"p-STAT3 EXPRESSION IN SQUAMOUS CELL CARCINOMA OF CERVIX."

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LIST OF ABBREVIATIONS USED

WHO	-	World Health Organisation
HPV	-	Human papilloma virus
p-STAT3	-	Phosphorylated Signal Transducers and Activators of Trasncription
SCC	-	Squamous cell carcinoma
SCJ	-	Squamocolumnar juction
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
TRIS Hcl	-	Trisaminomethane – Hydrochloric acid
DAB	-	Diamino Benzoic Acid
PBS	-	Phosphate buffer saline
DPX	-	Dibutylphathalate xylene
VEGF	-	Vascular endothelial growth factor
JAK	-	Janus tyrosine kinase

ABSTRACT

"P-STAT3 EXPRESSION IN SQUAMOUS CELL CARCINOMA OF CERVIX".

BACKGROUND : Cervical carcinoma is the fourth common cancer in women with global incidence of 13.3% and mortality rate of 7.3%. p-STAT3 is a protein belonging to transcription factors family, which plays an important role in normal cell differentiation, apoptosis, angiogenesis and gene expression. Dysregulation of antiapoptotic genes and inhibition of STAT3 activation and its constitutive activation leads to cancer cell growth in many organs. Its expression in cervical cancer tissues is evaluated by immunohistochemical(IHC) staining which can used for assessing the prognosis of patients and to explore its possible role in carcinoma.

OBJECTIVES : To observe the expression and intensity of p-STAT3 in histologically diagnosed cases of squamous cell carcinoma of cervix and its correlation with histological grading. Expression of Ki67 is also compared with p-STAT3 in the study.

METHODOLOGY : Paraffin embedded blocks from 30 squamous cell carcinoma patients who were histologically diagnosed were retrived from Department of Pathology ,J N Medical college, Belagavi. They were stained with Hematoxylin and eosin and further by p-STAT3 antibody and Ki-67 immunohistochemistry in KAHER'S basic research laboratory and were evaluated for percentage positive tumour cells and its intensity. Their expression was assessed using four tired semi-quantitative method. SPSS software was used for statistical analysis.

RESULTS : All the carcinoma cases show positive expression for p-STAT3. It was correlating with Broder's grading, and intensity in the study but was not statistically significant.Ki-67 showed statistically significant association with Broder's grading (p value 0.028). A statistical significance was observed with Ki-67 and p-STAT3 expression.(p 0.037).

CONCLUSION : We found p-STAT3 expression is positive in all cervical carcinoma patients and is associated with proliferative activity of neoplastic cell which is shown by its statistical association with Ki-67 in the study.

KEY WORDS : Squamous cell carcinoma, STAT3 (Signal Tranducers and Activators of Transcription), Ki-67, clinicopathological, histological grading.

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INTRODUCTION

Cervical carcinoma has ranked fourth among malignancies occurring in women in the world following breast, lung cancer and colorectal carcinoma[1]. It is the second most common neoplasia in India[1–3]. It is a largely preventable disease due to cytological based screening programme. It is caused by Human Papilloma virus (HPV) high risk type HPV-16.

According to World Health Organisation(WHO) 2019 estimated 604000 new cases of cervical cancer globally representing 6.6% of all female cancer deaths[1]. The mortality rate from 6.9% (2018) has increased to 7.3% (2019) which can be controlled through a systematic approach which includes prevention, early detection, effective screening and treatment programmes. Vaccines are available in present era against cancer causing types of HPV[1]

The commonest occurring site for squamous cell carcinoma(SCC) and cervical dysplasia is transformation zone. It is situated between original and new squamocolumnar junction located between ectocervical squamous epithelium and endocervical columnar epithelium[4]. Persistent infection with high risk HPV16, 18 is an important cause of high risk premalignant lesion and leads to subsequent development of invasive cervical cancer[5]. Though relationship between HPV and cervical cancer had been well established but molecular mechanism behind tumor metastasis and its progression is unclear. Being an era of molecular genetics, focusing on signal transduction cascades of which Signal Transducers and Activators of Transcription (STAT), particularly STAT3 which takes a crucial role in malignant transformation has been stressed upon.[6]

pSTAT3, a important member of STAT family consisting of STAT1 ,STAT2, STAT3, STAT4, STAT5 and STAT6 plays an indispensible role in normal cellular events like proliferation, cell differentiation, apoptosis, cell survival, and angiogenesis following growth factor, cytokine, and hormonal signaling[7–10].It is normally situated in cytoplasm and gets activated through phosphorylation. Its constitutive stimulation is found in many other organ cancer including lung , head and neck , prostate, ovary, breast.[7,11,12]

This STAT3 is activated by tyrosine(Tyr) phosphorylation residue at 705, leading to nuclear translocation, dimer formation, activation of target gene transcription and recognition of STAT3 specific DNA binding elements which includes tumor formation.[6]Antagonism of constitutively activated STAT3 can induce apoptosis and prevents cancer cell growth, indicating that constitutive activation of STAT3 signaling is prerequisite for cancer cell survival and growth.[6]

This study was undertaken to evaluate the expression of pSTAT3 by immunohistochemistry (IHC) in Squamous cell carcinoma of cervix, its expression to compare with histological grading.

Ki 67 is also included in the present study which is a nuclear protein present in nucleus of dividing cells and not in resting cells.[7,13] Ki 67 index seen in basal and parabasal layers of cervix show a gradual rise with the progression of disease from cervical intraepithelial neoplasia to cervical carcinoma.[7,14,15] The association between pSTAT3 and Ki 67 is also considered as important factor to know about prognosis in the present study.

AIMS AND OBJECTIVES

Primary objective:

1. To evaluate expression of p-STAT3 in squamous cell carcinoma of cervix .
2. To study the expression of Ki-67 staining.

Secondary Objective:

To compare p-STAT3 and Ki 67 expression.

REVIEW OF LITERATURE

EMBRYOGENESIS :

Sex differentiation is a complex process that involves many genes including anatomical genes. Understanding the complex process is the key to many developmental disorders encountered in the female.(16) The female genital system involves uterus with cervix, bilateral fallopian tube along with ovary and vagina. The genital tract is lined by mesoderm mostly except the germ cells which are endodermal in origin. The epithelial lining of vagina and vulva are ectodermal in origin.(16)

The paraneoplastic duct develops into genital duct of female under the influence of estrogen and absence of testosterone and mullerian hormone. This duct consists of three parts : 1. A cranial portion that opens into abdominal cavity, 2. A horizontal portion which crosses the mesonephric duct, 3. A caudal portion which fuses with the counter part from opposite side . The fused paramesonephric duct forms the future corpus of uterus and cervix along with upper portion of vagina. In males this paramesonephric duct is suppressed leaving a small portion giving rise to appendix of testis.(16)

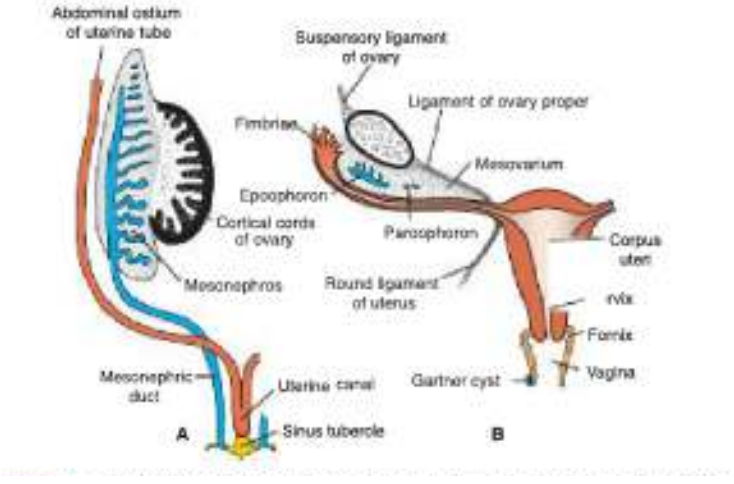


Figure 1 : Development of cervix(16).

ANATOMY OF CERVIX.

The main organ of female reproduction is uterus with cervix. Its adnexal structures include uterine tube, vagina and its ligaments. Uterus is pear shaped muscular organ located in the pelvis between rectum and urinary bladder.[17] Uterus is divided into following parts : a muscular corpus which forms above two-third and cervix which forms the lower one –third portion. The fibromuscular junction which is named as internal cervical os points out the junction made by corpus and cervix. The uterus is placed in position by various ligaments which include true and false ligaments. True/primary supports include: pelvic diaphragm, perineal body and urogenital diaphragm. Ligamentous true supports include transverse cervical ligaments of Mackendrodt, uterosacral ligaments, round ligament of uterus, pubocervical ligament and uterine axis. False support include broad ligament, uterovesical ligament and rectovaginal fold of peritoneum.[17]

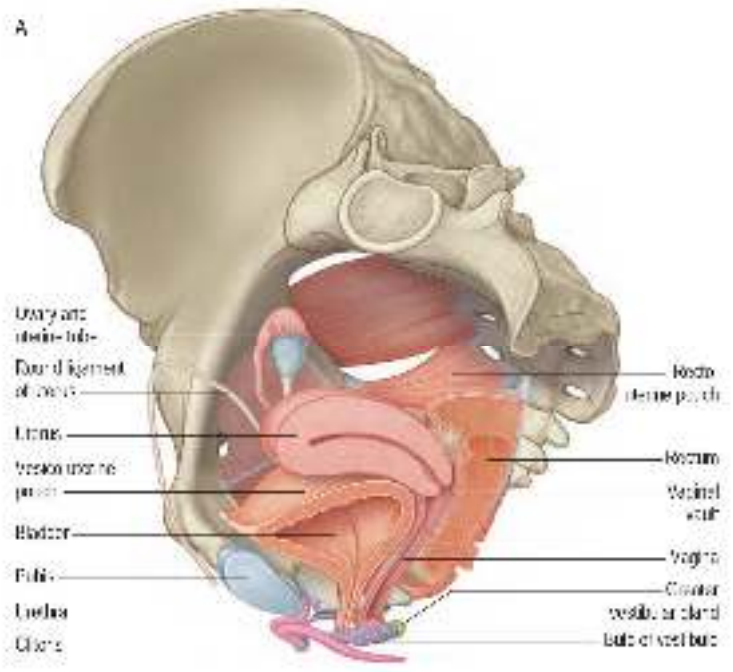


Figure 2 : Anatomical location of cervix(17)

The shape and size of the cervix is different in pregnant and non-pregnant women. In non-pregnant females it is more cylindrical compared to body of uterus and measures 3cm long & 2.5cm in diameter. In multiparity women, cervix is larger and bulbous compared to nulliparous women and have transverse slit like opening rather than circular shape as in nulliparous women.(17)

Uterine cavity communicates to vagina through cervix which is flattened from front to back. Upper end of cervix opens into uterine body through internal os, the lower end with vagina through external os.(18) The cavity of cervix is called cervical canal which is fusiform in shape .The portion of cervix that is situated outside the external os towards the vagina is ectocervix, and that lies above the external os within the canal is endocervix.(17) The upper third of cervix forms the narrow isthmus which is eventually pulled up into the uterine cavity during second month of pregnancy.(17)

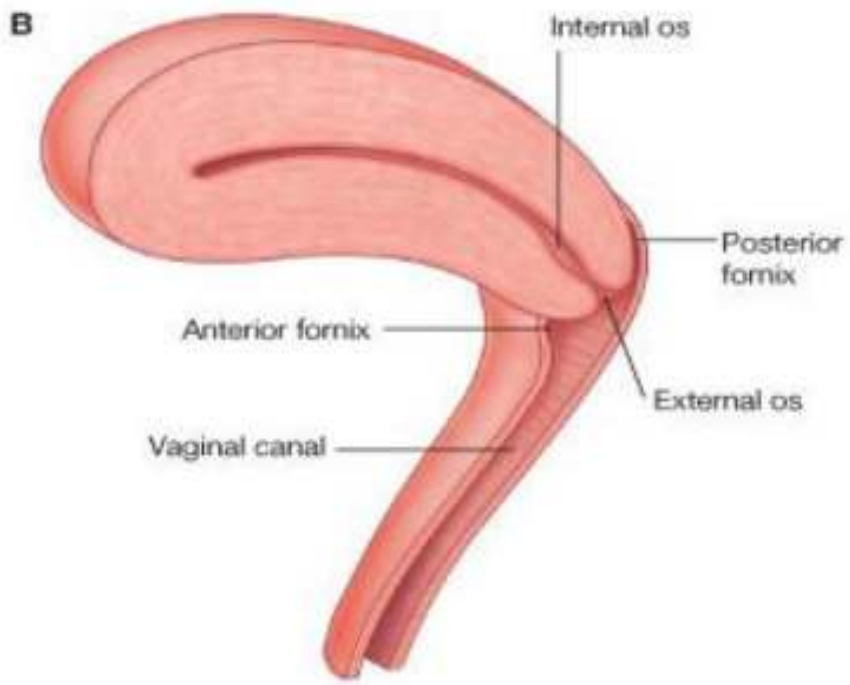


Figure 3 : Parts of cervix with uterus and vagina. (17)

The space between vagina and vaginal part of cervix is termed vaginal fornix. The external portion of cervix which enters the upper vagina divides the cervical region into supravaginal and vaginal parts.[18] The vaginal part is also known as portiovaginalis. A cellular connective tissue, parametrium separates supravaginal part and bladder anteriorly. Posteriorly it is related to rectouterine pouch containing coils of intestine and rectum. On either side it is related to ureter and uterine artery.[19]

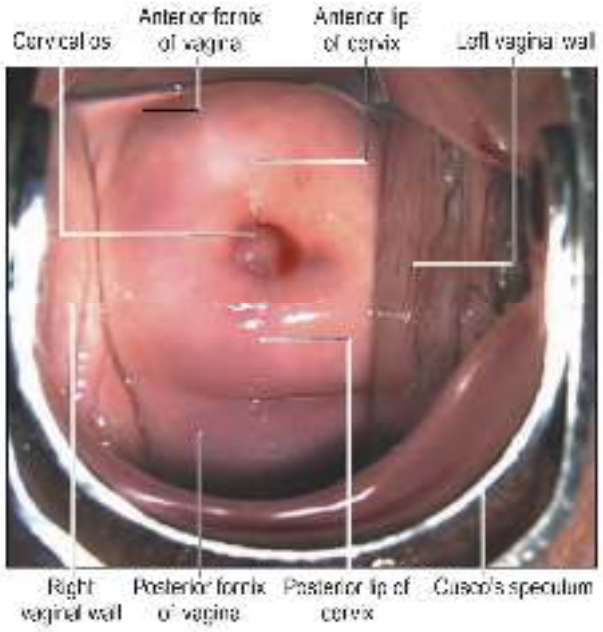


Figure 4 : Visulaiztion of cervix on speculum examination.[17]

Arterial supply : Uterus is supplied by uterine artery, a branch of anterior divison of internal illac artery. In broad ligament, uterine artery cross the ureter anteriorly at the cervico-uterine junction. A major branch of it ascends along the uterus tortuously within the substance of broad ligament.[17] It anastomosis with branches of ovarian artery. Second branch descends down to cervix and supply it, which later anastomose with branches of vaginal artery forming azygous arteries of vagina.

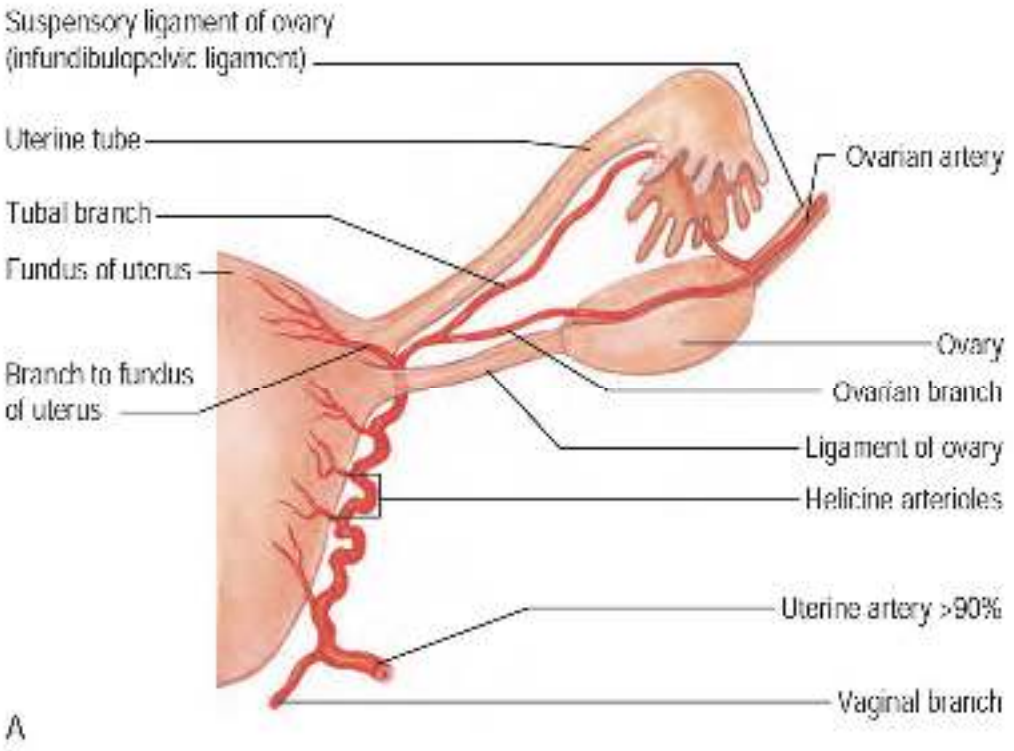


Figure 5 : Arterial supply of cervix.[17]

Venous supply : The venous drainage of uterus is contributed by internal illac veins. These venous plexus anastomoses with ovarian and vaginal venous plexuses.[17]

Lymphatic drainage : They are three groups of lymph nodes - external, obturator and internal iliac nodes. These lymphatics are present in superficial and deep regions of uterine wall. The lymphatic drainage from uterine fundus and fallopian tube accompany the lymphatic drainage of ovary into para-aortic lymph nodes.[20]

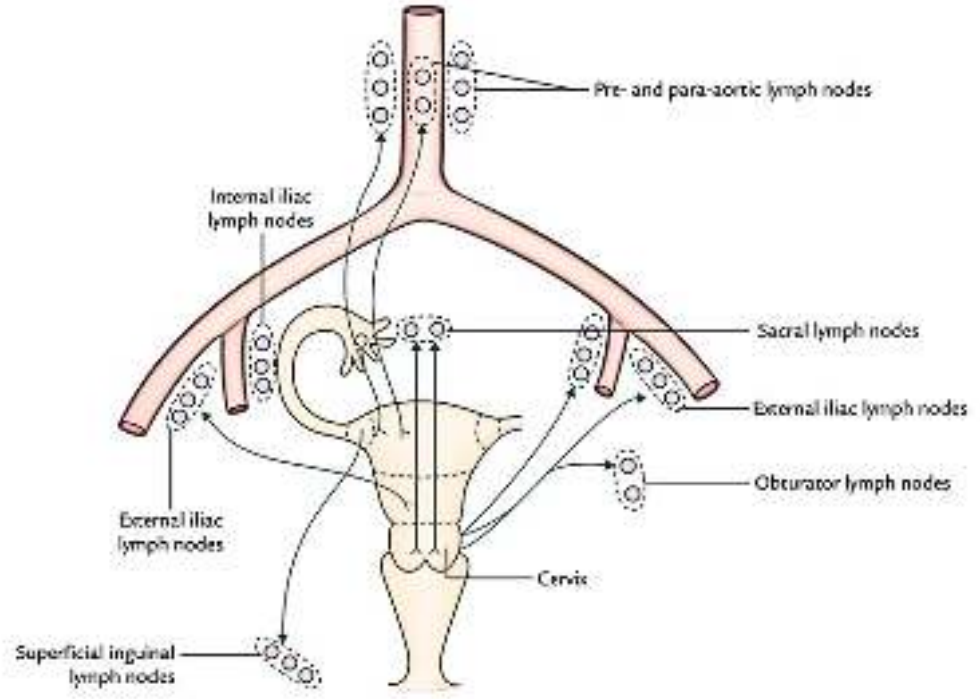


Figure 6 : Lymphatic drainage of cervix.(20)

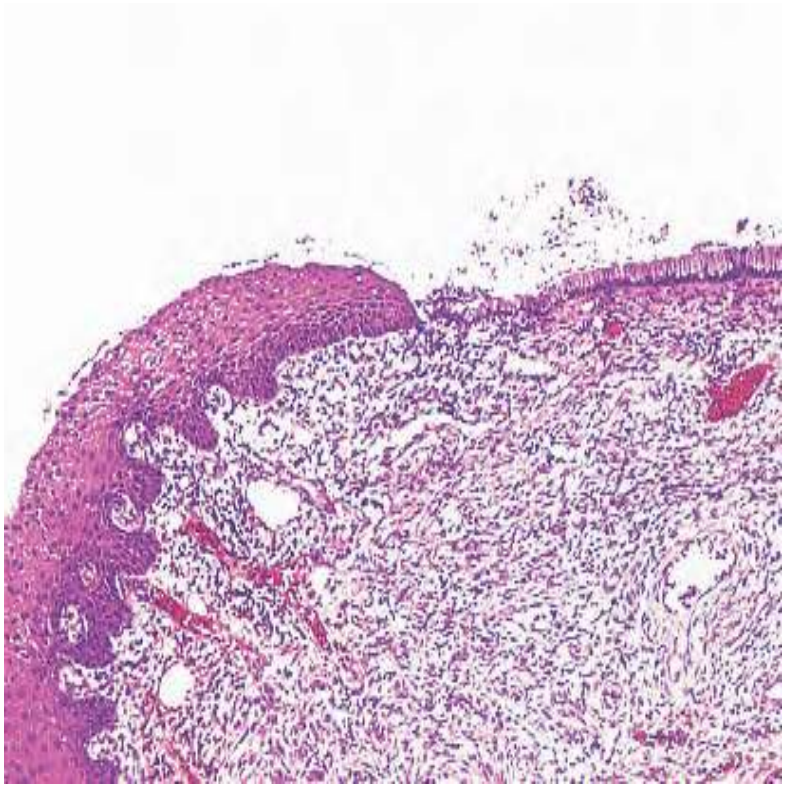
Nervous supply : The uterus is predominantly supplied by inferior hypogastric plexus. They supply uterine body and fallopian tube. This uterine nerve ends in myometrium and endometrium.(17) Cervix is innervated by plexus containing paracervial ganglia. Preganglionic efferent sympathetic fibres are derived from T12 to L4 segments. Parasympathetic preganglionic fibres are derived from S2 to S4 spinal regions and end in paracervical ganglia.(17)

Sympathetic innervation produces contraction of uterus along with vasoconstriction, while uterine inhibition and vasodilation are produced by presympathetic innervation.(17)

PHYSIOLOGY OF CERVIX : The cervix is under the influence of ovarian hormones mainly estrogen. It softens the tissue, stretches the cervix, dilates and makes the cervix thinned out in late pregnancy, favouring parturition. Another

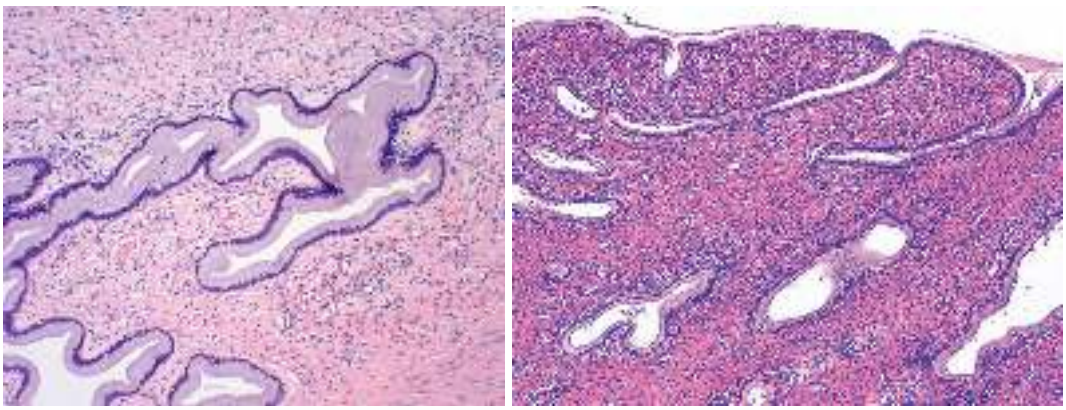
function of cervix is to allow spermatozoa into the genital tract at the time of ovulation for fertilization.[21]

HISTOLOGY OF CERVIX : The part of the cervix which is visible on vaginal examination is ectocervix is lined by squamous layer. The endocervix shows secreting tall columnar epithelium. The junction between them is called *squamo-columnar junction*(SCJ).[21]



Photoimicrograph 1 : Normal Squamocolumnar junction[21]

The bulk of cervix is mainly comprised of collagenous tissue .The cervical canal shows folded mucosa with superficial layer of mucous secreting columnar cells.[21] The endocervical glands are also lined by columnar cells. Morphologically squamocolumnar junction is of two types : One is original SCJ which is present at time of birth and the other is functional SCJ which is seen in reproductive age group.



Photomicrograph 2 :

a) normal Endocervical glands[22] b) fibrocollagenous stroma.[21]

The columnar epithelium can be replaced by squamous metaplasia. The region located between original SCJ and post pubertal SCJ is termed as *transformation zone*(TZ). Histologically it is characterized by metaplastic epithelium. During reproductive age and pregnancy, the transformation zone is seen on the exposed part of cervix. In older and post menopausal women, TZ is the corner stone for the pathogenesis of squamous cell carcinoma and precursor lesions of cervix.[22] The non-keratinized ectocervical squamous epithelium shows three zones histologically :

1. **Germinal cell or parabasal/basal layer** :

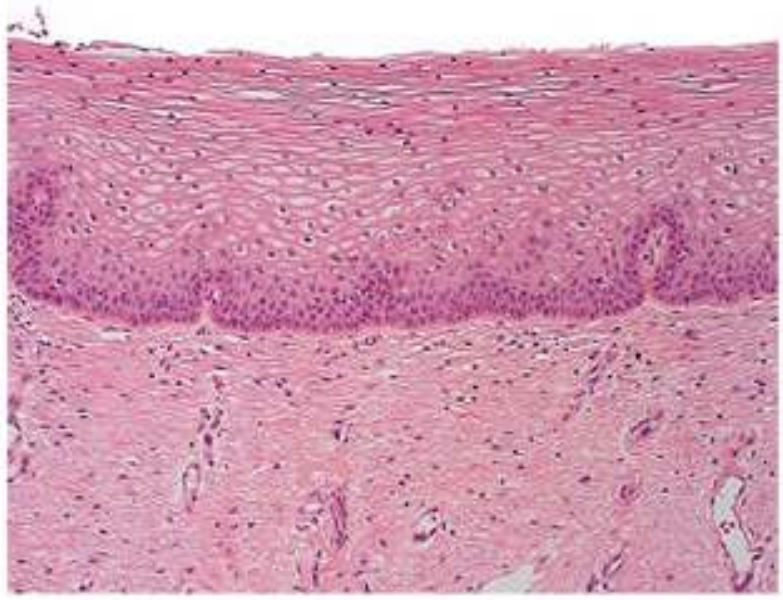
Basal cells are 10um in size with scanty cytoplasm, oval nuclei which are at right angles to basal lamina. The parabasal cells are large cells than previous cells. This layer is meant for epithelial regeneration.[22] Some of the receptors like estrogen receptor(ER), Her 2 neu, progesterone receptor(PR) are seen in this layer. Parabasal cells show mitotic figures, but not in basal cell layers. Markers such as proliferating cell nuclear antigen(PCNA), Ki-67 are positive in parabasal cells.[22]

2. **Intermediate cells** :

They have clear, vacoulated cytoplasm. These are undividing cells which are positive for periodic acid shciff(PAS) stain.[22]

3. **Superficial cells** :

These cells are flattened with large cytoplasm with pyknotic nuceli.[22]



Photomicrograph 3 : Histology of Ectocervix.[22]

HISTORY OF CERVICAL CARCINOMA

In 400BC, Hippocrates-The Greek physician identified warts and had mentioned about cervical carcinoma.[23] His attempt of treating it by a procedure trachelectomy is one of his achivements: although he could not treat the cancer. Aulus Cornelius Celsus also identified distinct type of warts like accrochordon(skin tags), thymion(genital warts) and myremecia (non genital warts).[24]

One of the most ancient Greek physician- Aretaeus, who practiced in 2nd and 3rd century BC, classified uterine carcinoma as superficial and deep ulcer, which would later infiltrate into the uterus. He mentioned about cancer type which doesn't present as ulcer but grows in uterus as mass.(24) He distinguished between these two lesions and declared that the symptoms and prognosis of cancer with ulcers has the most worst progression.

A surgeon named Rigoni-Sten, in Padua in the mid 19th century who had immense interest in epidemiology studied the death certificates of women dying from cervical carcinoma. He observed that uterine cancer was rare in celibate nuns. Epidemiologist belonging to 20th century observed that the cervical carcinoma was more predominant in female sexual workers and also in women whose partners had a higher number of sex partners or who approach sex workers. It was less in Jews.(24)

Zur Hausen and Giesmann in 1983, along with their fellow workers identified Human papilloma virus (HPV)16 in the premalignant lesions of genital cancer. In 1985, they demonstrated the presence of HPV-DNA in cervix cancer cells. Their studies formed as a base work for development of two vaccines - Gardasil and Ceravix in 2006 which were approved by Food and Drug Administration (FDA).(24)

One of the biggest achievement of inventing colposcope by Hinselmann in 1925, stood as important milestone. Similarly, evolution of Pap technique by Papanicolau, the launching of Pap screening test by himself and Traut, invention of specificed spatula by Ayre in 1946 for cervical scrappings were other important milestones in prevention strategy.(24) Universal standardization and documentation of screening test results by Bethesda system in 1980 and its improvisation in 2001 was also an important strategy in reporting cervical lesions and carcinomas.(24)

From the ancient times to present day, the progression and evolution in studied, along with screening techniques leading to early detection of carcinoma and decreased mortality rate.

CERVICAL INTRAEPITHELIAL NEOPLASIA

In early 1880, Sri John Williams quoted on the presence of non-invasive epithelial lesions and invasive squamous cell carcinomas of cervix. Later on in 1900's many studies were done with final outcome of term Carcinoma in situ in 1930's.[22] By the end of 1970, by Kolstand and Kelm, Koss et all, 1963 clearly stated that significant proportion of untreated patients diagnosed with carcinoma in situ developed invasive squamous cell carcinoma. Later the term dysplasia is given to the lesions intermediate between normal cervical epithelium and carcinoma in situ. Dysplasia was treated as potentially reversible process.[22] Cervical intraepithelial neoplasia(CIN) takes duration of 5-15 years to progress to cancer.

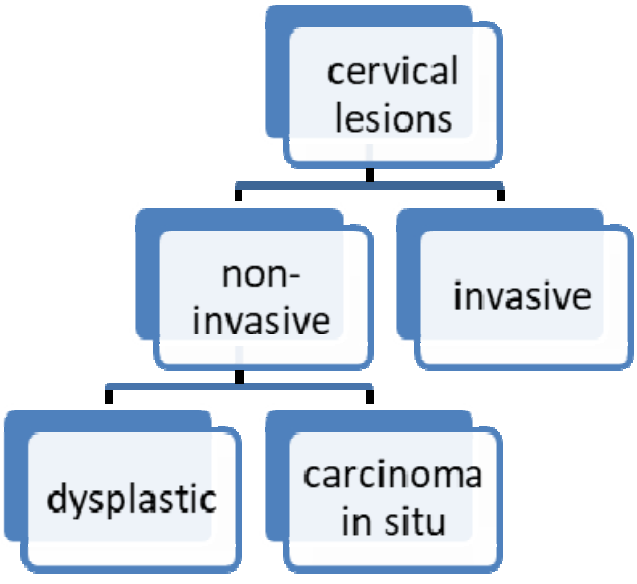


Figure 7 : Schematic classification of cervical lesions.[22]

Over time all the premalignant lesions progress to squamous cell carcinoma represents a single disease entity termed as ***Cervical intraepithelial neoplasia*** (CIN) by Richart in the year 1973.[22]

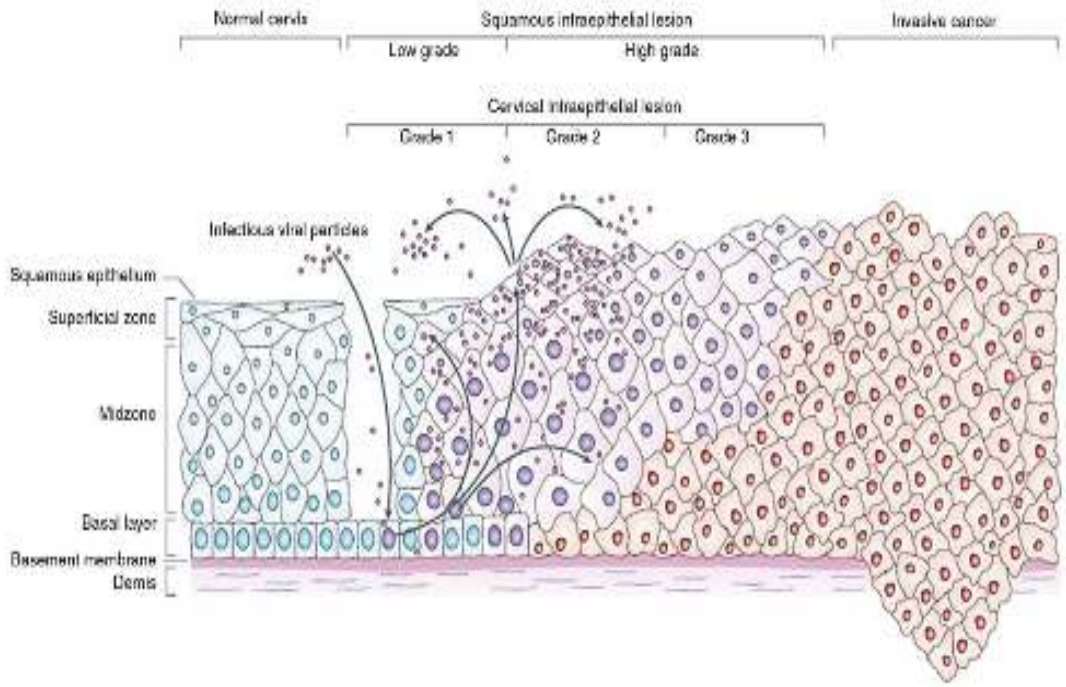


Figure 8 : Picture depicting changes in cervical epithelium from normal tissue to carcinoma.[22]

CLASSIFICATION OF CIN

The most widely accepted classification is by Bethesda. It divides into two groups. [18]

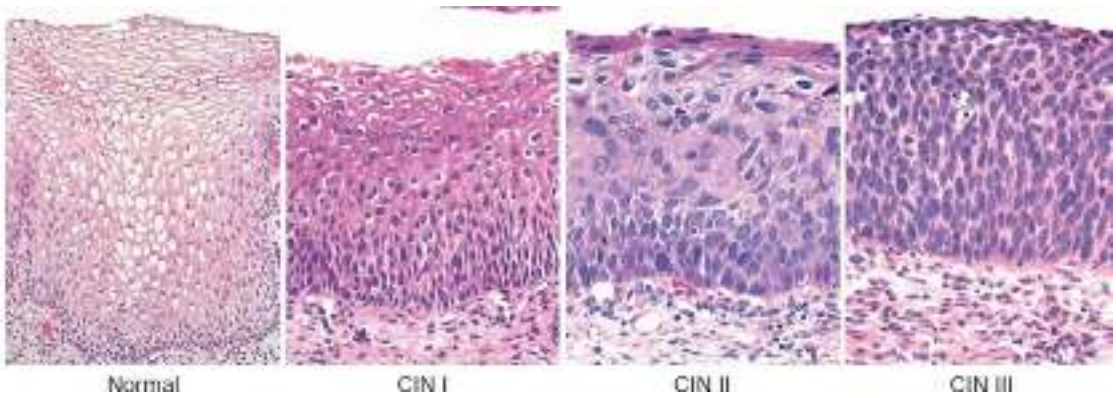
1. Low grade squamous intraepithelial lesion (LSISL)
2. High grade squamous intraepithelial lesion (HSIL)

Table 1: Bethesda System.[18]

OLDER CLASSIFICATION	WHO CLASSIFICATION	BETHESDA SYSTEM
Mild dysplasia	CIN 1	LSIL
Moderate dysplasia	CIN 2	HSIL
Severe dysplasia	CIN 3	HSIL

Low grade intraepithelial lesion : The most characterized feature of LSIL is HPV induced histologic and cytologic changes. These lesions are usually caused by HPV low risk variants. Significant nuclear atypia is utmost important.[22] It is identified by variation in nuclear size with nucleomegaly , hyperchromasia, irregular and wrinkling of nuclear border. Proliferation of basal/parabasl cells is seen extending upto lower third of the epithelium lining. Some of the cells show perinuclear cytoplasmic cavitations or halos along with thickening of cytoplasmic membrane, which is termed as koilocytic aytpia. It is a pathognomic feature of HPV infection.[22]

High grade intraepithelial lesion: Presence of atypia in almost all layers of squamous epithelium is seen. More than lower third of the epithelium is occupied by basal type cells. Nuclear pleomorphism, nuclear crowding and loss of polarity is noted. Cytoplasm is scant with increased nuclear : cytoplasm ratio.[25] Variability in size of nucleus (anisonucleosis) is one of the important feature of HSIL. If immature basaloid cells occupy upto two-third of the squamous epithelial lining it is termed as CIN 2. If it ocupies till the upper one-third of epithelial lining it is termed as CIN 3.[22]



Photomicrograph 4 : Cervical intraepithelial neoplasia -grades.[22]

SQUAMOUS CELL CARCINOMA OF CERVIX

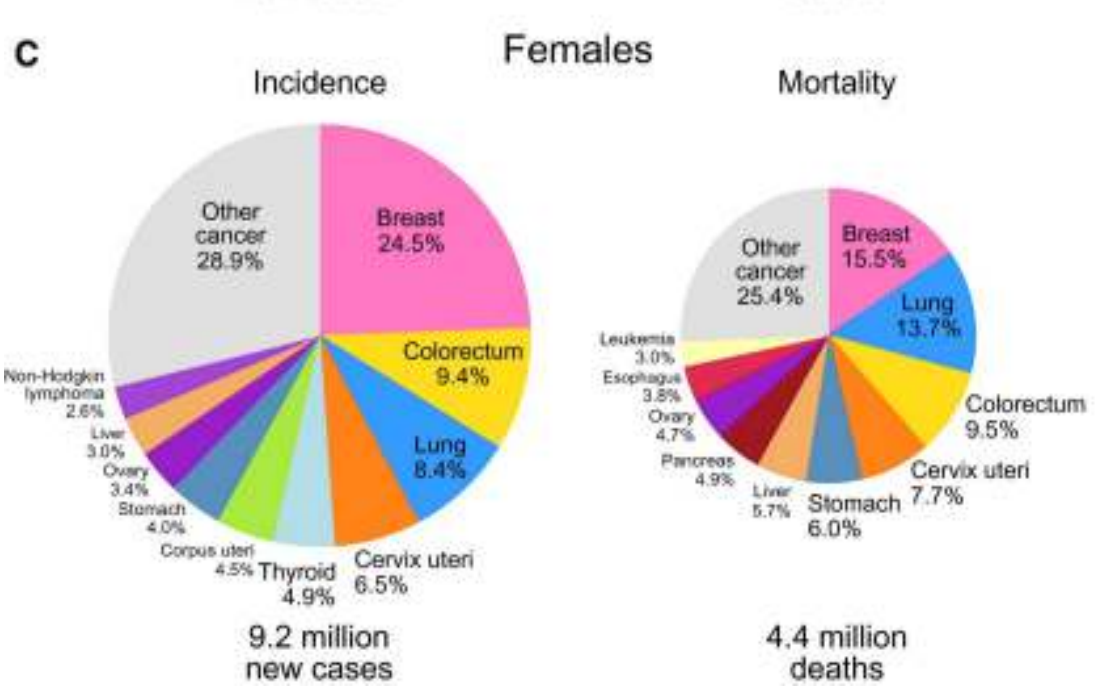
Burden of carcinoma cervix in the community:

Cervical cancer becomes fourth most commonly diagnosed cancer in women. Total estimated cases are 6,04,000 worldwide and also has become the leading cause of mortality with 3,42,000 deaths in 2019.[1]

It is 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.[26]

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.[27]

It is observed that women between 30 to 69 years of age account for 17% of cancer related deaths pointing out as major cause of cancer mortality in this age group.



Graph 1 : Pie chart depicting both incidence and mortality of carcinoma cervix globally[(1)]

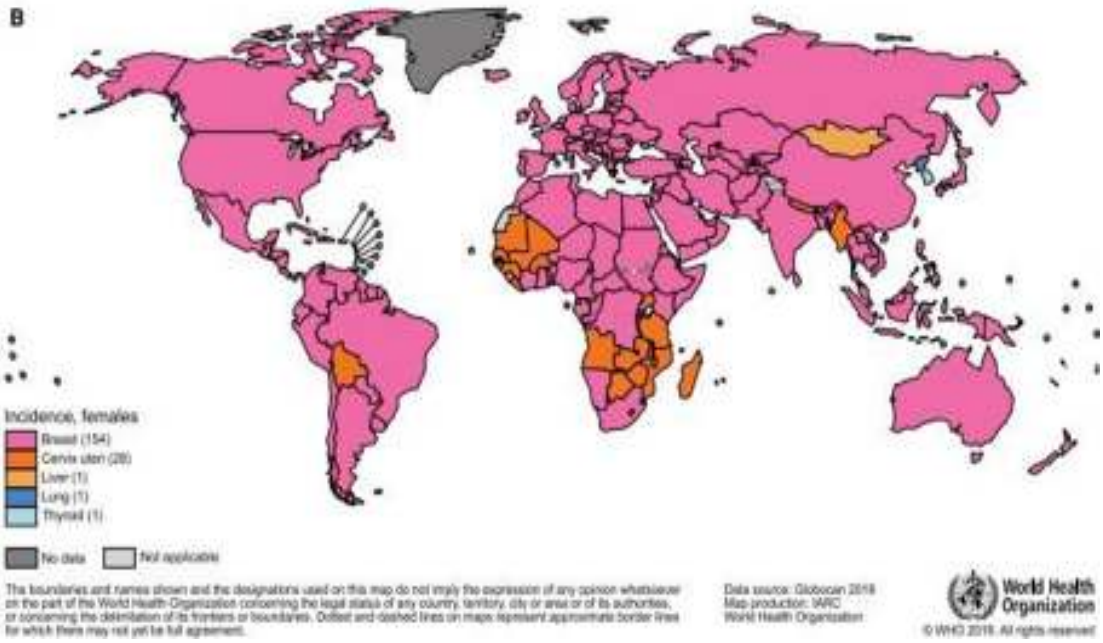


Figure 9 : Global map depicting the incidence rate of most common cancers.[(1)]

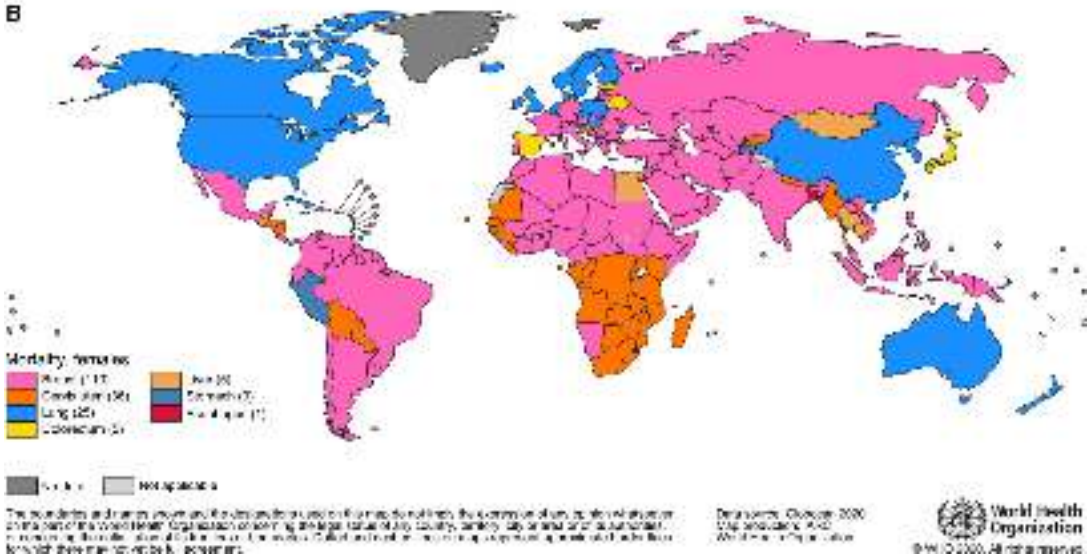
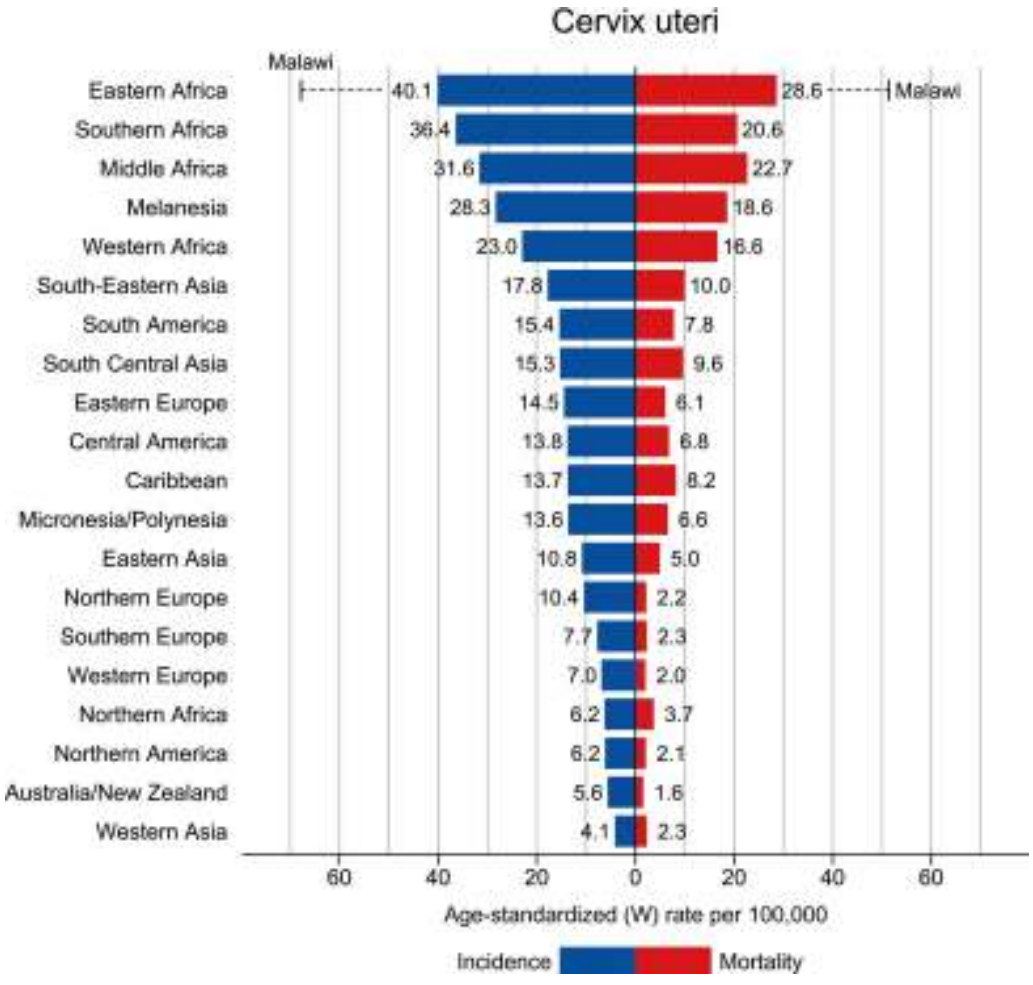


Figure 10 : Global map depicting the mortality rates of most common cancers.[1]

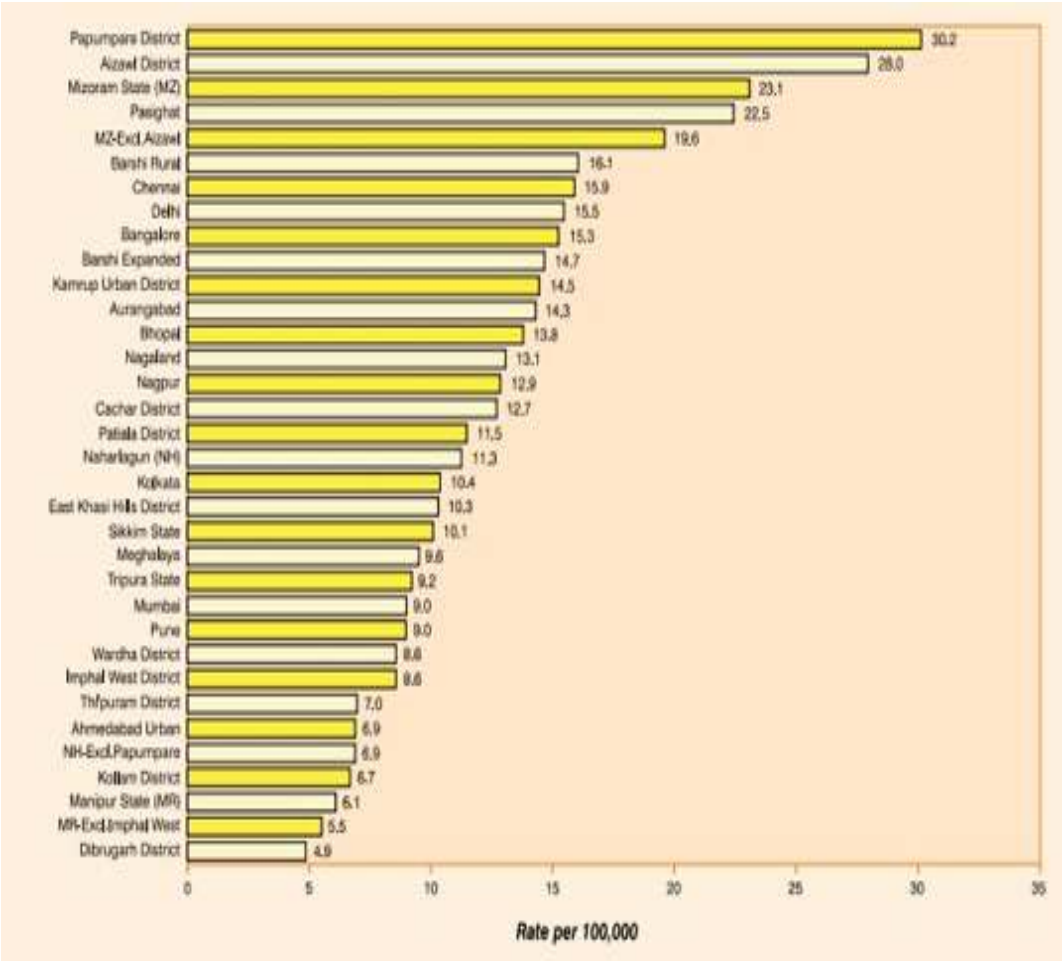


Graph 2 : Region specific mortality and incidence rates[1]

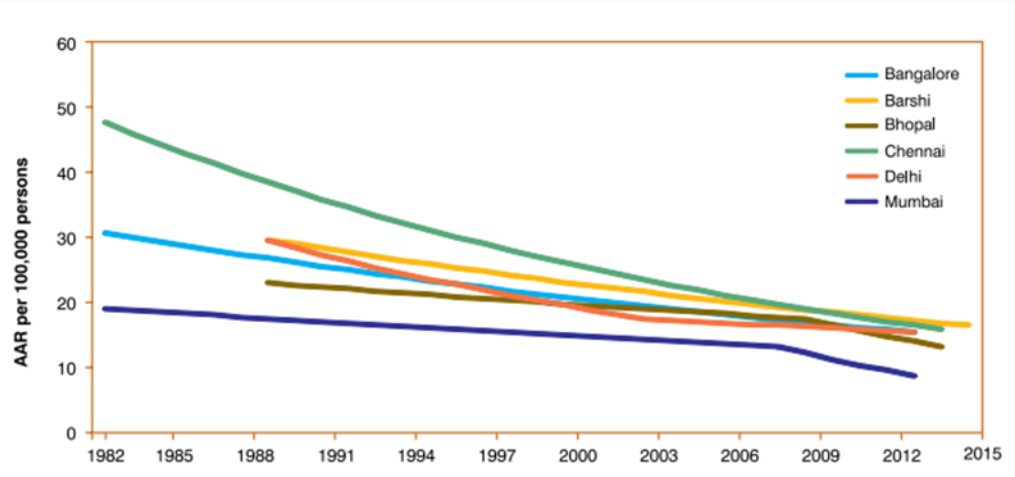
The above chart depicts that there are higher incidence rates in Africa and South east Asia.[1]

In India, according to the recent National Cancer Registry Program (NCRP), cervical cancer contributes to about 6-29% of all cancers in females.(27) Papumpare District (30.2), Aizawl District (28.0), Mizoram state (23.1) and Pasighat PBCR (22.5) occupied the top most places in a population-based cancer registry in a three year report from 2012-2014.[28]

The incidence rate which is age adjusted of cervical carcinomas was found to vary widely among registries.[28]



Graph 3 : Age adjusted incidence rates by NCRP[28]



Graph 4 : Trend of cervical cancer over time in India[28]

Though the above graph shows a decreasing trend for the incidence rates over a period of time for cancer cervix, it still persists to be the major 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.[29]

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.[29,30]

WHO ideally recommends screening of women between the age 30 to 49 years every 3 to 5 years either by VIA (visual inspection of cervix with 3-5% acetic acid) or by Pap tests, or every 5 years through HPV testing.[31]

According to World Health Organization (WHO) 2020, squamous cell carcinoma are categorized under epithelial tumors.[32] It is a invasive epithelial tumour composed of sheets of squamous cells with degree of differentiation, pattern of growth, and cell type. Squamous epithelial tumors are classified as follows:

- Mimics of squamous precursor lesion:
    - Squamous metaplasia
    - Atrophy of the uterine cervix
- Squamous cell tumors and precursors :
    - Squamous intraepithelial lesions of the uterine cervix.
    - Squamous cell carcinoma, HPV associated, of the uterine cervix.
    - Condyloma accuminatum
    - Squamous cell carcinoma, HPV independent, of the uterine cervix
    - Squamous cell carcinoma, NOS of the uterine cervix.

Most common type is the immature non keratinizing tumors associated with HPV-High risk type.[32,33]

The common histologic variant is squamous cell carcinoma (80%), followed by adenocarcinoma of cervix (10-12%) of all cervical cancers. Non – keratinzing type are associated with HPV.[32]

Invasive cancer has three variants pathologically according to Wentz and Reagan in 1958.[34,35] They are :

1. Large cell Non- keratinizng carcinoma.
2. Small cell carcinoma.
3. Large cell keratinizng carcinoma.

Non-Keratinizing tumors: Polygonal squamous cells with intercellular bridges are arranged in sheets or nests. No 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.[34,35]

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.[34,35]

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.[34,35]

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

Small cell neuroendocrine carcinoma

Small cell squamous 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. [28]

MODIFIED BORDER'S GRADE:

It was based on the amount of keratin, nuclear pleomorphism, and the mitotic activity[32,36]:

- **Well differentiated tumours**: These tumours show presence of squamous cells with abundant keratinisation with formation of pearls, few cells with intercellular bridges with mild pleomorphism and less mitotic activity.

- **Moderately differentiated tumours**: These tumours show neoplastic cells with less distinct cell outlines and cytoplasm compared to well differentiated tumours and increased nuclear pleomorphism and high mitotic figures.
- **Poorly differentiated tumours:** These tumours show small primitive cells with very scant cytoplasm, enlarged hyper chromatic nucleus and increased mitotic figures. Keratinization is occasional or absent. It simulates HSIL.

FIGO STAGING OF CARCINOMA CERVIX 2018.[32]

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 bullou 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

Figure 11 : The International Federation of Gynecology and Obstetrics (FIGO) Uterine cervical cancer staging

RISK FACTORS : [37–43]

1. Sexual activity :Early sexual activity (less than 16 years of age) and number of sex partners.
2. Age at the time of first pregnancy.
3. Sexually transmitted diseases: HPV, Chlamydia trachomatis, Herpes simplex virus.
4. Parity : With high parity there is increased risk for cancer of cervix.
5. Low socioeconomic class
6. Cigarrette smoking
7. Persistence infection with high oncogenic risk HPV.
8. Human Immunodeficiency Virus.
9. Immunosupressive co-morbid health conditions.
10. Certain Human leucocyte antigen (HLA) subtypes.
11. Oral contraceptive use-Doubles the risk of HSIL and invasive cancer
12. Intervals since last Pap smear.

SCREENING METHODS.

The methods used for detection of invasive cancer in early stage are : [44]

1. Pap smear testing.
2. Visual inspection of cervix after application of acetic acid.
3. Visual inspection with acetic acid(VIA) with magnification.
4. Visual inspection on application of Lugol's iodine
5. HPV -DNA testing.
6. Unaided visual inspection.

ETIOPATHOGENESIS :

Human papilloma virus is the main cause of many cervical diseases ranging from condyloma accuminata to squamous cell carcinoma. Cervical carcinoma is the most commom human cancer attributable to the effects of Human papilloma virus.[22]

HPV belong to papillomaviridae family. These are double stranded DNA tumor viruses with base pairs of 8000 in its genome. It is a non-enveloped virion measuring 45-55nm in diameter with a capsid comprising of 72 capsomeres.[22] More than 40 variants of HPV can infect anogenital tract of which 20 types are common. Oncogenic risk of different types of anogenital HPV are : [22]

LOW RISK – HPV 6,11, 42, 43, 44, 53.

HIGH RISK - HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68.

UNCLEAR RISK – HPV 26, 66, 73, 82.

Most of the HPV infection cause low grade squamous epithelial lesions, more than 90% of which spontaneously regress and about 10% become transforming infection with molecular changes. [2,31] The prevalence of HPV 16, 18 genotype show strong association with the risk to develop intraepithelial lesion, squamous cell carcinoma and cervical adenoma carcinoma.[2,31,32] HPV 16 prevalence increased with increasing grade of lesions i.e it is found more in CIN 3 than CIN 2 and CIN 1.[22] Understanding of genomic organization of HPV is utmost important in the pathogenesis of cervical dysplasia and carcinoma. HPV genome has three different regions :

An upstream regulatory region (URR) which is also known as long control region(LCR), second the early region and the late region.[22]

Table 2 : HPV Genome : Its regions and functions.[22]

REGION	CODING/NON CODING	FUNCTION
LCR	Non coding region	Viral replication and transcription
Early region	Coding region	Encodes protein involved in viral replication
Late region	Coding region	Encodes for structural protein of virus.

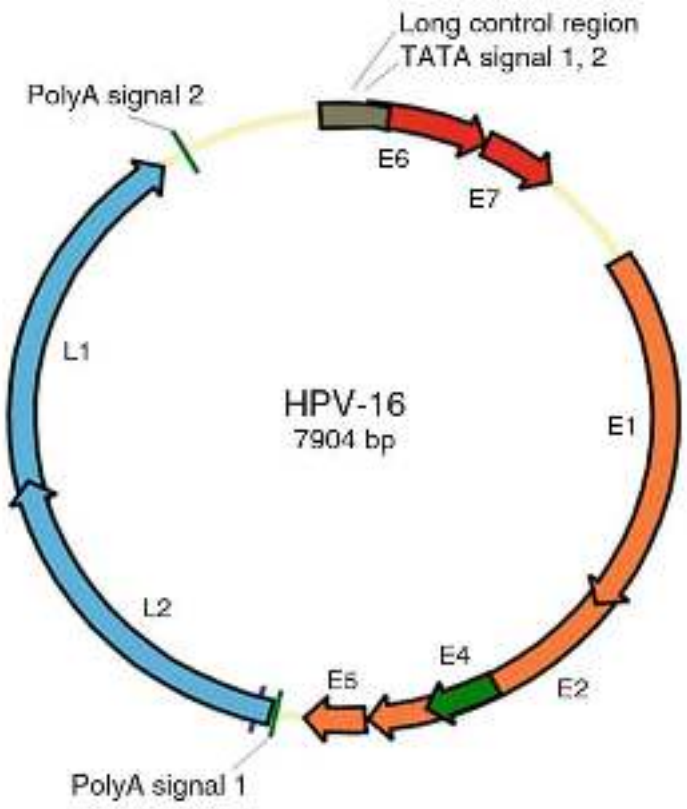


Figure 12 : Genomic organization of HPV.[22]

As HPV being a epithelotropic virus, productive papilloma virus infection begins when virions gain entry to basal cell layer through microwounds in the epithelium. Transforming of infectious condition to HSIL and invasive cancers occurs when virus gain accesses to squamous columnar junction.[22] The early HPV gene E and E2 support viarl DNA replication, makes the infected cells to remain for longer duration in the lesion. Viral DNA replication is dependent on host replication except for viral helicase E1. HPV 1, on entering the basal cells limits the differentiation of infected cells which is largely mediated by E6. In the lower epithelium, the expression of E7 stimulate proliferation of cells. E6 and E7 induce unorganized reentry of the cell into S phase of cycle, activating the host replication machinery for viral genomes amplification. E6 binds to p53 resulting in degradation of p53 ubiquitin dependent pathway by proteolytic enzymes resulting in blockage of apoptosis. Similarly, E7 attaches to retinoblastoma (Rb) gene, resulting in blocking of cell proliferation inhibiting function of these endogenous tumor suppressors. The end results of overexpression of both E6 and E7 leads to unrestricted proliferation of cells and blockage of apoptosis.[22,45]

Viral DNA multiplication occurs predominantly in the superficial and intermediate cell layers of stratified squamous epithelium. There is over expression of E6 as well as E7 protein when viral infected basal cells undergo maturation and move towards the surface. All these changes lead to assembling of intact virions in larger amounts in superficial cells, producing viral changes. This can be observed both cytologically and histopathologically. These changes include cytoplasmic vacoulization, acanthosis, koilocytosis, multinucleaton and nuclear atypia.

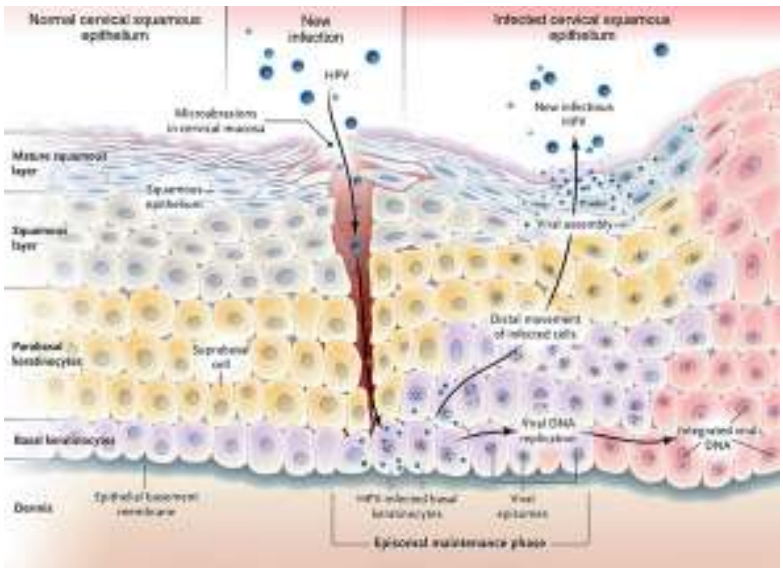


Figure 13 : Life cycle of HPV.[22]

PROGNOSTIC MARKERS IN CERVICAL CARCINOMA[18,46–49]

1. Clinical stage : includes time of presentation and age of patient.
2. Nodal status : It is a crucial predictor in prognosis.
3. Size of the largest involved node and total number of lymph nodes positive for metastasis.
4. Size of the primary tumor in its greatest dimension.
5. Depth of invasion :

RISK	SIZE OF TUMOR	DEPTH OF INVASION
LOW RISK	< 2 cm	irrespective
	2.1 – 3 cm	<= 1.5cm
INTERMEDIATE RISK	2.1- 3 cm	>1.5 cm
	>3cm	<=1.5cm and >=1.5cm

6. Endometrial extension : The survival rate is decreased by a factor of 10-20%.
7. Parametrial involvement : On microscopic examination.
8. Invasion of blood vessels.
9. Microscopic grading: Whether the degree of tumor differentiation as evaluated in routinely stained sections correlated with survival independently from staging is a controversial issue. The two types of grading used is Reagen-Ng and Broder's method.
10. Tumor associated tissue eosinophilia (TATE): Studies where done assessing the survival rate basing on mature eosinophils in inflammatory infiltrate of carcinoma. More number points out good prognosis by some authors and poor prognosis by others.
11. Keratin pearls : They have no predictive valve.
12. Cell proliferation index : High rates are associated with poor prognosis.
13. Angiogenesis : No evidence is seen between blood vessel involvement and prognosis.

BIOMARKERS IN CERVICAL CARCINOMA[50–52]

There are multiple markers which have been evaluated in cervical carcinoma. They are enlisted as follows:

1. HPV DNA : High risk(Hr) HPV DNA is seen in almost all cervical carcinoma and hence is not important in depicting the prognosis. However Hr-HPV variant or copy number differs among patient and could be utilised as prognostic cell biologic marker. Increased values of HPV E6/E7 mRNA expression decreases overall survival outcome.

2. Angiogenesis markers : Various markers like Vascular endothelial growth factor (VEGF), hypoxia inducible facto 1-alphaF(HF-α) and angiogenesis inhibitor thrombosporidin (TSP-1) are been evaluated. Strong expression of HIF-α marker indicates poor survival, VEGF is involved in neovascularization of tumor, leading to poor survival rate.
3. Apoptosis marker : Bax, Bcl -2, Bcl-xl, Murine double minute 2(MDM 2) and p 53 are some of the apoptotic markers. The expression of Bcl 2 enchances cell survival. MDM 2 inactivates tumour suppressor gene p53 enchancing cell survival. Expression of Bcl -2 favours better survival.
4. Cell cycle regulation markers : Cyclin dependent kinase (CDKs), regulates cell cycle which in turn is mediated by cyclins and its inhibitors (Cip, Ink 4, Kip inhibtors such as p16, p27 and p 21). Loss of CDK inhibitors and disregulated CDK can lead to carcinogenesis and proliferation of tumor.
5. DNA charecteristics : Tumor cell with specific DNAploidy and DNA index have disease biological behavior and may have prognostic impact. Proliferation index, which is recorded as the percentage of G2 and M plus the percentage of S phase fraction of cells has been related to poor survival.
6. Epidermal growth factor receptor (EGFR) : EGFR and Her 2μ are receptors of tyrosine kinase, from EGFR family. Few studies demonstrate moderate / strong EGFR and positive C-erb-2 immunostaining as bad prognosis while other studies disagree this relation.
7. Metastatic markers : CD 44 is a transmembrane protein, a member of adhesion molecules family. CD 44 V6 immunostaining depicts few survival outcome by very few studies. Products of NM 23 gene is

nucleoside diphosphate (NDP) kinase and its reduce expression has been associated with high metastatic potential of tumor cells. Positivity of NM 23 gene indicates less chance of survival in early cervical carcinoma.

8. Proliferation markers : Proliferation of tumor cells is an specific characteristic behavior. Markers like BM 28/ Mini chromosome maintenance protein 2 (HsMCM 2), Ki 67 are evaluated. No relation with survival rate has been observed.
9. Serum tumor markers : Antigen for Squamous cell carcinoma(SCC-Ag) can be detected in cytosol of normal squamous cells, but in very minimal amount. It is analyzed using immunoassays. Patients with high pre-operative serum SCC-Ag level indicates poor survival. Other makers like CA-125 are also used. Combined use of CA-125 and SCC-Ag, which is called double tumor marker (DTM) is used to assess the survival of patient.
10. Miscellaneous markers : C-Met which is a tyrosine protein kinase Met or Hepatocyte growth factor receptor(HGFR) overexpression indicates poor survival. Other markers like platelet derived growth factor (PDGF), Fragile histidine trial(FHT), Urokinase type plasminogen activator (UPA), and Plasminogen activator inhibitor type 1(PAI-1) shows no relation with survival in cervical carcinoma patients.

REVIEW OF IMMUNOHISTOCHEMISTRY(IHC)

A method used for localizing and identifying specified antigens in tissues or cells based on antigen-antibody reaction is called ***immunohistochemistry or immunocytochemistry.*** The main tool is binding of an antibody with its antigen at a light microscopic level. Starting back from 1940, history of IHC can be studied, when Coons developed an immunofluorescence method to detect antigen in frozen section. However this method was applied in surgical pathology in early 1990s. Later on few technical developments lead to various applications of IHC in dialy use. Auraneas, Nakane and his colleagues developed enzymatic label(horseradish peroxidase) allowed visualization of the known antibody by light microscopy in the presence of appropriate colorogenic substrate system. (32)

In 1974, antigen processing is demonstrated from formalin fixed paraffin embedded (FFPE) tissues for first time by Taylor and Burns. Critical issue is the need to attain greater sensitivity. Maximum sensitivity would allow staining of FFPE tissue from a simple one step direct conjugate method to multiple step detection method such as biotin streptavidin (B-SA), Avidin biotin conjugate (ABC) and peroxidase antiperoxidase (PAP). These would eventually lead to amplication method and highly sensitive polymer based labeling system.(42) Its use in diagnostic and surgical pathology has evolved so much that the use of single or multiple IHC stains is now routine in daily practice. It has been adapted for assessment of prognosis and as predictive markers.(42)

Huang introduce enzymatic digestion prior to IHC staining to unmask few antigens that had been altered during formalin fixation. In 1991, antigen retrieval technique developed by Fraentel-Conrat, Shi et al was thrown into light basing on

few biochemical studies.[53] Antigen retrieval(AR) technique is an easiest method than enzyme digestion which is done by heating routine paraffin tissue sections at higher degree temperature before the procedure of IHC staining. After AR pre-treatment, staining intensity of IHC has increased dramatically. Worldwide application of AR-IHC in pathology made IHC staining easy and expanded its application in molecular pathology.[53]

p-STAT3 MARKER

pSTAT-3 marker implies signal transducers and activators of transcription(STAT) proteins which belong to transcription factors family. This family is comprised of seven different members STAT 1, STAT 2 , STAT3, STAT 4, STAT 5A and 5B and STAT 6.(12,54) The STAT3 gene is situated on the long (q) arm of chromosome 17 at 12.31.[12] These transcription factors undergo activation by series of extracellular signaling protein such as, growth factors(epidermal growth factor , insulin like growth factor 1), cytokines (IL-6) and hormones that bind to surface receptors of cell, leading to phosphorylation forming homo to hetero dimers, consequently causing translocation to the nucleus resulting in disturbing physiological cell process like cellular differentiation, proliferation, angiogenesis[55] and apoptosis, migration depending on the signal tissue and cellular context. It is both cytoplasmic and nuclear antigen.[12,56]

STRUCTURE OF STAT 3 marker. (37)

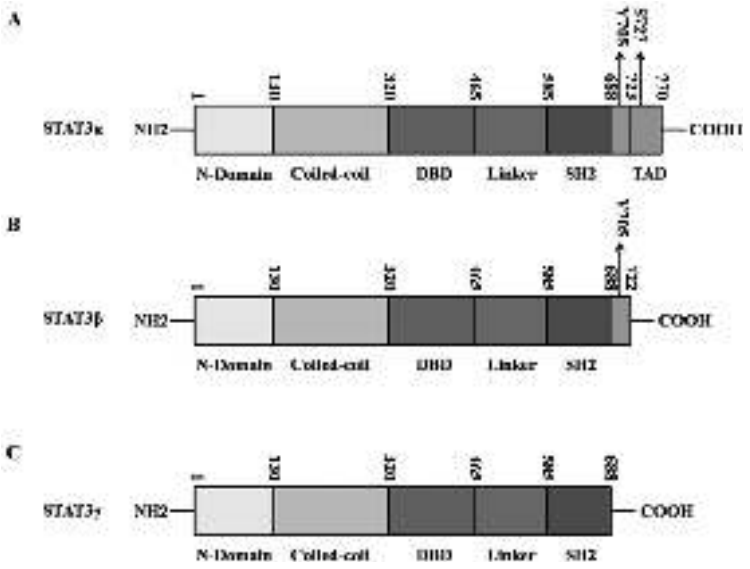


Figure 14 : Schematic structure of p-STAT3 protein.(37)

STAT3 alpha is full length STAT3 protien, STAT3 beta is truncated form of STAT3 alpha which does not contain TAD and s727 while STAT3 gamma is STAT3 alpha with does not have TAD, s727 and Y705.(37)

Table 3 : Parts of p-STAT 3 protein and their functions.

PARTS OF STAT3 PROTEIN	FUNCTIONS
ND : N-Terminal Domain	Dimer formation
CCD : coiled-coil domain	Acts as dimeration tag,Nuclear location signal
DBD : DNA binding domain	Binds to specific DNA sequence
(SH)2 : Src homolgy	Mediates docking of STAT to phosphorylated tyrosine residues.
TAD : C terminal transcriptional activation domain	Gene expression

NORMAL MECHANISM OF JANUS TYROSINE KINASE(JAK)/STAT PATHWAY.

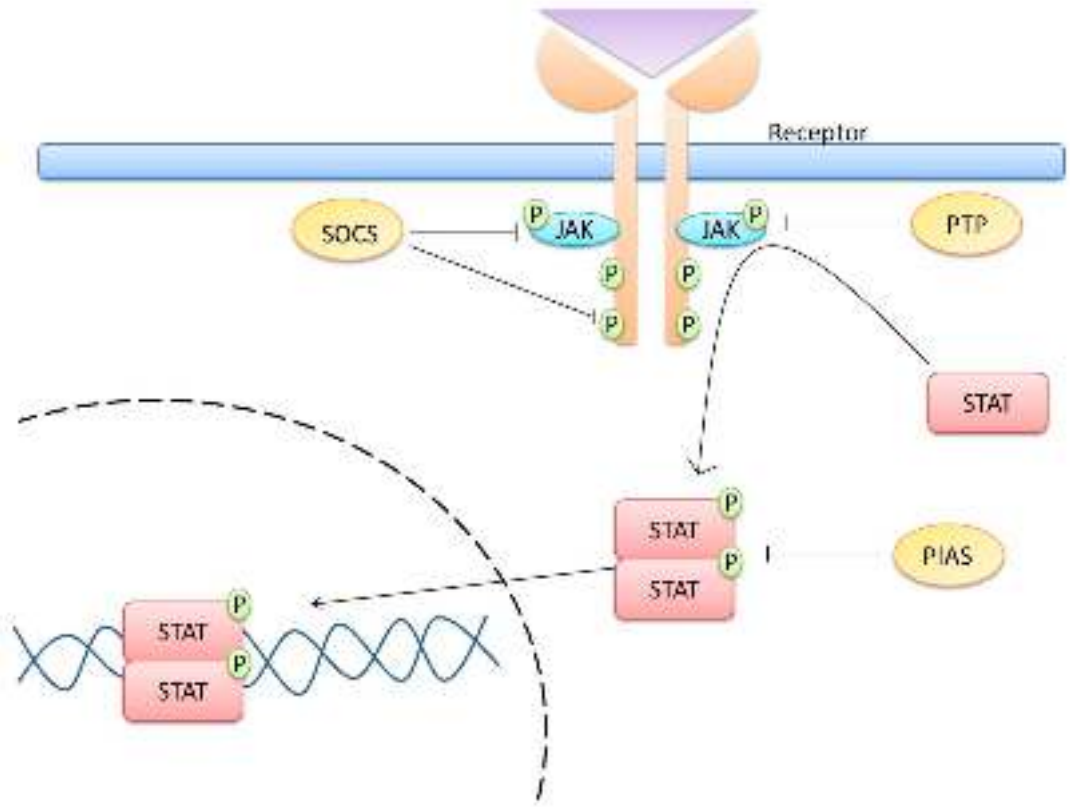


Figure 15 : Mechanism of JAK-STAT pathway. [37,38]

STAT 3 is found to be mostly associated with uncontrolled growth leading to neoplasm. Its activation is seen in many other multiple cancers which include ovary, breast, lungs, head and neck, and prostate.[38–42]

MECHANISM IN TUMOROGENESIS :

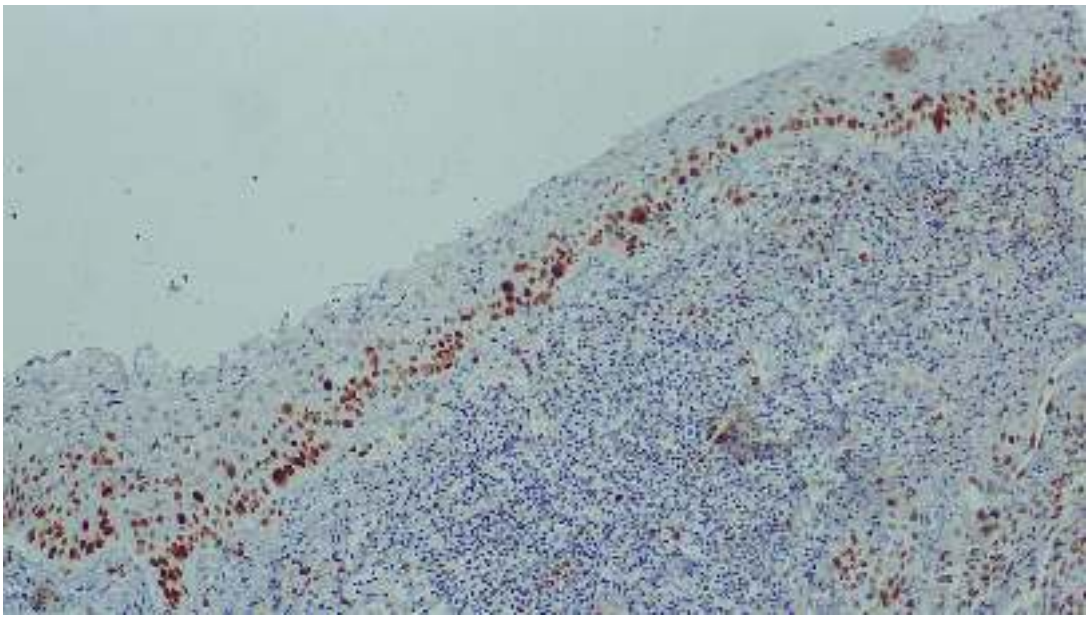
1. Dysregulation of genes which encode the antiapoptotic protiens Mcl-1, Bcl-xl and Bcl-2 as well as proteins involved in proliferation, cyclin D, and Myc by participation of persistently activated STAT3 is the reason for oncogenesis.[59]
2. It regulates adaptive and innate immune responses during development of cancer.[60]
3. It induces tumor angiogenesis by upregulating Vascular endothelial growth factor(VEGF) expression.[61]

Ki-67 MARKER

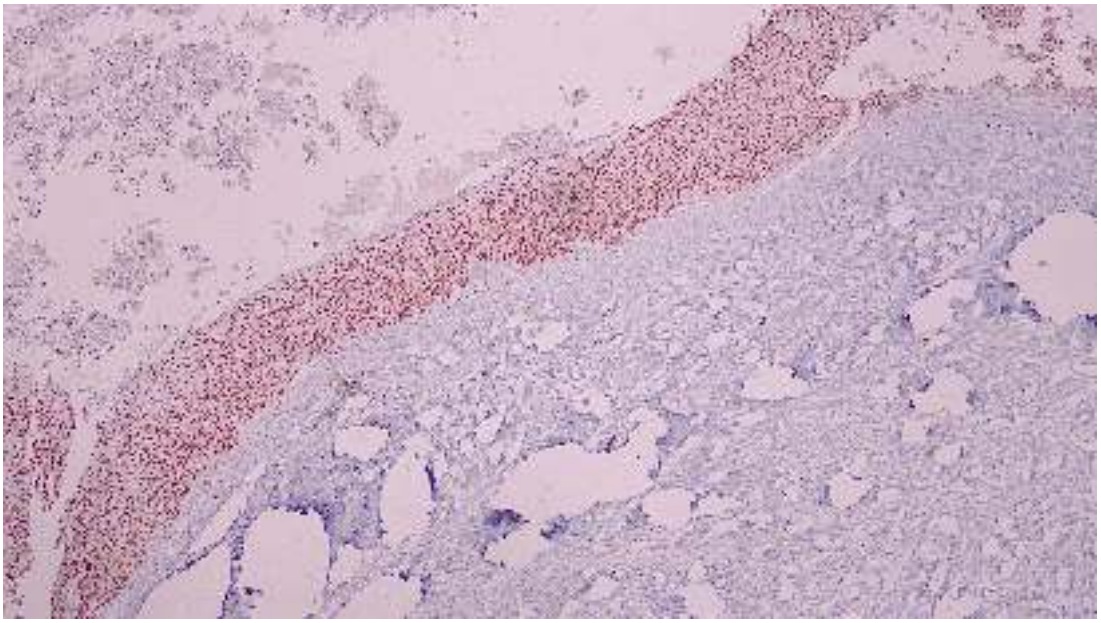
Uncontrolled tumor proliferation activity is one of the cause for tumor formation. This molecular basis of tumor aggressiveness can be separated by a proliferation markers Ki-67. It is a nuclear antigen expressed during all phases of active cell cycle.(G1, S, G2, M) except in G0.[62] It depicts the cell proliferation status. It can be detected by both qualitative and quantitative methods i.e monoclonal antibody in immunohistochemical assay, electron microscopy, ELISA, flowcytometry and IHC.[62–64]

Ki-67 is confined to the dividing cells only which can be either normal physiological or tumor cells. It is not expressed in resting cells. In normal cervical squamous mucosa, parabasal epithelial cells are positive for Ki-67, as dividing immature cells are present in deeper layers. As the cells grow upwards, it matures resulting in failure of uptake of Ki-67.[7,13] It is considered as an independent predictor of CIN and CIN progression to cervical carcinoma.[13] During HPV infection, the transition of cell from G1 phase to S phase is stopped by retinoblastoma(Rb) protein

and Rb itself is decomposed. As an end result, Ki-67 expression is observed during proliferation of cell which occurs due to infection by human papilloma virus .[41]



Photomicrograph 5 : Ki- 67 expression by basal cells of normal cervical epithelium. 10x.



Photomicrograph 6 : Show Ki-67 expression in all the layers of ectocervix in cervical carcinoma.(4x)

REVIEW OF pSTAT 3 MARKER:

Several studies were done to assess the pSTAT3 expression in squamous cell carcinoma.

A study was conducted by Sheau-Fang Yang et al from Taiwan Graduate institute of Medicine, Taiwan to evaluate the role of p-STAT 3 and association with Ki-67 on all cervical intraepithelial neoplasia. They observed a strong expression of p-STAT3 in cervical intraepithelial neoplasia than in normal cervical tissue. In addition they also observed a significant association with ki-67.[7]

Another study conducted by Shirish shukla et al at Institute of cytology and prevention oncology, Uttar Pradesh analysed STAT3, HPV 16, E6, E7 and p53 expression on HPV infected cervical cell lines concluded saying that HPV 16, E6, E7 positive cells revealed higher levels of p STAT 3 expression than HPV negative cells indicating a strong association between p STAT3 and E6/E7 which leads to cervical cancer.[44–46]

Sobti R.C, Neha Singh et all study shows a significant level of mRNA expression of p-STAT3 was found in majority of cervical cancer cases compared to control.[12] They conducted this study on biopsies samples obtained from 100 patients diagnosed with cervical cancer of various stages. Similar study conducted by C-L Chen et al from Centre for Childhood cancer, Columbus USA studied expression of STAT3 activation in cervical and endometrial cancers. They examined cervical cancer tissue utilizing tissue microassay slides and cancer cell lines.[12]

A study conducted by S Takemoto et al in 2009 from Department of Obstetrics and Gyneacology, from Kurune Univeristy School of Medicine , Japan observed 56.8% positive expression of p-STAT3 in 125 cases of squamous cell of cervical cancer patients who undergone extended hysterectomy along with pelvic lymphadenectomy. They further studied metastasis to lymph node, invasion into lympho-vascular space and diameter of the tumor.(65)

Some of the studied were done on genetic background on STAT 3 expression to prove that abnormal expression /activation of STAT family is the cause of cervical cancer .Some of such studies are taken as strengthening pillar to correlate with my present study.

Wang Kana, Zhou Bin et l from Gynecology and Obstetrics Department and laboratory of Molecular Translational Medicine, Sichuan, China conducted a study to suggest that STAT 3 polymorphism was associated with less susceptibility, poor differentiation and parametrial invasion in Chinese women diagnosed with cervical cancer in year 2011. This study was done on 275 cervical cancer patients and 340 normal patients. Techniques of polymerized chain reaction (PCR-restriction fragment length polymorphism and DNA sequencing analysis were the modalities used in their study. (66) They concluded that allele G of rs4796793 was associated with susceptibility and with few pathological features of cervical cancer. Polymorphism of the above mentioned single nucleotide was associated with STAT 3 level but not with STAT 5A/5B suggesting that this single nucleotide peptide is one gene that was associated with cervical cancer.(66)

Another study conducted by Zhou Ying, Li Min et al from Department of Pathology, Laboratory of Molecular Medicine China on all cervical carcinoma. They studied on mechanism which resulted in failure of control over STAT 3 activity. Retinoid-interferon induced mortality-19 (GRIM-19) gene is a tumor suppressive protein recognised by genetic technique in the interferon/retinoid-induced cell death pathway.[67] They showed that decrease in GRIM-19 protein levels were reduced in human cervical cancers. They reported a significant downward regulation of GRIM-19 protein levels in several cervical cancers which in turn resulted in increased expression of STAT 3 and its downstream genes. Restoration of GRIM-19 levels in cancer cells proportionately restores growth suppression in vivo. Cell cultures, IHC staining, HPV DNA testing, RT-PCR, immunobloting were the technique used in their study.[67]

A recent study conducted by Ethan L.Morgan and Andrew Macdonld at Tumour Biology section and School of Molecular and Cellular Biology UK in 2020 studied on targeting the JAK/STAT pathways directly or indirectly. They proposed a double stranded shorter decoy oligonucleotide which potentially inhibits STAT3 in Head and Neck SCC in vivo which can be used intravenously. Further study is carried on to implement the drug in all HPV causing malignancies. They recently demonstrated that Ruxolitinib and Fedratinib which are two JAK inhibitors were beneficial in HPV positive cervical cancer cells.[68–70]

METHODOLOGY

The study was done at 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/418).

Study Design: Observational Cross Sectional study

Study Period: January 2019 – December 2020

- One-year prospective 2020
- One-year retrospective 2019

Study population: Old (2019) and new (2020) specimens of cervical biopsy received in the histopathology laboratory (diagnosed as squamous cell carcinoma of cervix on histology) at Department of Pathology, Jawaharlal Nehru medical college and Dr Prabhakar Kore Hospital and MRC.

For retrospective cases, data as well as tissue blocks were retrived from storage.

Inclusion criteria: All the new histopathologically diagnosed cases of squamous cell carcinoma by cervical biopsies.

Exclusion criteria:

Inflammatory lesions of cervix-cervicitis

Adenocarcinoma of cervix.

Hystercetomy done for other causes like Dysfunctional uterine bleeding, fibroids.

Cervical intraepithelial neoplasia.

Metastatic lesions of cervix.

Sample Size: 30 .

Total of 30 paraffin embedded tissue blocks were collected during the specified time interval.

For the new cases clinicals data was obtained from patient's outpatient card and requistions received to department along with biopies. For retrospective cases, data was obtained from records and blocks were retrieved from the department.

Sampling procedure :

All the 30 formalin fixed paraffin embedded blocks were collected. From each block three sections of two to three microns thickness are obtained using rotatory microtome (Leica RM2245). Each section is floated on tissue floatation bath maintained at 50-55-degree Celsius. The first section was taken on regular glass slide coated with Egg albumin as adhesive which was stained with Hematoxylin and Eosin. The remaining two sections were taken on commercially available glass slides coated with Poly-L-lysine for immunohistochemistry with p-STAT3 and Ki 67 markers.

After staining with Haematoxylin and Eosin, 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 :

Both nuclear and cytoplasmic staining was considered positive for p-STAT3 expression and complete nuclear staining is considered positive for Ki-67 expression.

The percentage of dysplastic cells which are immunoreactive for p-STAT3 are grades by four tired semi-quantitative system : [7]

PERCENTAGE OF TUMOUR CELLS POSITIVE FOR P-STAT3 and Ki-67 EXPRESSION	**GRADE**
< 25% positive	Grade 1
26-50 % positive	Grade 2
51- 75% posistive	Grade 3
>76% positive	Grade 4

More than 1000 cells expressed in 3 to 4 different high power field areas of tumor are analyzed for each smaple.[7]

GRADING OF INTENSITY OF P-STAT3 marker[7]

Low intensity	Grade 1/Grade 2
High intensity	Grade 3/Grade 4

Both nuclear and cytoplasmic expression of p-STAT3 is correlated and with Ki-67 expression also. Further it is correlated with histopathological grading.

ANALYSIS PLAN:.

All the data collected was analyzed using SPSS statistical software (version 20.0) and Microsoft word and excel have been used to generate tables and graphs. Comparison between p-STAT3 expression (both cytoplasmic and nuclear) and Ki-67 expression was made using Fisher's Exact test.

$P < 0.05$ was considered statistically significant.

RESULTS

Present study was conducted at Department of pathology, Jawaharlal Nehru Medical college, KAHER Belagavi. 30 cases of cervical biopsy which were histopathologically diagnosed as SCC during the time period January 2019 to January 2020 were studied. All the cases were evaluated for Hematoxylin & Eosin stain, followed by p-STAT3 and Ki-67 expression and were compared with the histological grading.

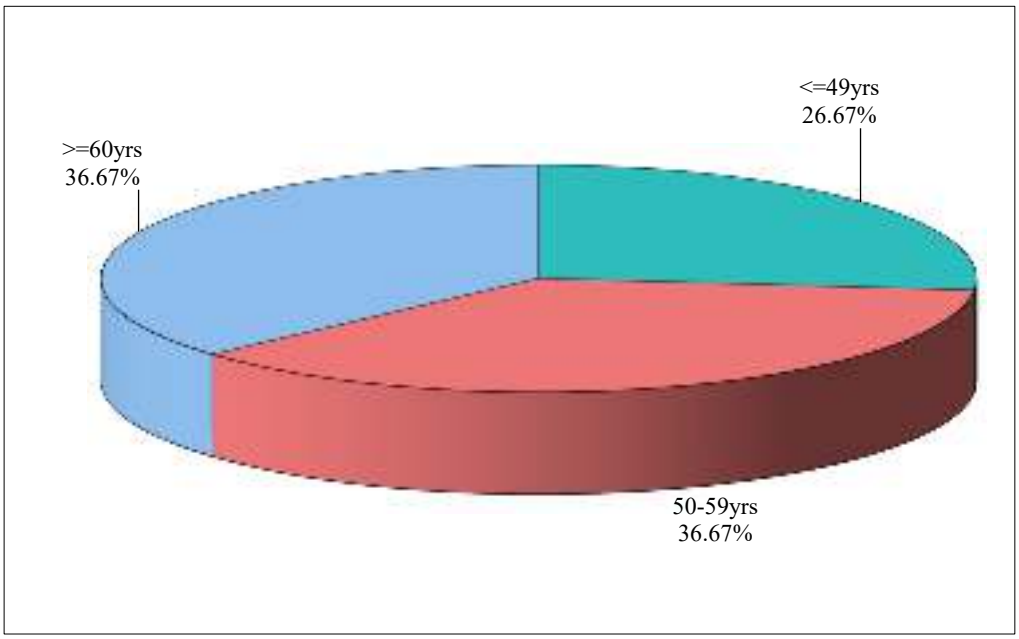
All the cases were cervical punch biopsies.

In our study, age of the patients ranged between 35 to 70 years with mean age of 53.93 ± 9.56 years. The peak incidence of carcinoma was seen between 51-60 years of age. (Table 4, Graph 5)

Table 4: Age wise distribution of cases

Age groups	Number of cases	% of cases
<=49yrs	8	26.67
50-59yrs	11	36.67
>=60yrs	11	36.67
Total	30	100.00
Mean ± SD	53.93 ± 9.56	

Graph 5: Age wise distribution of cases.

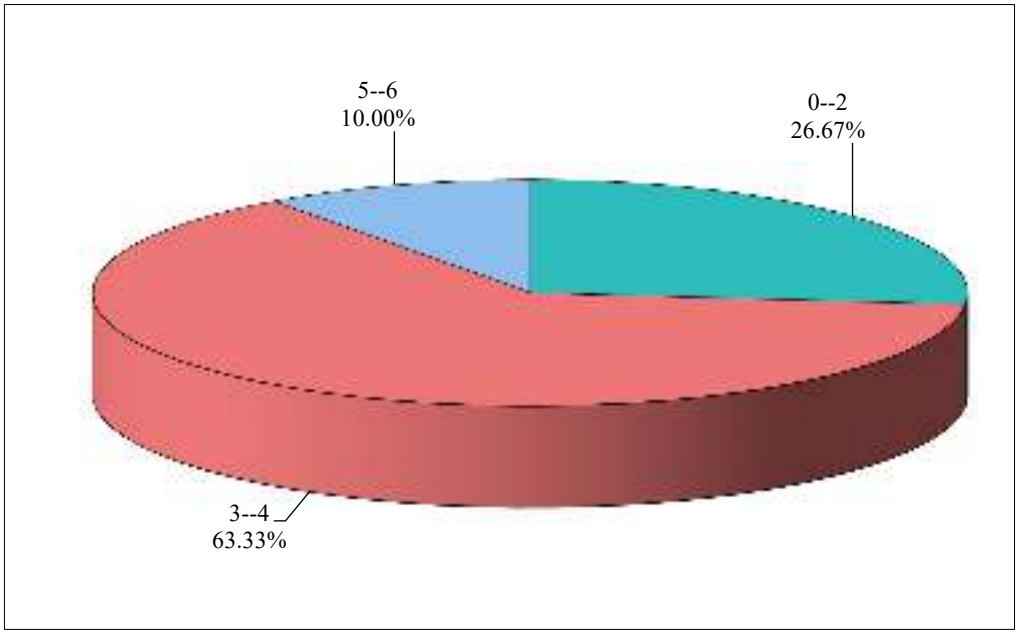


All the patients were married. Of 30 cases, 8 patients (26%) were of parity 0 to 2, 19 patients (63.33%) were of parity 3 to 4 and 3 patients (10%) were of parity more than 5 to 6. Maximum number of cases had parity of 3 to 5 (63%). (Table 5, Graph 6)

Table 5: Parity wise distribution of cases

Parity	Number of cases	% of cases
0--2	8	26.67
3--4	19	63.33
5--6	3	10.00
Total	30	100.00

Graph 6: Parity wise distribution of cases

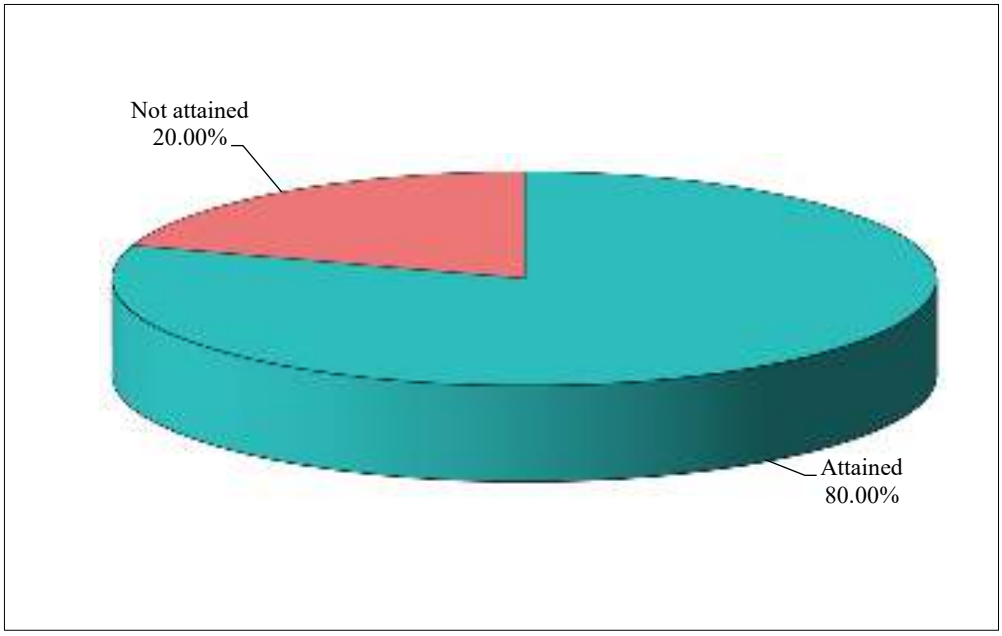


Our study includes 6 women (20%) in reproductive age group and 24 (80%) were postmenopausal women (80%). It was observed that cervical cancer is common in postmenopausal women. (Table 6, Graph 7)

Table 6: Distribution of cases based on menopausal status

Menopause	Number of cases	% of cases
Attained	24	80.00
Reproductive age group	6	20.00
Total	30	100.00

Graph 7:Distribution of cases based on menopausal status

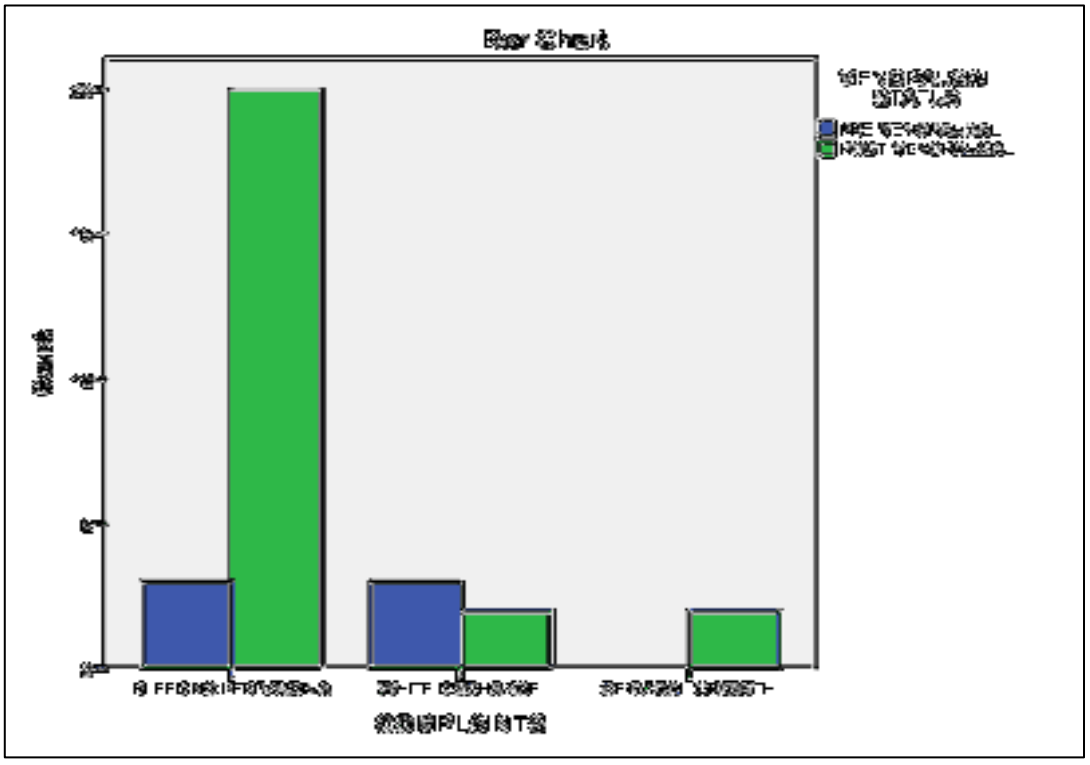


Majority of the patients presented with symptom of per vaginal bleeding(76%).Remaining cases complained of white discharge (16%) and cervical growth (6.6%) On clinical examination, there was a visible cervical polyp in one patient, and cervical growth in two patients. (Table 7, Graph 8)

Table 7 : Distribution of cases based on complaints.

Symptoms	Pre menopausal	Post menopausal	% of cases
Bleeding per vagina	3	20	76.6%
White discharge	3	2	16%
Cervical growth	0	2	6.6%

Graph 8 : Distribution of cases based on complaints



Histopathological examination:

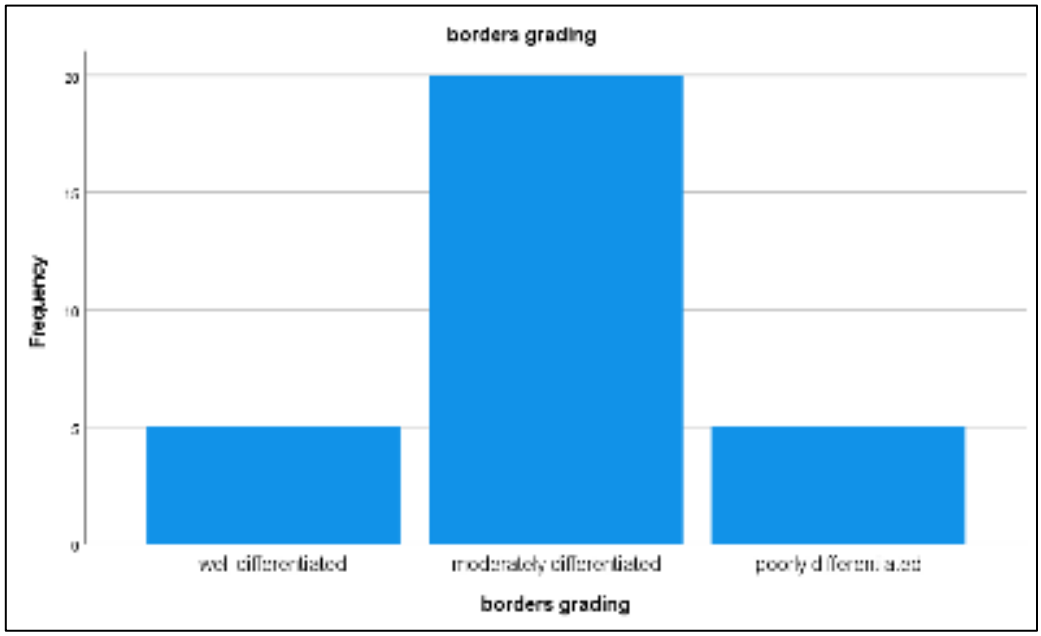
Histopathologically all cases were typed according to WHO 2018 classification, the modified Broder's grading was given and histological subtyping was done according to Wentz and Reagan et al.

Of all the cases of carcinoma of cervix studied, 5 cases (16.7%) were well differentiated, 20 cases (66%) were moderately differentiated and 5 cases (16.7%) were poorly differentiated carcinoma. Maximum number of cases were moderately differentiated carcinoma in the present study.(Table 8, Graph 9)

Table 8: Distribution of SCC according to Broder's grade

Grade	No of cases	Percentage
Well	5	16.7%
Moderately	20	66.7%
Poorly	5	16.7%
Total	30	100%

Graph 9: Distribution of SCC according to Broder's grade.

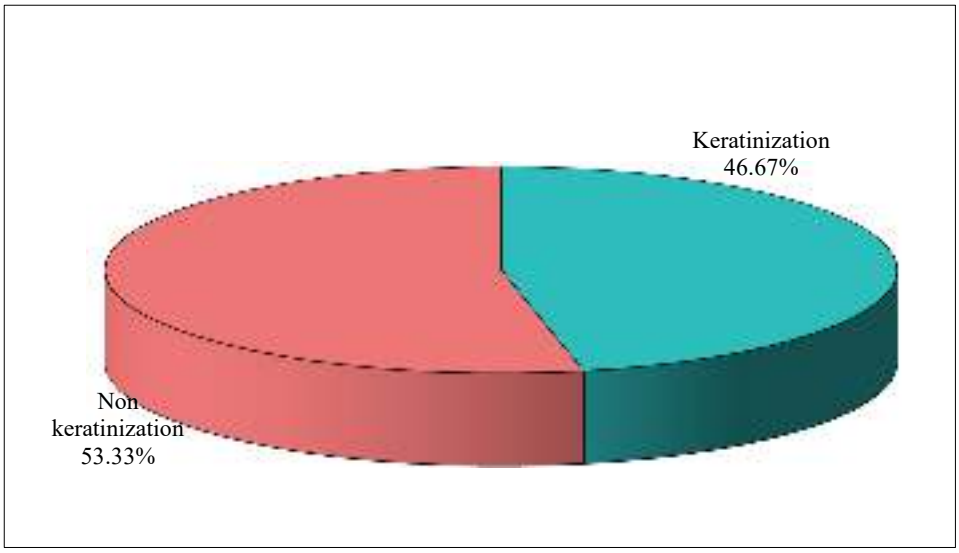


All the cases were further subtyped into large cell non-keratinizing (LCNK), small cell type and large cell keratinizing (LCK). Out of 30cases, 16 cases (53%) were LCNK and 14 cases (46.67%) were LCK. .No case of small cell type was found. (Table 9,Graph 10)

Table 9 : Distribution of cases based on histological type

Histological type	Number of cases	% of cases
Keratinization	14	46.67
Non keratinization	16	53.33
Total	30	100.00

Graph 10: Distribution of cases based on histological type



p-STAT3 IMMUNOHISTOCHEMISTRY

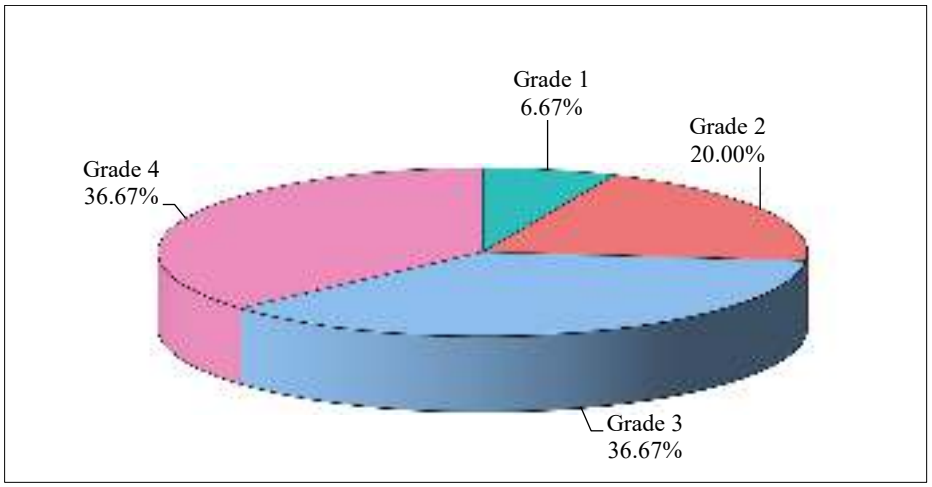
All the 30 cases of SCC cervix were subjected to immunohistochemistry staining with pSTAT3. The cases were graded into four grades based on percentage of positive tumor cells along with intensity of staining according to Sheau-Fang et al.[7]

All the 30 cases showed positive p-STAT3 expression. Percentage of positive cells with pSTAT3 expression was studied. Out of 30 cases, Grade 3 and Grade 4 was seen in 11 (36.67%) cases each, 6 cases (20%) showed Grade 2 and the remaining 2 cases (6.67%) showed Grade 1.(Table 10,Graph 11).

Table 10 : Distribution of cases according to p-STAT3 grading

P-STAT3 Grading	Number of Cases	% of Cases
Grade 1	2	6.67
Grade 2	6	20.00
Grade 3	11	36.67
Grade 4	11	36.67
Total	30	100.00

Graph 11: Distribution of cases according to p-STAT3 grading.

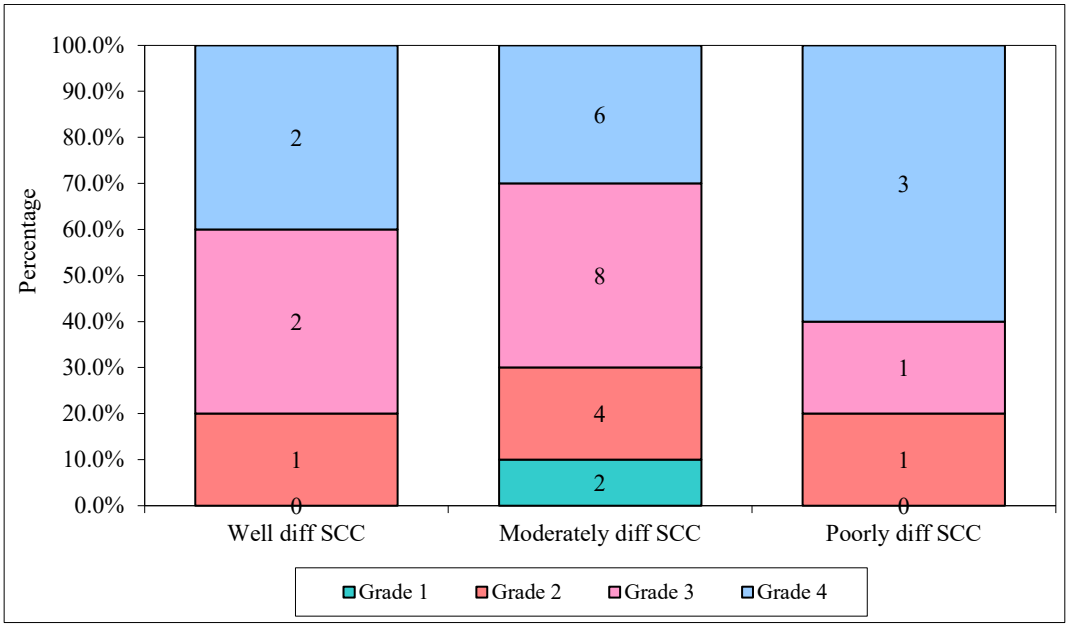


Expression of p-STAT3 was correlated with Broder's grading. Out of 5 poorly differentiated carcinoma patients, 3 cases (60%) showed grade 4, remaining 2 cases showed grade 1 and grade 3 respectively. Among 20 cases of moderately differentiated carcinoma, 8 cases (40%) showed grade 3, 6 cases (40%) grade 4, 4 cases (20%) showed grade 2 and remaining 2 cases (10%) showed grade 1. Total of 5 cases of well differentiated carcinoma, grade 3 and grade 4 was seen in 2 cases (40%) each and the remaining case (20%) showed grade 2 positivity. Broder's grading was not statistically correlating with p-STAT3 expression. (p value 0.87) (Table 11,Graph 12)

Table 11 : Correlation of P-Stat3 score with Broder's grade

Broder's grade	P-Stat3 score										χ^2	p-value
	Gr-1	%	Gr-2	%	Gr-3	%	Gr-4	%	Total	%		
Well diff SCC	0	0.00	1	20.00	2	40.00	2	40.00	5	16.67	2.455	0.874
Moderately diff SCC	2	10.00	4	20.00	8	40.00	6	30.00	20	66.67		
Poorly diff SCC	0	0.00	1	20.00	1	20.00	3	60.00	5	16.67		
Total	2	6.67	6	20.00	11	36.67	11	36.67	30	100.0		

Graph 12 : Correlation of P-Stat3 score with Broder's grade.

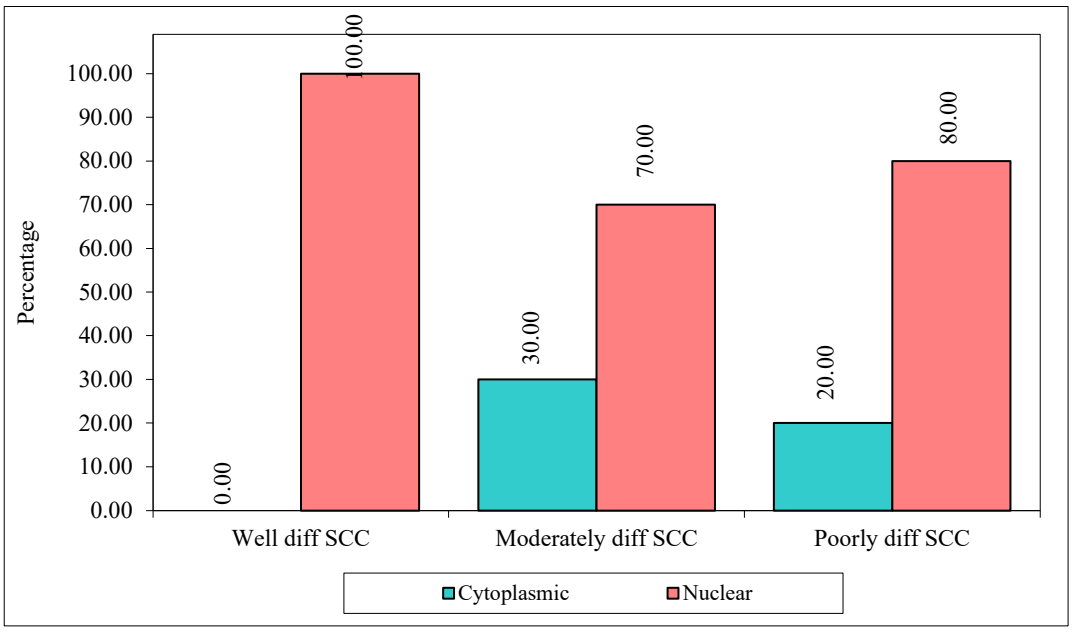


p-STAT3 expression and cytoplasmic/nuclear staining was correlated. All cases of well differentiated carcinoma cases(100%) showed nuclear staining, 14 of 20 cases (70%) of moderately differentiated carcinoma showed nuclear staining and rest 6 cases (30%) showed cytoplasmic staining, 4 of 5 cases (80%) of poorly differentiated carcinoma showed nuclear staining and remaining 1 case (20%) showed cytoplasmic staining. No significant correlation was noted(p value = 0.35)(Table 12,Graph 13).

Table 12 : Correlation of P-Stat3 cytoplasmic/nuclear staining with Broder's grading

Broder's grading	P-Stat3 expression						χ^2	p-value
	Cytoplasmic	%	Nuclear	%	Total	%		
Well diff SCC	0	0.00	5	100.00	5	16.67	2.050	0.359
Moderately diff SCC	6	30.00	14	70.00	20	16.67		
Poorly diff SCC	1	20.00	4	80.00	5	66.67		
Total	7	23.33	23	76.67	30	100.00		

Graph 13 : Correlation of P-Stat3 cytoplasmic/nuclear staining with Broder's grading

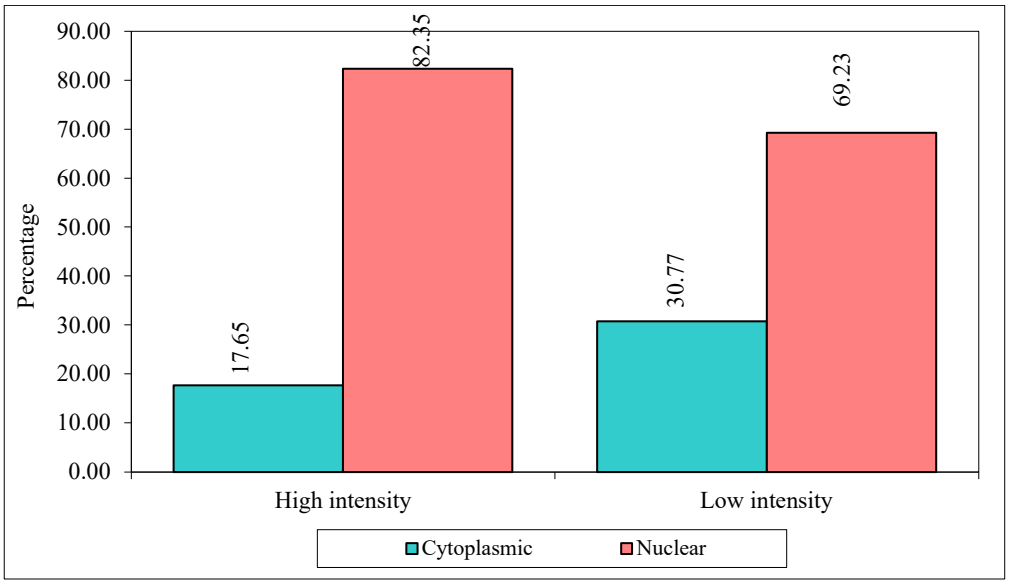


Comparing the intensity of staining with nuclear/cytoplasmic staining, both high (14 of 17 cases ,82.35%) and low intensity cases (9 of 13 , 69.23%) showed predominantly nuclear staining, which was not statistically significant(p value 0.40).(Table 13, Graph 14)

Table 13 : Correlation of P-Stat3 expression with intensity

Intensity	P-Stat3 expression						χ^2	p-value
	Cytoplasmic	%	Nuclear	%	Total	%		
High intensity	3	17.65	14	82.35	17	56.67	0.709	0.400
Low intensity	4	30.77	9	69.23	13	43.33		
Total	7	23.33	23	76.67	30	100.00		

Graph 14 : Correlation of P-Stat3 expression with intensity



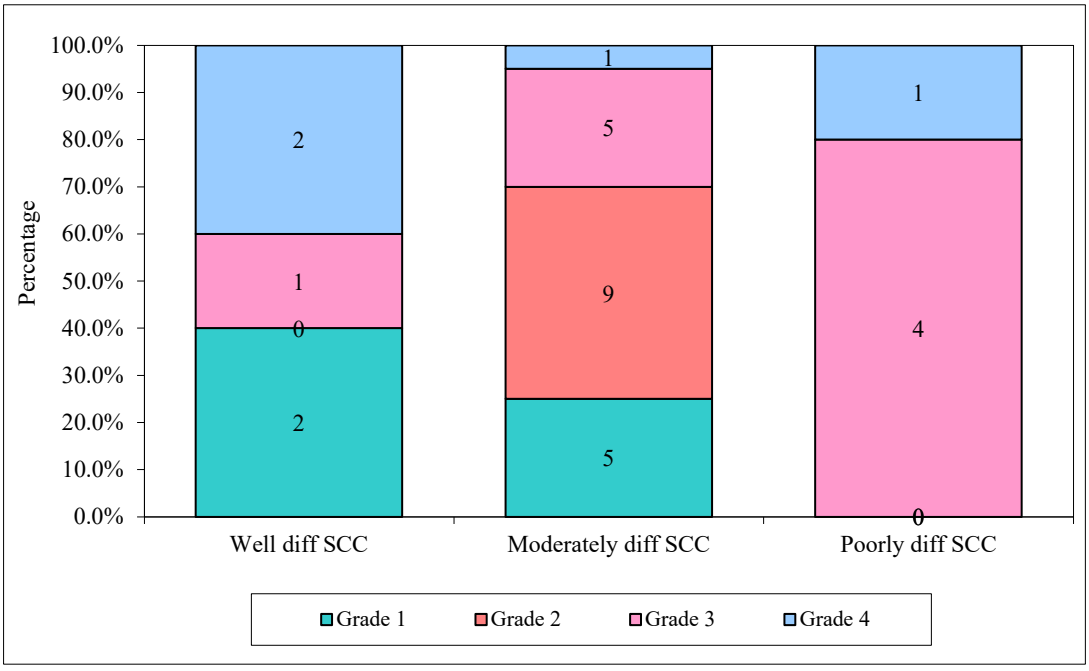
KI-67 IMMUNOHISTOCHMISTRY

Correlating Ki-67 expression with Broder's grading, One case(20%) of well differentiated carcinoma showed grade 3; 2 cases(40%) each showed grade 1 and grade 4. Out of 20 moderately differentiated carcinoma cases, 9 cases (45%) showed grade 2, 5 cases(25%) each showed grade 1 and grade 3 and remaining one case (5%) showed grade 4. 4 of 5 cases (80%) of poorly differentiated carcinoma showed grade 4 and one case (20%) showed grade 1. A statistically significant correlation was noted between Broder's grading and Ki-67 expression with p value 0.028*.(Table 14,Graph 15)

Table 14 : Correlation of Ki67 with Broder's grading

Broder's grading	Ki67 score										χ^2	p-value
	Gr-1	%	Gr-2	%	Gr-3	%	Gr-4	%	Total	%		
Well diff SCC	2	40.00	0	0.00	1	20.00	2	40.00	5	16.67	14.111	0.028*
Moderately diff SCC	5	25.00	9	45.00	5	25.00	1	5.00	20	66.67		
Poorly diff SCC	0	0.00	0	0.00	4	80.00	1	20.00	5	16.67		
Total	7	23.33	9	30.00	10	33.33	4	13.33	30	100.0		

Graph 15 : Correlation of Ki67 with Broder's grading

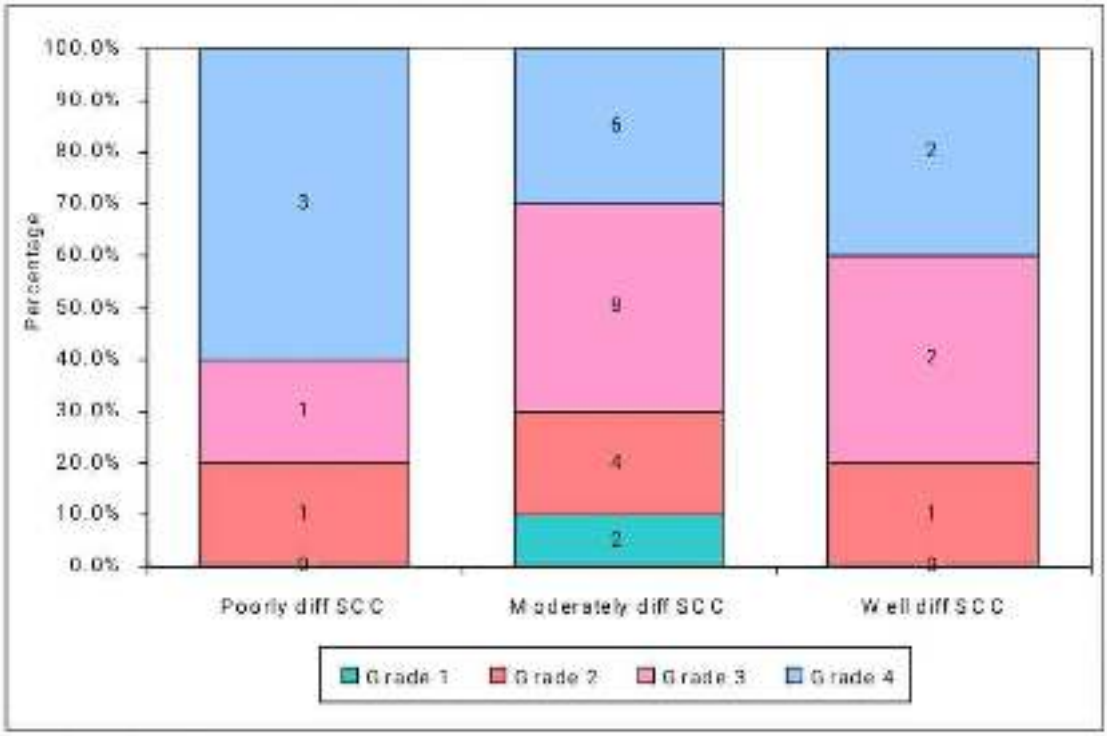


Correlate the p-STAT3 and Ki-67 expression in all the cases of SCC irrespective of Broder's grading, histological type, it was observed that with increase in grade of p-STAT3, Ki-67 expression also increased which was of statistical significant with p value 0.037*(Table 15, Graph 16)

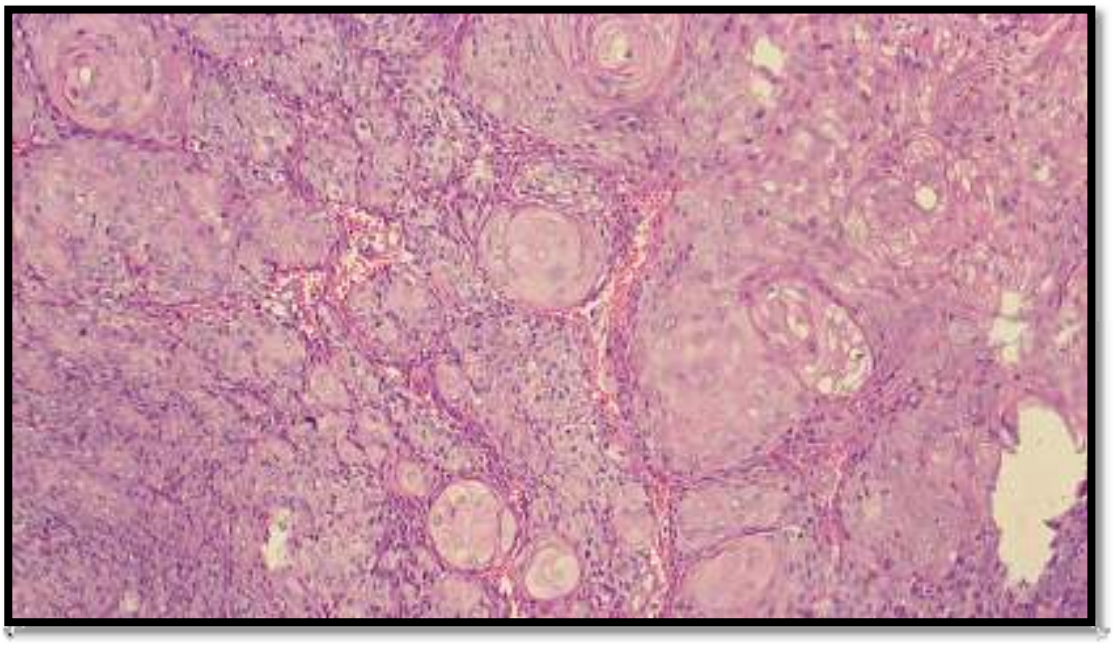
Table 15: Correlation of Ki67 with P-Stat3 grades

Ki67	P-Stat3 score										χ^2	p-value
	Gr-1	%	Gr-2	%	Gr-3	%	Gr-4	%	Total	%		
Grade 1	0	0.00	3	42.86	4	57.14	0	0.00	7	23.33	11.875	0.037*
Grade 2	0	0.00	1	11.11	3	33.33	5	55.56	9	30.00		
Grade 3	2	20.00	2	20.00	4	40.00	2	20.00	10	33.33		
Grade 4	0	0.00	0	0.00	0	0.00	4	100.0	4	13.33		
Total	2	6.67	6	20.00	11	36.67	11	36.67	30	100.0		

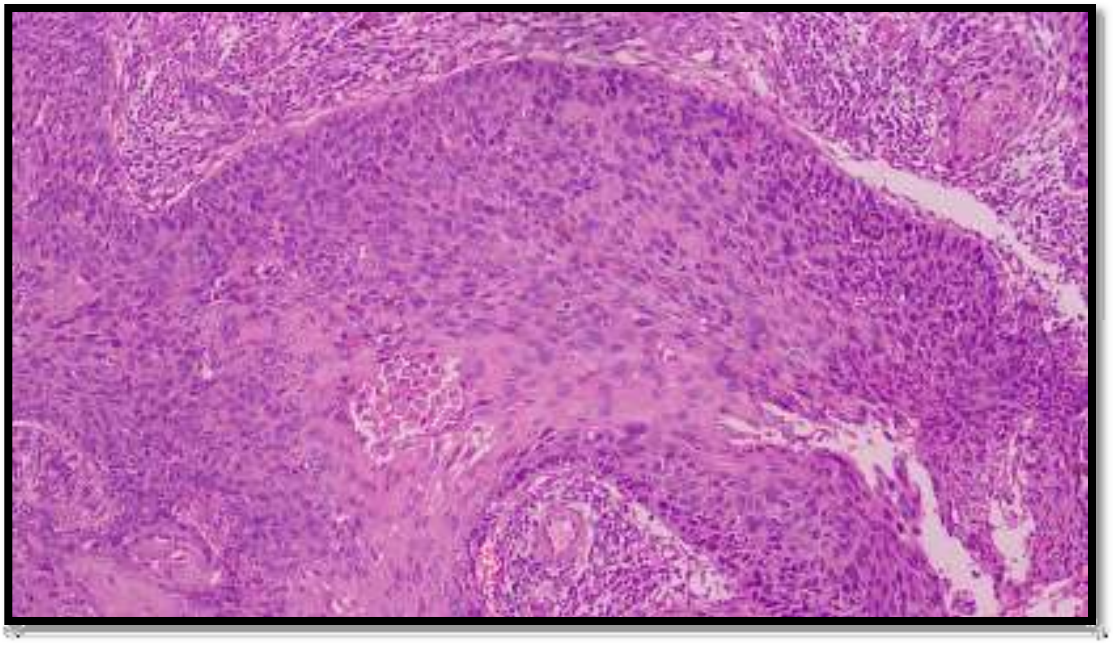
Graph 16: Correlation of Ki67 with P-Stat3 grade



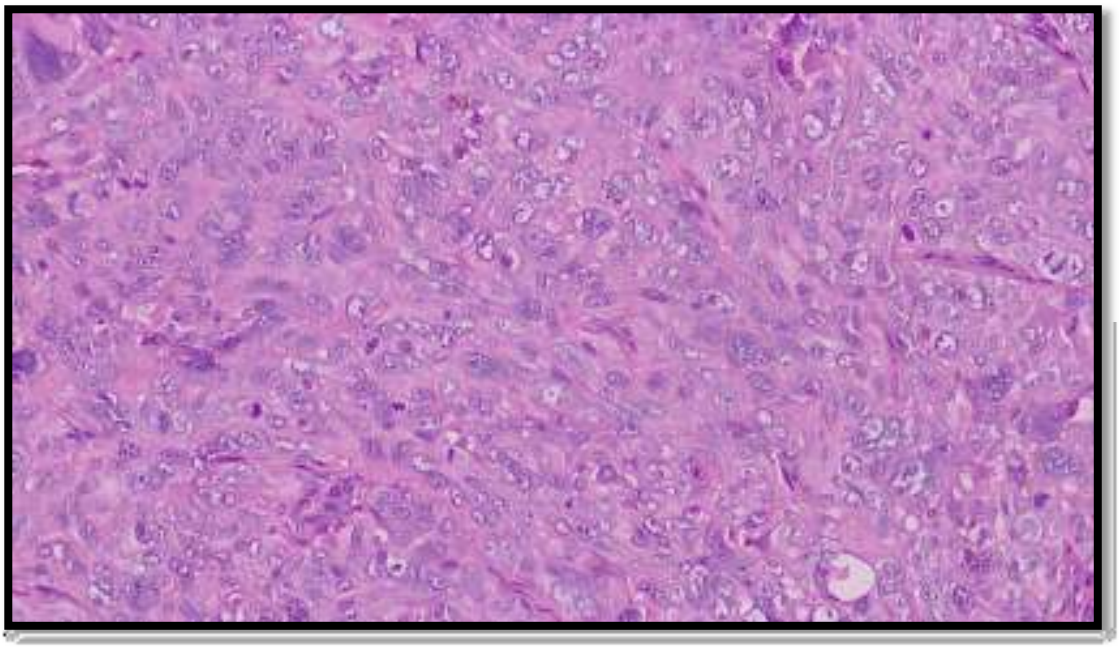
MICROPHOTOGRAPHS



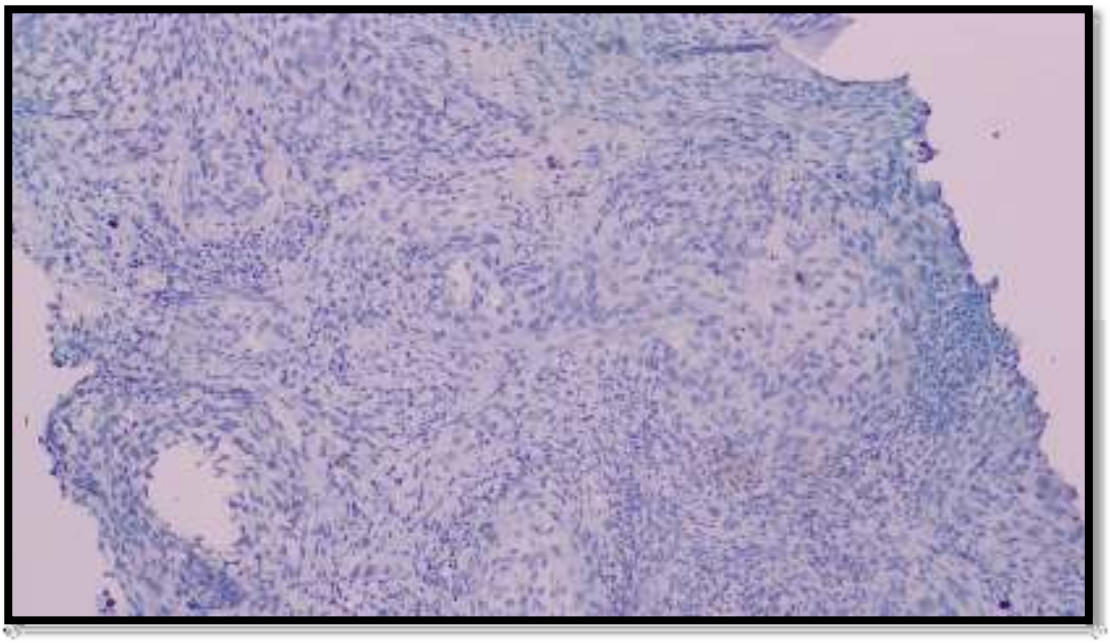
Photomicrograph 7 : Shows well differentiated SCC with keratin pearls (H&E, 10x)



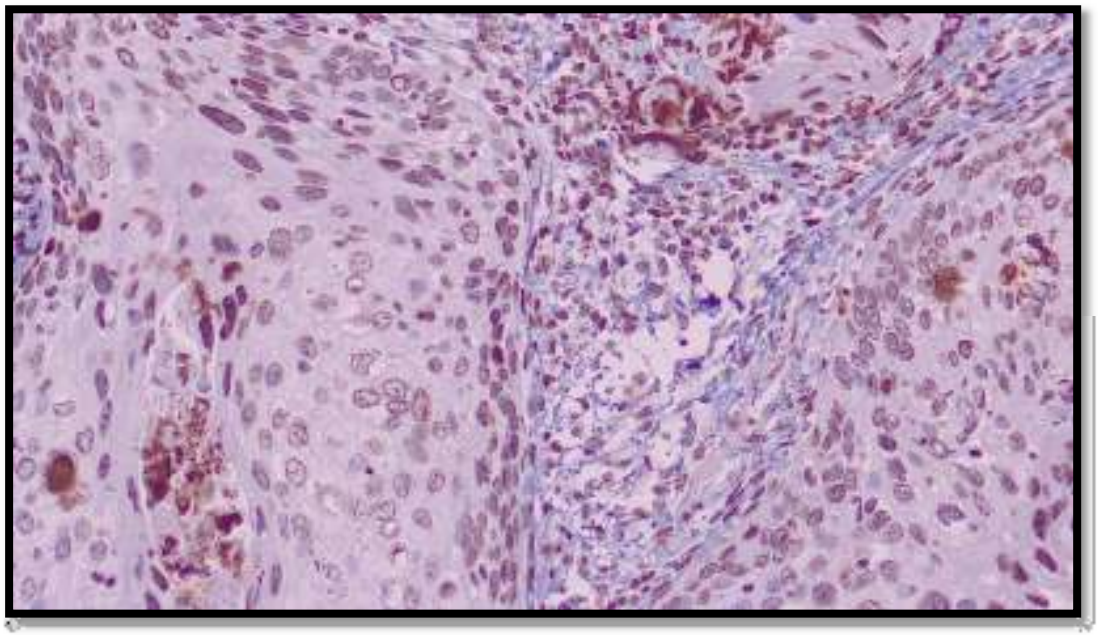
Photomicrograph 8: Shows moderately differentiated SCC (H&E, 10x)



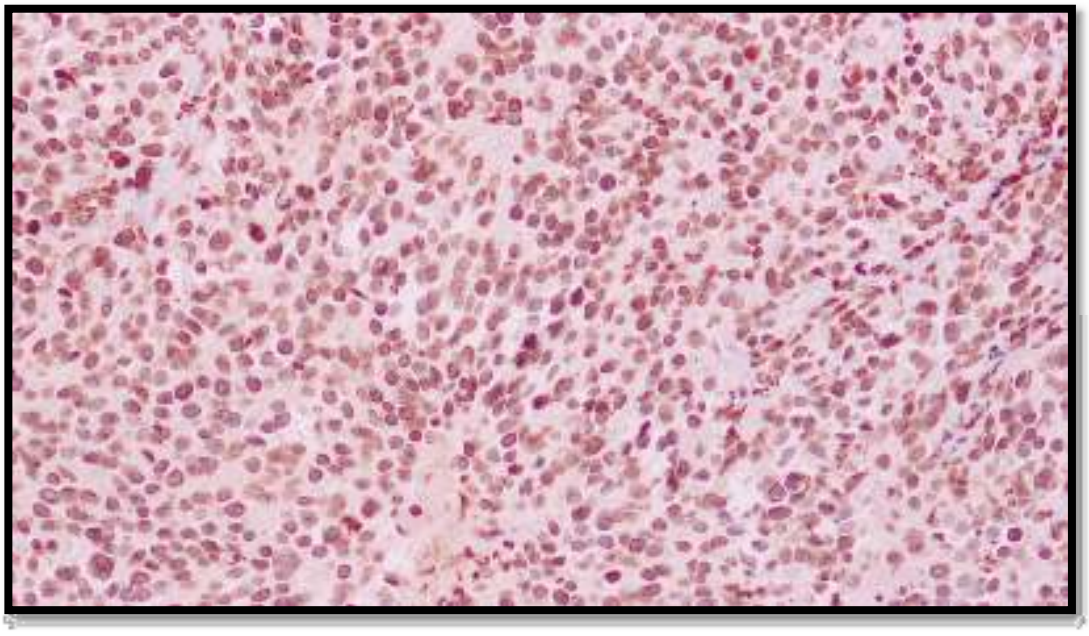
Photomicrograph 9 : Shows poorly differentiated SCC (H&E, 20x)



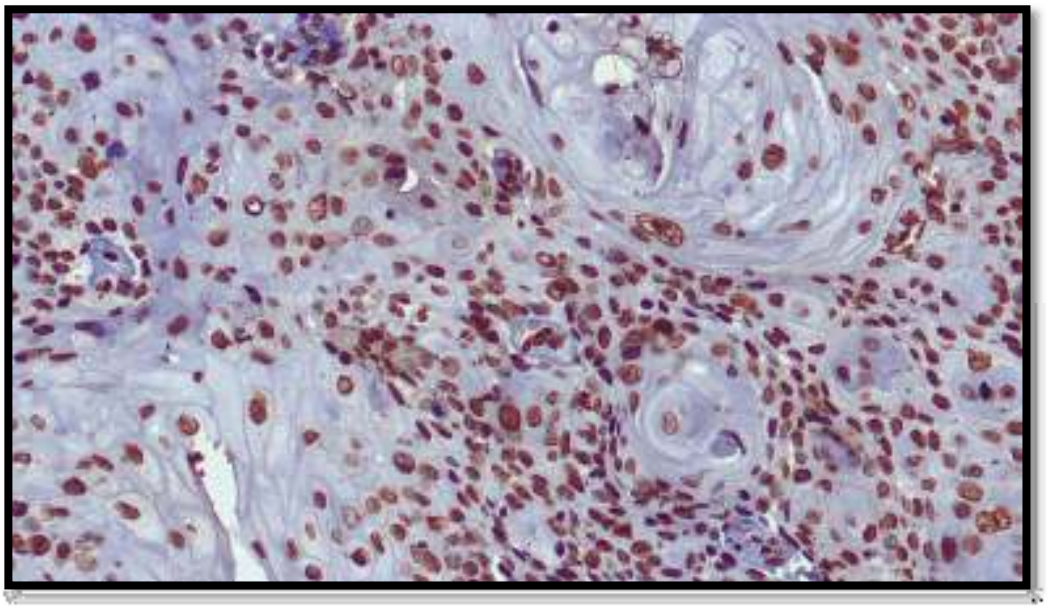
Photomicrograph 10: Shows IHC Grade 1 (0-25%) p-STAT3 positivity with low intensity in moderately differentiated SCC.(10x)



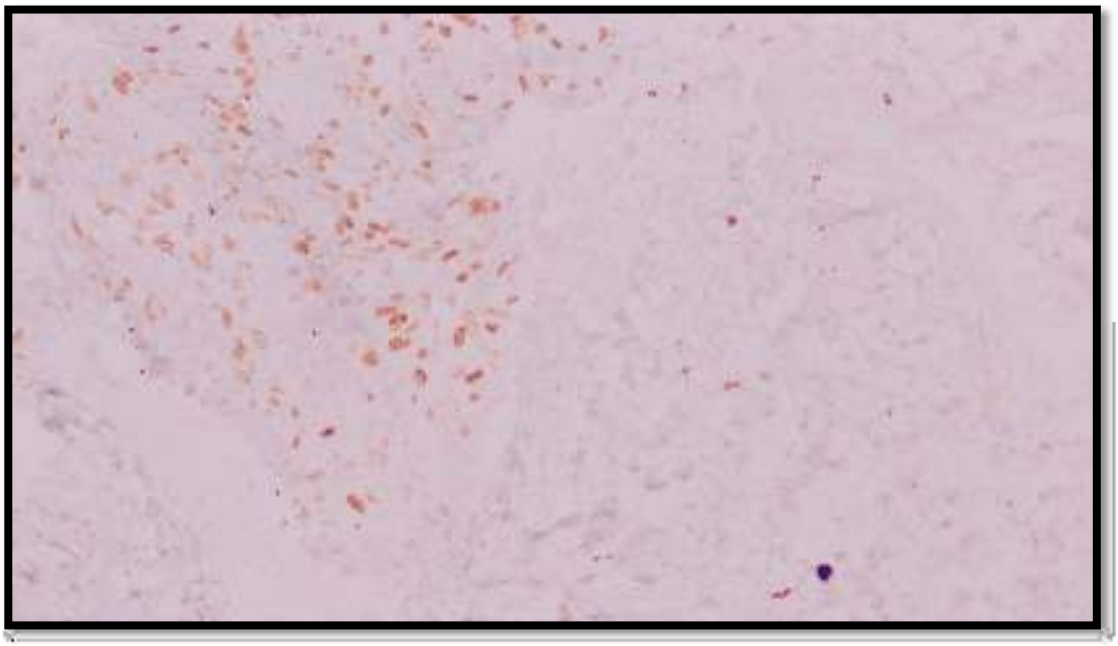
Photomicrograph 11 : Shows IHC Grade 2 (26-50%) p-STAT3 positivity with low intensity in well differentiated SCC(20x)



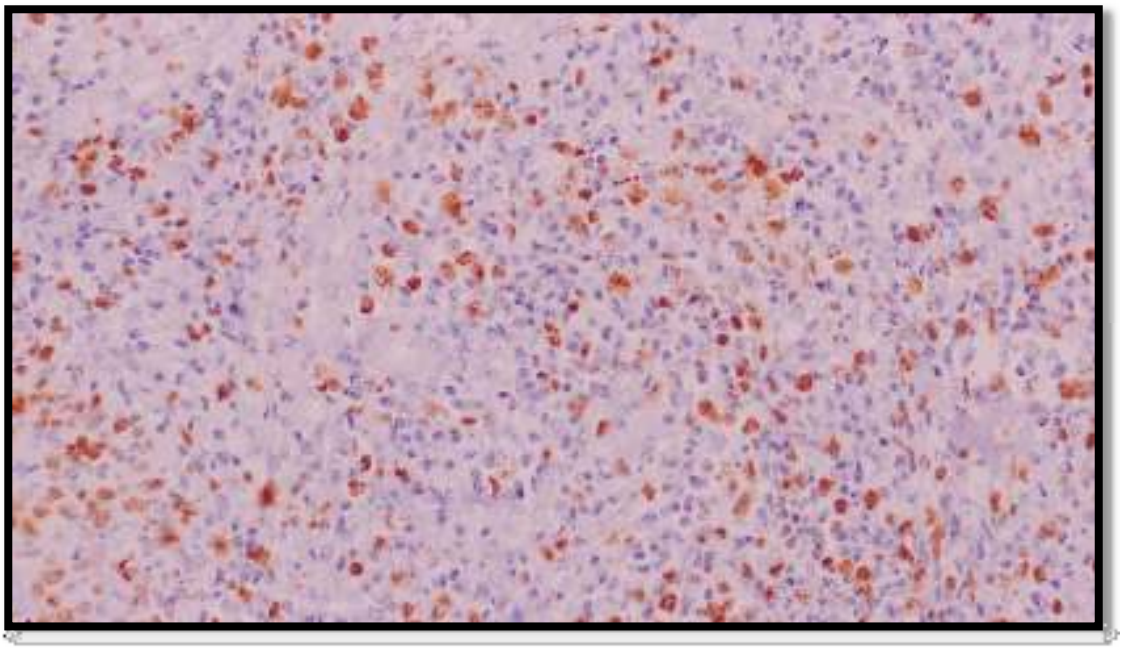
Photomicrograph 12: Shows IHC Grade 3 (51-75%) p-STAT3 positivity with high intensity in moderately differentiated SCC.(20x)



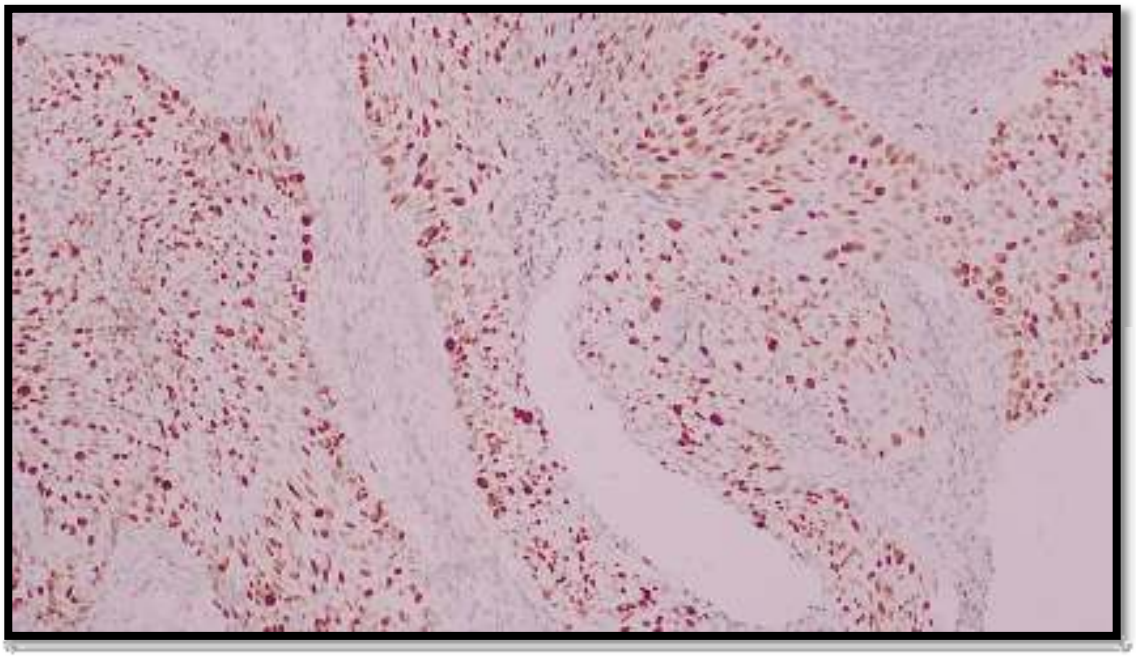
Photomicrograph 13 : Shows IHC Grade 4 (>75%) p-STAT3 positivity with high intensity in well differentiated SCC.(20x)



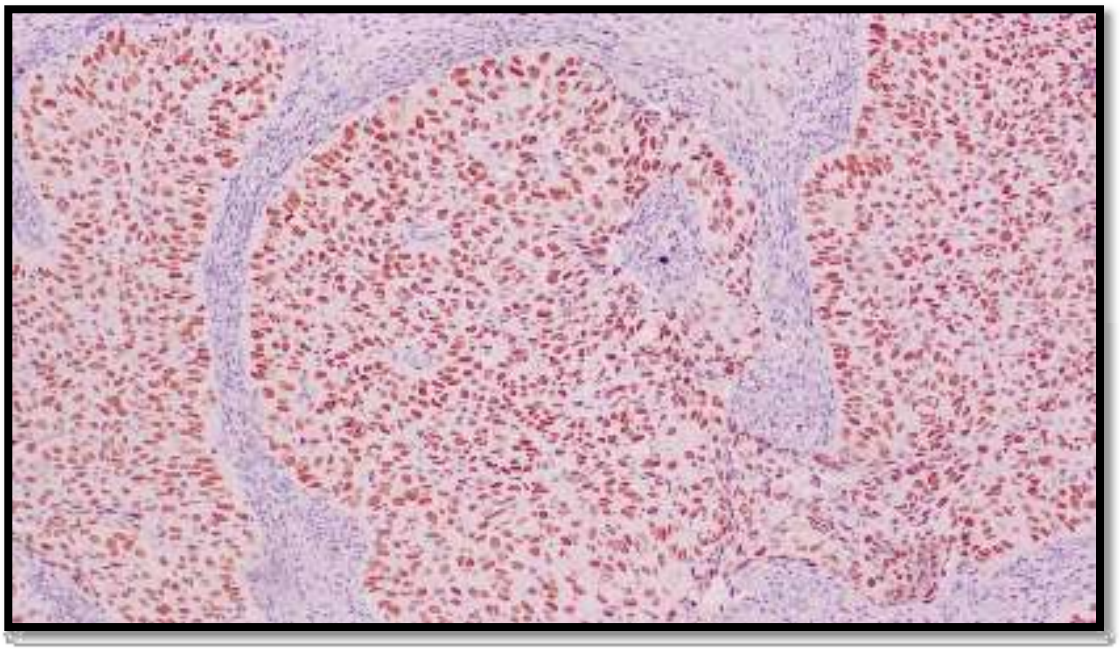
Photomicrograph 14: Shows IHC Ki-67 Grade 1(0-25%) positivity in moderately differentiated SCC.(20x)



Photomicrograph 15 : Shows IHC Grade 2 (26-50%) Ki-67 expression in moderately differentiated SCC. (10x)



Photomicrograph 16 : Show IHC Grade 3 (51-75%) Ki-67 positivty in moderately differentiated SCC. (10x)



Photomicrograph 17 : Shows IHC Grade 4 (>75%) Ki-67 poitivity in poorly differentiated carcinoma SCC.(10x)

DISCUSSION

Immunohistochemical expression of p-STAT3 was seen only in neoplastic cells of cervical epithelium. Its positive expression is never seen in normal squamous cells of cervix. It stains both nucleus and cytoplasm of neoplastic cells. All the cervical punch biopsies were taken to evaluate p-STAT3 and its expression is correlated with Ki-67 index and histological grading.

Mean age of patients was 53.93 ± 9.56years and the peak incidence was observed between 51-70 years of age. This finding was similar with the studies conducted by Shukla et al[71] 2010 (50.3 years), Sobti R C et al[12] 2009 (50years), Chen et al 2007 [4] (47± 12.46 years). One study by Kana Wang et al[46] 2011 had shown mean age of 42.13±1.4 years .(Table 4,Graph 5)

Maximum patients were of parity 3 to 4 with a mean parity of 3.50 ± 1.93 which is similar to study done by Sobti R C et al[12] 2009, Raju K et al [72]2019,Rajaran S et al[73]2006. In the study done by Annika K.Lindstrom et al[74] in Sweden most of the patients were of parity 2.7. (Table 5, Graph 6)

Highest number of patients were in postmenopausal age group. It was in similarity to the study done by Chen at al[6] 2007 and Sobti et al[12] 2009,Madhumati et al [75] 2012(Table 6,Graph 7)

The commonest clinical symptom among patients was bleeding per vagina which was similar to the study done by Schalkwyk V L et al [76]2008, Sandhu JK et [77]2012, Harries J et al [78]2020, Mwaka D M et al[79] 2015.

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

It was observed that most of the cases were moderately differentiated carcinoma (66.7%), which was correlating with study done by Hua Wang L.L et al.[11] It was in contrast to study conducted by Shukla et al [71] where well differentiated carcinoma cases were more in number, while study done by Wang et al[66] showed more number of poorly differentiated carcinoma.(Table 8,Graph 9)

These observations were correlated with multiple studies done to assess p-STAT3 using different modalities. Current study showed p-STAT3 positivity in all 30 cases (100%) of SCC of cervix using IHC. This finding was similar to study done by Hua Wang et al[11] 2018 who reported 100% positivity (58/58 cases) using IHC and real time polymerise chain reaction , Ying Zhou et al [67]showed 80% (16/20) using IHC and polymerise chain reaction, Shukla et al 2010[71] 71 %(30/50) based on immunoblotting technique, IHC and gel shift assay. Choi C H et al [80] 2009 reported 68.9% (20/29) based on microarray slides, IHC and western blot method. Where as studied done by Takemto S et al[65] 2009 showed 56.8% (71/125) , Zhang P et al [81]2014 43%(19/43), Chen at al [6] got 24% (25/104) positivity using multiple techniques in their studies.

Results obtained are dependent on different modalities used for identifying the expression and the grading criteria taken into consideration which varies from one study to other. For example, Choi et al[80] considered tumor positivity when >50 % cell showed p-STAT3 expression, Chen at al reported low positivity rate (25/104) compared to our study because their data included adenocarcinoma and micro

invasive carcinoma. Similarly, study done by Takemoto et al[65] considered all cases showing nuclear staining as positive. Current study showed majority of cases(76.67%) with nuclear staining and in remaining cases(23.3%) cytoplasmic staining is seen in predominant foci along with nuclear staining which was similar to study done by Sheau Fang Yang et al.[7]

p-STAT3 expression did not show significant correlation statistically with Broder's grading which is in accordance to the study done by Takemoto et al[65], Wang et al[66], Chen C L et al[6]. Whereas study done by Shukla et al[71,82,83], Sobti R.C et al[12] showed significant association with Broder's grading.

Most of the cases showed grade 3 and show grade 4 positivity of 11 cases each. Majority of poorly differentiated carcinomas showed grade 4 (3/5 cases i.e 60%), most of moderately differentiated carcinomas showed grade 3 (8/20 cases i.e 40%), where as grade 2 and grade 3 was seen in 2 cases of well differentiated carcinoma each(40%) which is not statistically significant(p value 0.87). Some of the well differentiated carcinomas showed high grade positivity and few poorly differentiated carcinomas showed low grade positivity which can be explained further by studying HPV status in those patients according to Shukla et al.[71] 23 out of 30 cases (76.67%) show nuclear positivity irrespective of Broder's grading with no statistical significance (p value 0.35). Nuclear positivity is also dependent on HPV status according to study done by Shukla et al[71], Sobti R C[12]. Multiple studies done by them, proved p-STAT3 expression is directly proportional to HPV status of the patient.

Shukla et al[71,82,83] analysed p-STAT 3 expression in both cervical and pre-cancer lesions. They concluded saying that in order for p-STAT3 activity to be recorded, expression of viral genome E6/E7 is required to interact with host cell signaling in turn activates signaling cascade. Mere presence of viral DNA doesn't not give positive for p-STAT3. Increased positive expression of p-STAT3 in our study could be due to geographic variation as HPV 16 is most prevalent in Indian women with prevalence of HPV16 sequences in 83% of cancers[84–86]. However assessment of HPV status was not undertaken in our study. Henceforth, p-STAT3 can be included as prognostic factor for assessing the progression of the disease and survival outcome of the patient

Correlation of nuclear/cytoplasmic staining with intensity of expression of p-STAT3 showed high intensity staining in majority of cases with nuclear staining but was not significant statistically. However, Sheau-Fang et al have reported $p < 0.05$ quoting that nuclear staining is more sensitive.[87,88](Table 13,Graph 14).

Ki-67 expression show statistical association with Broder's grading with p value 0.028 in accordance with study done by Qin Yu et al.[89–91] However, Anucta E et al[92] had not reported statistical significance between Ki-67 and histologic type, grading, lymph node invasion.

Statistical significance is noted with Ki-67 and p-STAT3 expression(p value 0.037) as in accordance to the study done by Sheau Fang Yang et al[7]. The possibility of p-STAT3 overexpression is due to cell differentiation loss or increased cell turnover.

CONCLUSION

Our study evaluated the expression of p-STAT3 and its association with Ki-67 in 30 cases of squamous cell carcinoma of cervix .Its expression was observed with various pathological parameters. p-STAT3 (ser727) was graded using a four tired semi quantitative system.

All the 30 cases show p-STAT3 positivity. Most of the moderately differentiated carcinoma cases showed grade 3, maximum of the poorly differentiated cases were grade 4 and well differentiated cases showed grade 3 and 4 positivity.

Grading of p-STAT3 correlated with Broder's grading but was not statistically significant. Ki- 67 expression was seen statistically correlating with Broder's grading.

Cases with higher grade of p-STAT3 showed higher grade of Ki-67 which was statistically significant.

In conclusion, we found p-STAT3 expression is positive in all cervical carcinoma patients and is associated with proliferative activity of neoplastic cell which is shown by its statistical association with Ki-67 in the study.

SUMMARY

1. In current study, 30 histopathologically diagnosed cases of cervical squamous cell carcinomas (punch biopsies) were studied from the time period January 2019 to December 2020 at Department of Pathology, JNMC, Belagavi.

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

3. Paraffin embedded blocks of all 30 cases were subjected to H & E stain and further followed by immunohistochemical staining for p-STAT3 and its correlation with Ki-67 expression and pathological parameters.

4. The peak incidence of cervical cancer was observed between the age group 51- 70 years with the mean of 54 years.

5. Most of the women were of parity 3 to 4 (63%) and presented with bleeding per vagina (76.6%).

6. Squamous cell carcinoma was seen most commonly in postmenopausal women (80%).

7. Most of the patients (66.7%) were moderately differentiated carcinomas according to Broder's grading system and most common subtype was large cell non- keratinizing type (53%).

8. All the cases showed positivity for p-STAT3 immunostaining. High p-STAT3 i.e grade 4 was seen more in poorly differentiated carcinoma cases and grade 3

was seen in moderately differentiated SCC, with no statistical significance (p value 0.87).

9. Most of the cases have shown grade 3 and grade 4 staining and maximum cases (76%) show nuclear staining with high intensity with no statistical correlation (p value 0.40).

10. Ki-67 expression was more in poorly differentiated carcinoma compared to moderately and well differentiated carcinoma which show statistical significance (p value 0.028).

11. There is a significant correlation between grading of percentage of positive tumor cells between p-STAT3 and Ki-67 with p value 0.037. Increase p-STAT 3 expression is associated with increased Ki-67 expression.

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ANNEXURES

ANNEXURE I

INFORMED CONSENT FORM

p-STAT3 EXPRESSION IN SQUAMOUS CELL CARCINOMA OF THE CERVIX.

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 Pediatrics, 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 CERTIFICATE

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 (Govt)
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/418 **Date: 29/08/2020**

To.

REG NO: BN0119005

PG student in Pathology,
J.N.Medical College,
BELAGAVI.

Sub: Institutional Ethical Clearance for the study.

With reference to the above, we wish to inform you that your proposed research project titled **"p-STAT3 EXPRESSION IN SQUAMOUS CELL CARCINOMA OF CERVIX"**, is ethical and justifiable. The proposed research project has been cleared by the JNMC Institutional Ethics Committee on Human Subjects Research.

(Dr. Anita Dalal)
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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ANNEXURES -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:

BRODER'S GRADE:

H & E STAINING :

IHC DIAGNOSIS:

IHC MARKER	**PERCENTAGE OF POSITIVE TUMOR CELLS**	**GRADE**	**NUCLEAR/CYTOPLASMIC STAINING**	**INTENSITY**
p-STAT3				
Ki-67				

ANNEXURE -IV

HEMATOXYLIN AND EOSIN STAINING PROTOCOL.

- Deparaffinize in Xylene I and II and III changes. [III change use warmed xylene] (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

Stock Solution: - HEMATOXYLIN :

Harris Hematoxylin -1 L

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: p173-187.

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 alchohol) 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 p-STAT3 and keep for 30 minutes.
- 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 substrate 15minutes 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 hematoxylin (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 of solution 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

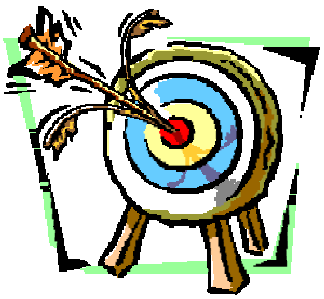
KEY TO MASTERCHART

B	-	Biopsy
WD	-	White Discharge
WDSCC	-	Well Differentiated Squamous Cell Carcinoma
MDSCC	-	Moderately Differentiated Squamous Cell Carcinoma
PDSCC	-	Poorly Differentiated Squamous Cell Carcinoma
P	-	Present
AB	-	Absent
BPV	-	Bleeding Per Vagina
CG	-	Cervical Growth
M	-	Menopausal
A	-	Attained
NA	-	Not Attained
CF	-	Clinical Features
BG	-	Broder's Grading
HIST	-	Histological Subtype
LCK	-	Large Cell Keratinzation
LCNK	-	Large Cell Non Keratinization
INT	-	Intensity
N	-	Nuclear Staining
C	-	Cytoplasmic Staining

MASTER CHART

SL.NO	AGE	IP NO	B	CF	M	PARITY	BG	HIST	p-STAT3	N/C	INT	Ki-67
1	60	97956	5383	BPV	A	P3L3	MDSCC	LCK	3	C	low	1
2	52	987555	5762	BPV	A	P6L6	WDSCC	LCNK	2	N	low	1
3	55	851332	247	BPV	A	P3L3	MDSCC	LCNK	4	N	high	4
4	65	786316	881	BPV	A	P3L3	MDSCC	LCNK	2	N	high	3
5	50	889360	4102	BPV	A	P2L2	MDSCC	LCK	4	N	high	2
6	60	979561	5382	BPV	A	P2L2	MDSCC	LCK	3	C	high	3
7	55	839728	7406	WD	A	P3L3	MDSCC	LCK	1	C+N	low	3
8	45	931936	1431	BPV	A	P5L5	PDSCC	LCNK	4	C	high	4
9	35	836616	7400	BPV	NA	nulli	PDSCC	LCNK	4	N	high	3
10	46	29953	5108	WD	NA	P3L3	PDSCC	LCNK	4	N	high	3
11	65		1526	BPV	A	P3L1	MDSCC	LCNK	3	N	high	2
12	61	4224	3308	BPV	A	P3L3	MDSCC	LCNK	4	N	high	2
13	40	971714	4768	WD	NA	P3L3	MDSCC	LCNK	4	N	high	2
14	45	855969	674	BPV	A	P3L2	PDSCC	LCNK	3	N	high	3
15	70	862779	1321	CG	A	P4L4	MDSCC	LCNK	1	N	high	3
16	55	21631	2928	BPV	A	P3L3	WDSCC	LCK	4	N	low	4
17	50	4183	686	BPV	A	P2L2	PDSCC	LCNK	2	N	high	3
18	60	817370	5220	BPV	A	P4L4	MDSCC	LCK	3	C	high	2
19	55	891171	4246	BPV	A	P3L3	MDSCC	LCK	2	C	low	1
20	69	814541	4910	BPV	A	P3L3	MDSCC	LCK	3	N	high	3
21	45	97810	5209	BPV	NA	P2L2	MDSCC	LCNK	3	N	high	2
22	55	30923	4508	BPV	A	P2L2	MDSCC	LCK	3	C	low	1
23	55	815858	5100	WD	A	P3L3	MDSCC	LCNK	2	N	high	1
24	38	11574	3036	BPV	NA	P2L2	MDSCC	LCK	4	N	low	2
25	63		4078	CG	A	P5L5	MDSCC	LCK	3	N	low	1
26	37	17112	1690	WD	NA	P3L3	WDSCC	LCK	3	N	low	1
27	68	1013253	1177	BPV	A	P5L4	MDSCC	LCNK	2	N	low	2
28	52	1013131	1148	BPV	A	P2L2	MDSCC	LCNK	4	N	low	2
29	52	1012855	1179	BPV	A	P3LE	WDSCC	LCNK	4	N	low	4
30	60	1000138	519	BPV	A	P4L4	WDSCC	LCK	3	N	low	3

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