21	52	BPV	5	POSM	W	N	1	1	1
22	72	BPV	3	POSM	M	N	3	3	9
23	65	BPV	3	POSM	M	N	3	3	9

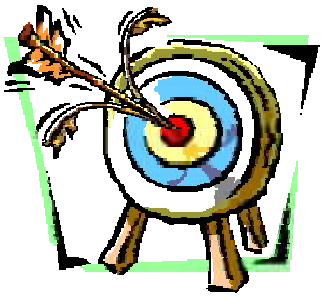
24	45	BPV	4	PM	M	N	2	3	6
25	60	BPV, WDPV	3	POSM	M	N	2	3	6
26	55	BPV	3	POSM	M	Y	2	2	4
27	50	BPV	3	POSM	M	N	3	3	9
28	52	BPV, CG	3	POSM	M	N	2	3	6
29	70	BPV	3	POSM	M	N	2	3	6
30	55	BPV	2	POSM	M	Y	1	3	3
31	69	CG	3	POSM	M	N	3	3	9
32	60	BPV	3	POSM	M	N	3	2	6
33	35	BPV, PA, MPA	3	PM	P	Y	1	1	1
34	45	BPV	3	POSM	P	N	3	3	9
35	40	BPV	3	PM	M	Y	3	3	9

ANNEXURE-VIII

KEY TO MASTER CHART

M	-	MODERATELY DIFFERENTIATED
W	-	WELL DIFFERENTIATED
P	-	POORLY DIFFERENTIATED
Y	-	YES
N	-	NO
PM	-	PREMENOPAUSAL
POSM	-	POSTMENOPAUSAL
BPV	-	BLEEDING PER VAGINA
WDPV	-	WHITE DISCHARGE PER VAGINUM
PA	-	PAIN ABDOMEN
BP	-	BACK PAIN
CG	-	CERVICAL GROWTH
MPA	-	MASS PER ABDOMEN
CF	-	CLINICAL FEATURES
BG	-	BRODER'S GRADING

Introduction



Objectives

Review of Literature

Methodology

Results

Discussion

Conclusion

Summary

Bibliography

Annexure-I

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Annexure-II

Annexure-III

Annexure-IV

Annexure-V

Annexure-VI

Annexure-VII

Annexure-VIII