SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION AND RESEARCH
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SIGNIFICANCE OF EXPRESSION OF EPITHELIAL MESENCHYMAL TRANSITION MARKERS IN SQUAMOUS CELL NEOPLASMS OF UTERINE CERVIX – A LABORATORY OBSERVATIONAL STUDY

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Title: Significance of Expression of Epithelial Mesenchymal Transition Markers in Squamous Cell Neoplasms of Uterine Cervix – A Laboratory Observational Study

ABSTRACT

Introduction :-

Cervical cancer is the fourth most common cause of cancer in women worldwide. Cervical cancer is a major health problem worldwide with developing countries like India contribute maximum to the global burden.

Epithelial Mesenchymal Transition is a phenomenon related to carcinogenesis which is characterize by morphological change in neoplastic epithelial cells to mesenchymal cells. This eventually leads to increase motility, increase invasiveness in neoplastic cells. Cytokeratin 19 is a basal cell marker, Vimentin is a cytoskeletal protein while RhoC contributes to reorganization of cytoskeletal. RhoC has also role in targeted therapy for different cancers.

Objectives :-

1. To observe expression of the 3 epithelial mesenchymal transition markers (cytokeratin 19, vimentin and RhoC) in normal, HSIL and squamous cell carcinoma.

2. To correlate expression of the 3 epithelial mesenchymal transition markers (cytokeratin 19, vimentin and RhoC) among normal, HSIL, squamous cell carcinoma and histological grade and clinical/radiological stage of the disease.

Materials and Methods :-

Seventy cases are taken. Out of 70, 10 cases were of normal cervix, 30 cases were of HSIL and 30 cases of newly diagnosed squamous cell carcinoma of cervix. Cinicopathological findings like age, parity, chief complaint, per vaginal, per speculum findings, grading and FIGO staging were obtained. Immunohistochemistry was performed for 3 markers :- Cytokeratin 19, Vimentin and RhoC. Correlation between expressions of Cytokeratin 19, Vimentin and RhoC with the study groups are analyzed. A p value of <0.005 was considered statistically significance.

Results :-

Mean age ± Standard deviation among normal, HSIL and SCC groups were 46.2 ± 16.12 years, 49.10 ± 10.13 years and 56.27 ± 9.29 years respectively. This difference of mean age across normal, HSIL and SCC was statistically significant. A weak basal cell positivity was seen in 80% cases with normal cervix. 93.3% and 100% cases of HSIL and SCC were positive for the expression of CK19. This difference of expressions of CK19 among HSIL and SCC of cervix was statistically insignificance (Fischer's exact test, p value=0.492). All the cases of normal cervix was negative for Vimentin expression. 33.3% and 73.3% cases of HSIL and SCC respectively showed positive vimentin expression. This difference of expressions of Vimentin across normal, HSIL and SCC was statistically significant (χ^2 = 19.496, p <0.001). 10%, 20% and 83.33% cases of normal cervix, HSIL and SCC showed positive expressions for RhoC

respectively. This difference in the expressions of RhoC across normal, HSIL and SCC was statistically significant (χ^2 = 30.241, p <0.001). No statistically significant difference between expressions of vimentin and RhoC and Histopathological grade (χ^2 = 1.327, p = 0.515 and χ^2 = 2.030 , p = 0.362 respectively) and the expressions of Vimentin and RhoC and FIGO staging were noted (χ^2 = 0.292 , p = 0.589 and χ^2 = 0.055, p = 0.815 respectively). Comparison between expression of CK19 and Histopathological grade and FIGO staging was not possible because all the cases of CK19 were positive for CK19 expression.

Conclusion :-

Epithelial mesenchymal transition is an important phenomenon that promotes invasion, migration in cervical cancer. Increase expression of CK 19 in HSIL and SCC of cervix highlights role of basal cells of cervix in cervical carcinogenesis.Vimentin and RhoC in cervical cancer promotes the phenomenon of epithelial mesenchymal transition and eventually leads to malignant transformation, increase motility and increase invasiveness.

Further multicentric studies with a larger sample size are required. Further studies on role of RhoC in targeted therapy for cervical cancer is recommended.

Keywords :- Epithelial Mesenchymal Transition, CK19, Vimentin, RhoC

INTRODUCTION

Cervical cancer is the fourth most common cancer in women worldwide after breast cancer, colorectal cancer, Lung cancer. Incidence of cervical cancer in India is 14.7 cases/1,00,000 population and constitutes about 17% of all cancers worldwide. [1] In Kolar, Cervical cancer contribute to 17.55% of all cancers.[2]

Squamous cell carcinoma (SCC) accounts for approximately 80% of cervical cancer, whereas adenocarcinoma accounts for about 20%.[3]

Cervical cancer occur in a stepwise fashion. The full spectrum of cancer progression includes normal cervix, Low Grade Squamous Intraepithelial Lesion (LSIL), High Grade Squamous Intra epithelial Lesion (HSIL), locally invasive and distant metastatic cancer. [4]

During the progression of epithelial cancers, epithelial cells lose their epithelial characteristics and acquires mesenchymal characteristics. The phenomenon is called epithelial mesenchymal transition (EMT) which is the most important mechanism of cancer metastasis which determines the prognosis of the disease. [5]

The epithelial mesenchymal transition phenomenon is widely studied in head and neck cancers, breast cancers but not fully studied in cervical carcinomas. However only a few studies are published in English literature regarding epithelial mesenchymal transition with the respect to cervical cancer. Hence, we have taken up this study where cytokeratin 19 (CK 19) is epithelial marker, vimentin is mesenchymal modulator and Ras homolog gene family member C (RhoC) is epithelial mesenchymal transition activator and cytoskeletal modulator. The concept can also be considered for targeted chemotherapy. [6]

OBJECTIVES

1. To observe expression of the 3 epithelial mesenchymal transition markers (cytokeratin 19, vimentin and RhoC) in normal, HSIL and squamous cell carcinoma.
2. To correlate expression of the 3 epithelial mesenchymal transition markers (cytokeratin 19, vimentin and RhoC) among normal, HSIL, squamous cell carcinoma and histological grade and clinical/radiological stage of the disease.

REVIEW OF LITERATURE

Relevant anatomy :-

Uterus consists of 3 parts :- Fundus, Body, Cervix. Cervix is the lower most part of uterus, funnel-shaped and protrudes to upper vagina. Cervix measures 2.5 to 3 cm in length in adult nulliparous females. Vagina is attached to the lower most part of cervix in a circumferential and oblique manner. Attachment of vagina divides cervix into three potions :- Upper, Supervaginal and lower vaginal portions. The vaginal portion of cervix is called ectocervix which has convex and elliptical surface, have anterior and posterior fornices and has external os in the centre. The external-os is connected to endocervical canal/endocervix which is elliptical in shape and measures 8 mm in greatest diameter. [7]

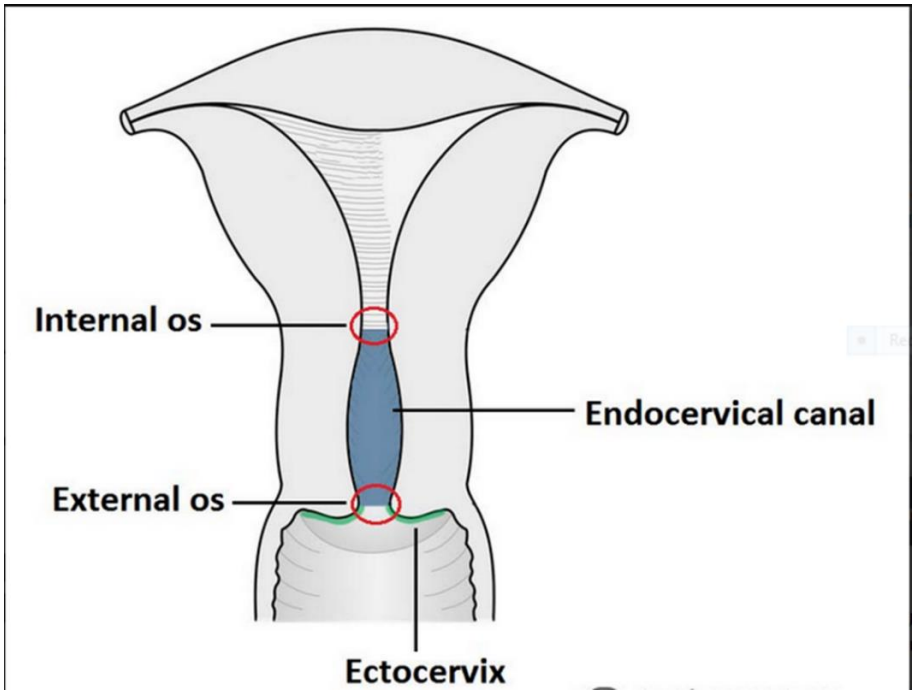


Figure 1. Schematic diagram showing normal histology of female genital tract[7]

Histology :-

Cervix is composed of fibrous, muscular and elastic tissue. The lining of cervix differs in ectocervix and endocervical canal. [8]

Ectocervix is lined by nonkeratinizing stratified squamous epithelium similar to that of vaginal lining epithelium. The lining epithelium of ectocervix is divided into three layers :- [8]

1. Basal/Parabasal cell layer/ Germinal cell layer
2. Intermediate cell layer
3. Superficial cell layer

1. Basal/Parbasal cell layer/ Germinal cell layer :- It consist of two cell types :- True basal cells and parabasal cells. The true basal cells is about 10 µm in diameter rest on basal lamina, consist of scant cytoplasm and oval nuclei. The parabasal cells are

larger than basal cells and forms 1 to 2 cell layers over basal cell layer. Parabasal cells are larger than basal cells and contain more cytoplasm. [8]

2. Intermediate cell layer/Mid Zone :- This layer consist of intermediate cells having clear and vacuolated cytoplasm.These cells are PAS positive. [8]
3. Superficial cell layer :- Superficial cells are mostly differentiated flattened cells having larger amount of pink, eosinophilic cytoplasm and pyknotic nuclei. [8]

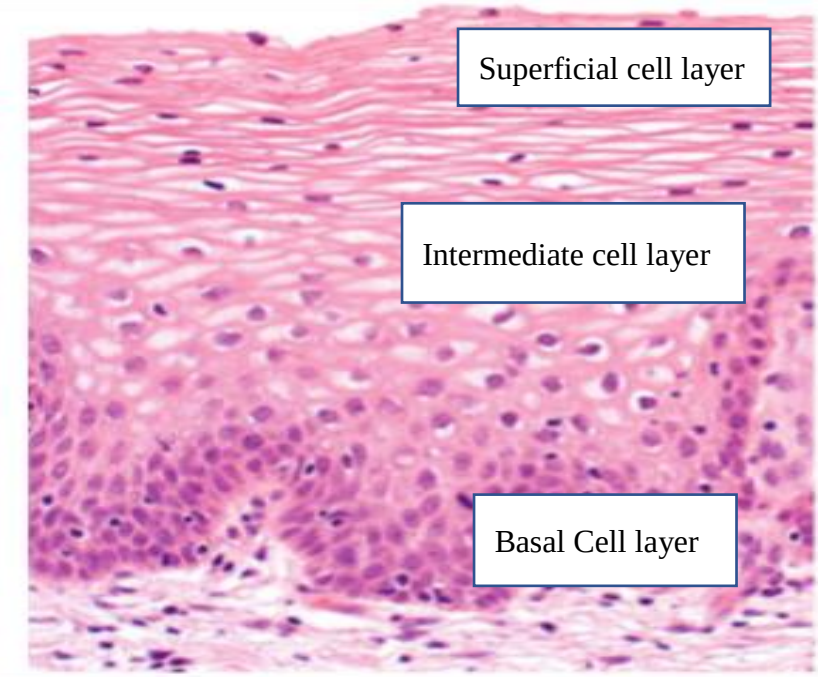


Figure 2 showing normal histology of cervix[8]

Endocervical canal is lined by single layer of mucin-secreting columnar epithelium and subepithelium is consist of compound, tubular racemose endocervical glands. Endocervical glands are formed by deep, cleft-like infoldings of the surface epithelium. These endocervical glands are lined by columnar epithelium with basal nuclei and fine granular cytoplasm with mucinous droplets. [8]

Transformation Zone (TZ):- Squamocolumnar junction (SCJ) of cervix between stratified squamous epithelium of ectocervix and mucin secreting columnar epithelium of endocervix. There is continuous remodelling of SCJ due to uterine growth, cervical enlargement and hormonal status. At birth and premenarchal years, original SCJ is located at level of external-os. At 1year of age, during menarche, reproductive age and at the time of pregnancy as uterus increases in size result in eversion of endocervical columnar epithelium to ectocervix which leads to SCJ below external-os. With time columnar epithelium at SCJ undergo squamous metaplasia and SCJ again ascend to external os. This new SCJ is called functional SCJ/physiological SCJ/ New SCJ. The region between original SCJ and functional SCJ is called transformation zone (TZ). TZ consist of squamous metaplastic cells. Nearly all squamous neoplasm of cervix starts at new SCJ. Distribution of cervical precursor lesion extent to region of TZ. [8]

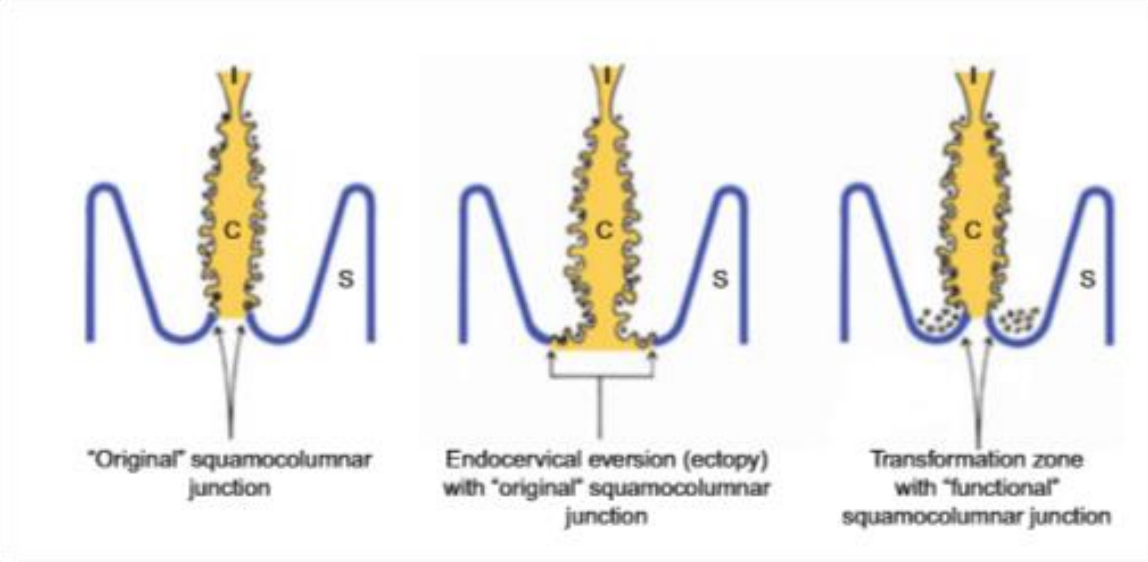


Figure 3. Changes in squamocolumnar junction of cervix[8]

Table 1. WHO Classification of tumours of uterine cervix[9]

Epithelial Tumours	
Squamous tumors and precursors	**Squamous intraepithelial lesions** Low-grade squamous intraepithelial lesion High-grade squamous intraepithelial lesion **Squamous cell carcinoma, NOS** Keratinizing Non-keratinizing Papillary Basaloid Warty Verrucous Squamotransitional Lymphoepithelioma-like **Benign squamous cell lesions** Squamous metaplasia Condyloma acuminatum Squamous papilloma Transitional metaplasia
Glandular tumours and precursors	**Adenocarcinoma in situ** **Adenocarcinoma**

	Endocervical adenocarcinoma, usual type Mucinous carcinoma, NOS Gastric type Intestinal type Signet-ring cell type Villoglandular carcinoma Endometrioid carcinoma Clear cell carcinoma Serous carcinoma Mesonephric carcinoma Adenocarcinoma admixed with neuroendocrine carcinoma **Benign glandular tumours and tumour-like lesions** Endocervical polyp Müllerian papilloma Nabothian cyst Tunnel clusters Microglandular hyperplasia Lobular endocervical glandular hyperplasia Diffuse laminar endocervical hyperplasia Mesonephric remnants and hyperplasia Arias Stella reaction Endocervicosis Endometriosis Tuboendometrioid metaplasia

	Ectopic prostate tissue
Other epithelial tumors	Adenosquamous carcinoma Glassy cell carcinoma Adenoid basal carcinoma Adenoid cystic carcinoma Undifferentiated carcinoma
Neuroendocrine tumours	**Low-grade neuroendocrine tumour** Carcinoid tumour Atypical carcinoid tumour **High-grade neuroendocrine carcinoma** Small cell neuroendocrine carcinoma Large cell neuroendocrine carcinoma
Mesenchymal tumours and tumour-like lesions	
Benign	Leiomyoma Rhabdomyoma Others
Malignant	Leiomyosarcoma Rhabdomyosarcoma Alveolar soft-part sarcoma Angiosarcoma Malignant peripheral nerve sheath tumour **Other sarcomas** Liposarcoma

	Undifferentiated endocervical sarcoma Ewing sarcoma **Tumour-like lesions** Postoperative spindle-cell nodule Lymphoma-like lesion
Mixed epithelial and mesenchymal tumours Adenomyoma Adenosarcoma Carcinosarcoma	
Melanocytic tumours Blue naevus Malignant melanoma	
Germ cell tumours Yolk sac tumours	
Lymphoid and myeloid tumours Lymphomas Myeloid neoplasms	

Precursor squamous lesions of the cervix

Reversible proliferation of dysplastic cells without breach of basement membrane and have potential of malignant transformation when left untreated is categorized as precursor lesions.

There are 3 classification system for the precursor squamous lesions of cervix. These classification systems are based on degree of dysplasia, extent of involvement, grade of lesion and natural course of progression. [10]

The 3 systems of classifications are :-

1. **Dysplasia**
2. **Cervical Intraepithelial Neoplasia (CIN) :-** It take account of the fact that every cervical lesions are homogenous. Further study demonstrate the heterogenous nature of cervical lesions which CIN system failed to explain. Studies showed that cervical precursor lesions are actually two disease prcesses. One is productive infection of HPV and other is neoplastic processes. So, all CIN-1 will not progress to CIN-2, CIN-3 and finally invasive carcinoma. [10]
3. **Squamous Intraepithelial Lesions (SIL)** as proposed by The Bethesda System for reporting cervical cytology. This is the most recent classification system and widely used now-a-days. Now WHO also incorporated 2-tier SIL classification. It take account of hetrogenousity of lesions. Two tier SIL system is biologically more relevant and histologically more reproducible as compared to CIN system, Low Grade Squamous Intraepithelial Lesions (L-SIL) are more homogenous having higher rate of spontaneous regression and lower rate of malignant transformation as compared to High Grade Squamous Intraepithelial Lesions (H-SIL). H-SIL are more heterogenous with respect to types of associated HPV, clonality. [10]

Table 2. Terminolgies used to define precursor squamous lesions of uterine cervix.

Dysplasia	**CIN**	**SIL**
Mild dysplasia	CIN-1	L-SIL
Moderate dysplasia	CIN-2	H-SIL
Severe dysplasia/ carcinoma-in-situ	CIN-3	

Squamous Intraepithelial Lesions (SIL)

Mostly seen in females in reproductive age-group.

Divided into :-

1. Low Grade Squamous Intraepithelilal Lesion (L-SIL)
2. High Grade Squamous Intraepithelilal Lesion (H-SIL)

Low-Grade Squamous Intraepithelial Lesion (L-SIL)

Definition :-

WHO define L-SIL as squamous intraepithelial lesion having clinical and morphological features of productive HPV infection. The risk of concurrent/future cancer is low. Usually asymptomatic and are detected on routine screening. [11]

Gross :-

Grossly not apparent as majority of lesions are flat. Exophytic and papillary lesions may be present. Papillary lesions are equivalent to Condyloma accuminata. [12]

Microscopy :-

Presence of mildly dysplastic basal cells in the lower one-third of epithelium (CIN-1). No breach in basement membrane. Mitotic activity is limited to lower one-third of epithelium. Absence of atypical/abnormal mitoses. Cells of intermediate layer show normal maturation and differentiation. These cells shows koilocytic changes which is characterized by presence of cytoplasmic halo around nucleus and nuclear abnormalities like hyperchromasia and nuclear membrane abnormlities like bi/ multi lobation. Koilocytic change is more prominent in upper layer of epithelium. [13]

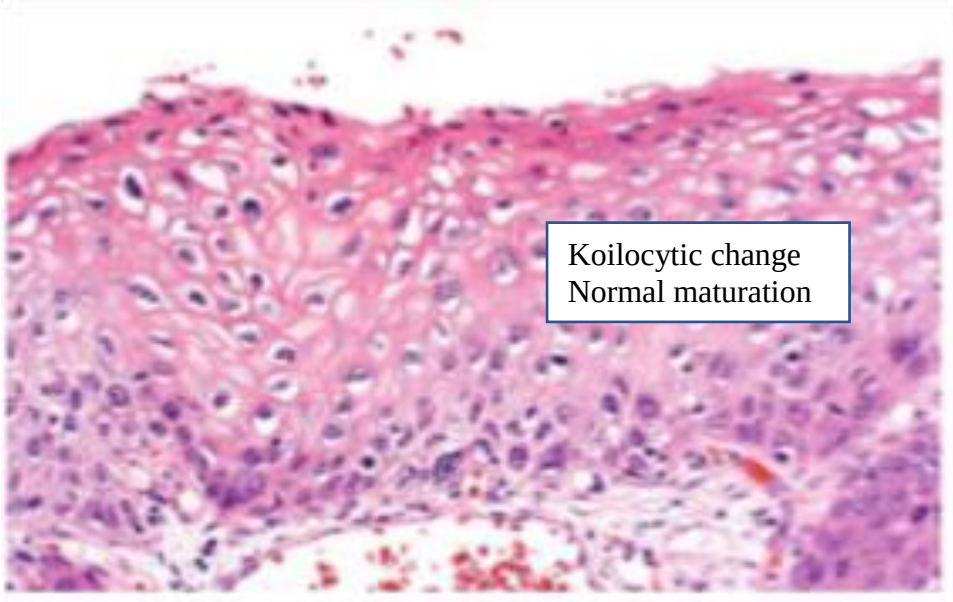


Figure 4. Histomorpholgy of L-SIL[9]

Prognosis :-

Most cases undergo spontaneous regression. Progression of L-SIL to H-SIL depends on multiple factors :- [14]

1. Types of HPV :- High risk is associated with HPV-16. [14]
2. Older age[14]
3. Immunosuppression [14]
4. Smoking. [14]

A patient with a post-colposcopic histopathological diagnosis of L -SIL has a 10% chance of harbouring HSIL due inappropriate biopsy site. [14]

High Grade Squamous Intraepithelial Lesion (H-SIL)

Definition :-

According to WHO, squamous intraepithelial lesion carrying significant risk of progression to cancer are called H-SIL. H-SIL are asymptomatic lesions detected by cytology, colposcopy and histology.[15]

Gross :-

Majority of lesions are not visible. Some lesions may be exophytic. Presence of bleeding and ulceration increase the suspicion for cancer. [16]

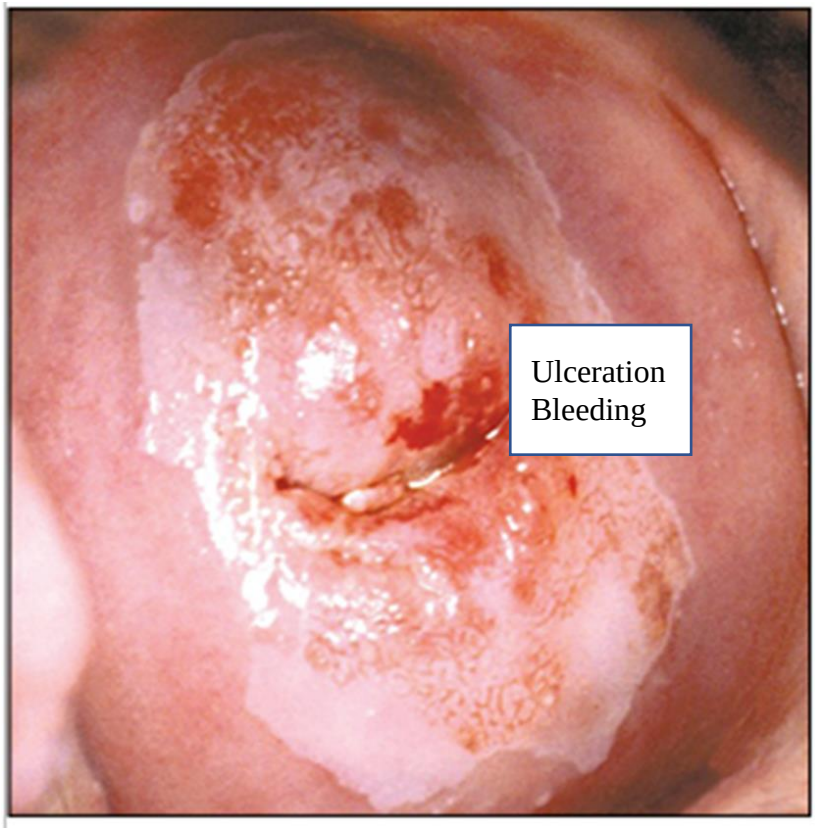


Figure 5. Colposcopic appearance of High Grade Squamous Intraepithelial Lesions[16]

Microscopy :-

Proliferation of dysplastic basal/parabasal cells to middle – third and upper-third of epithelium with intact basement membrane. [17]

Proliferation of squamous cells most frequently in the zone of metaplasia and near the current squamocolumnar junction. These cells show increased nuclear size, irregular nuclear membranes, and increased nucleo-cytoplasmic ratios with multiple mitotic figures. Abnormal mitoses are seen in intermediate and superficial layers of epithelium. [17]

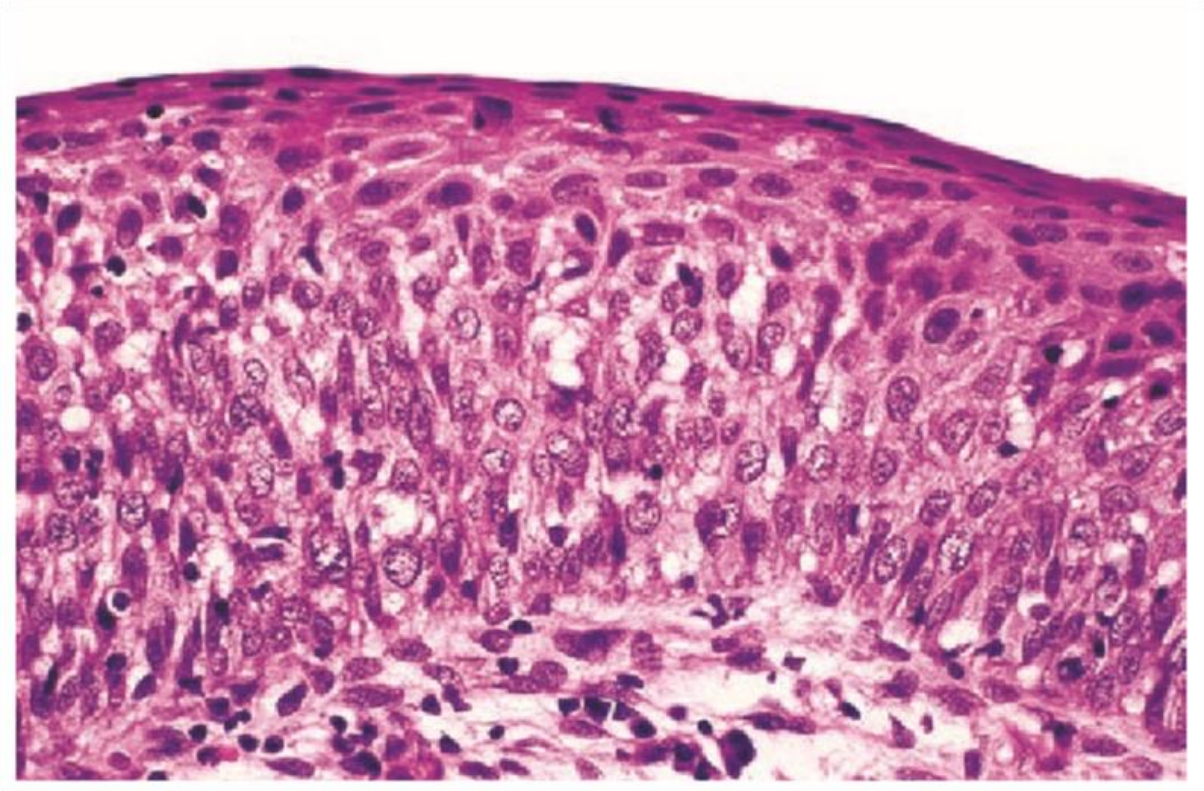


Figure 6. Histomorphology of High Grade Squamous Intraepithelial Lesions[13]

Prognosis :-

High-risk (HR) HPVs are found in over 90% of cervical HSILs. No biomarkers are yet proven to predict transformation of H-SIL to cervical cancer. Most patients are cured by cryotherapy, laser ablation, loop electro-surgical excision procedure (LEEP) or surgical conization. The size of the lesion, completeness of the excision/ablation, and status of margins are factors which predict recurrence. [18] HPV DNA testing 12 months post therapy is better predictor of recurrent or residual disease. [19]

Cervical Cancer

Incidence :-

Cervical cancer is the fourth most cause of cancer in females worldwide. As per 2018 estimates about 5,70,000 new cases of cervical cancer cases are present worldwide. Cervical cancer contributes to about 3,11,000 deaths worldwide. India contributes to about 16.5% of all cancers in female worldwide. About 97,000 new cervical cancer cases are diagnosed thus making cervical cancer as second most common cause of cancer in females after breast cancer. Cervical cancer contributes 7.5% of all cancer death in female. [1]

Risk factors :-

Human Papilloma Virus (HPV) is the most prevalent risk factor for cervical cancer. About 99% of all cervical cancer is associated with HPV. The other risk factors expose cervical epithelium to injuries and infection. Risk factors reflects role of the socio-economic status and personal hygiene in development of cervical cancer.

The risk factors for cervical cancer are :-

1. **HPV :-** HPV infection in sexually active person is the most attributable risk factors for cervical cancer. [20] (Role of HPV in cervical cancer will be further discussed)
2. **Age of first sexual intercourse :-** First intercourse at younger age or proximity to menarche increases risk for cervical cancer. There is two-fold increase risk for cervical cancer with age of first intercourse before 18 years of age. [21]
3. **Multiple sexual partners :-** Risk doubles with two partners and triples with six or more partners. [22]

4. **Parity :-** Early parity before 18 years of age and multiple pregnancies are associated with cervical cancer. [23]
5. **Smoking:-** It is postulated that immunocompromised state in smokers may lead to progression of HPV infection to cervical malignancy. [24]
6. **Tobacco use :-** Tobacco product alters DNA of cells of cervix thus leading to malignant transformation. [24]
7. **Sexually Transmitted Infections (STI) :-** STI with Chlamydia and genital herpes increases risk of HPV infection. [25]
8. **Coinfection with Human Immunodeficiency Virus (HIV) :-** Co infection with HIV leads to immunosuppression which in turn increase risk for persistent HPV infection. [25]
9. **Immunocompromised state :-** Decrease in immune-clearance of HPV infection leads to increase risk of cervical cancer. [25]
10. **Oral Contraceptive Pills (OCPs) :-** Prolonged use of OCPs for more than 5 years increases risk of cervical cancer. There is about 2 fold increase in risk for every 5 years of OCPs use. [25]
11. **Presence of precursor lesions of cervix :-** Increase risk for malignant transformation in HSIL (CIN 1,2). LSIL (CIN 1) is associated with low risk. [25]

Pathogenesis of cervical cancer

Persistant oncogenic HPV infection lies in the centre of the pathogenesis of cervical cancer. More than 90% of cervical cancer are associated with HPV infection. Globally HPV-16 are most detectable virus in cervical cancer followed by HPV-18. [26]

Human Papilloma Virus (HPV) :-

HPVs are non-enveloped DNA virus belongs to papillomavirus family. About > 100 HPV types are known, out of them 15 are oncogenic. They are highly tissue specific as well as lesion specific. Oncogenic HPVs are classified under 'alpha' genus exclusively infects oropharyngeal, cervical mucosa. [27]

Oncogenic HPVs are divided into 2 types based on propensity of their lesions for malignant transformation :- [27]

1. Low -risk HPVs :- Includes HPV-6, 11, 40, 42, 43, 44, 54, 61, 72, 81
2. High-risk HPVs :- Includes HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56,58, 59, 68, 82. HPV-16 and 18 are associated with 70% of cervical carcinoma cases.

Genome of HPV is approximately 8 kb and are covalent, closed, circular. They encode for eight genes :- E1, E2, E4, E5, E6, E7, L1 and L2 and 1 long control region (LCR) which act as binding site for cellular transcription factors, E1 and E2. E1 and E2 controls viral replication and gene expression. [27]

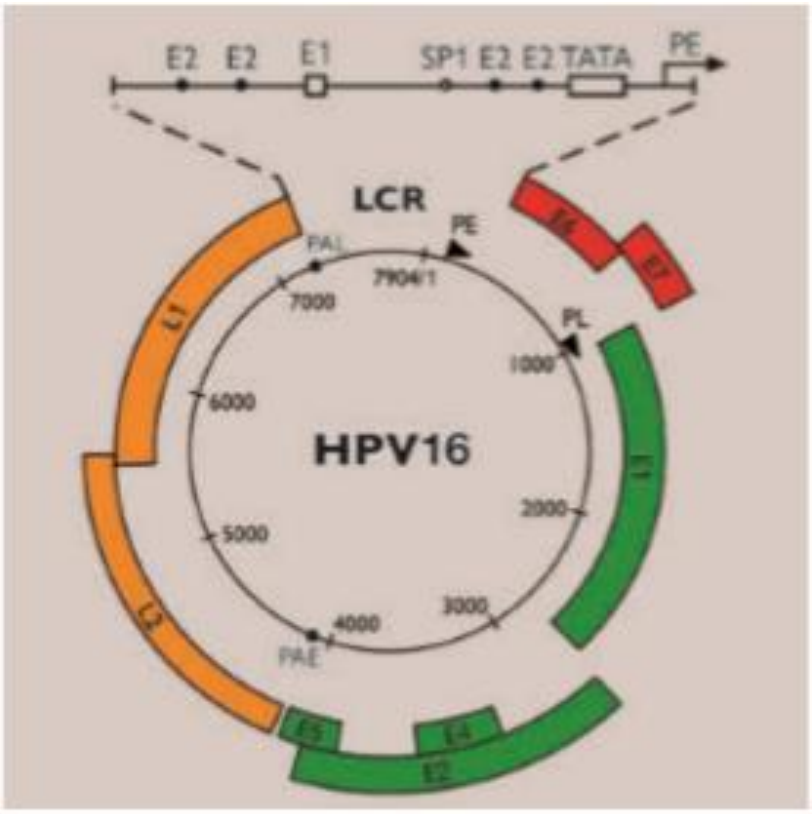


Figure 7. Genome of HPV16[9]

Cervical cancer develop in following phases :- [28]

Phase 1 :- HPV acquisition

Phase 2 :- HPV persistence

Phase 3 :- progression to cervical cancer precursors (correspond to HSIL/CIN2, 3)

Phase 4:- progression to invasion

Phase 3 and Phase 4 are highly variable among women and takes decades.

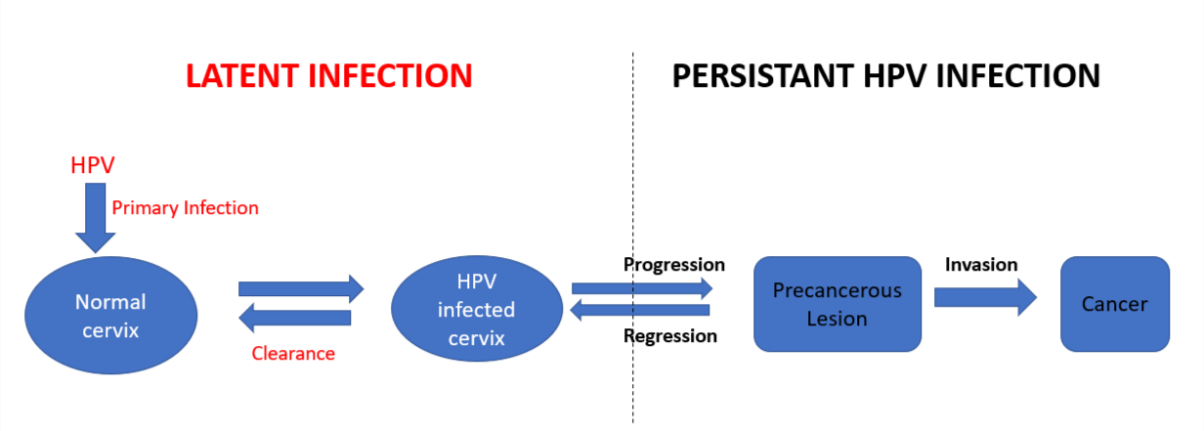


Figure 8. Phases of cervical carcinogenesis[28]

HPV infection :-

HPV is acquire through sexual activity. Numbers of sexual partners increase risk factor for HPV acquisition. Prevalence of HPV infection is extremely high among adolescents and young women. HPV infect immature basal cells of squamous epithelium and immature metaplastic cells present at squamocolumnar junction. Any breach in continuity of superficial and intermediate cell layer exposes the basal cells to HPV infection. HPV proliferates in maturing squamous cells. [28]

HPV persistence :- Majority of HPV infections regress spontaneously. HPV type (HPV-16), immunodeficiency , smoking, multiparity, long-term oral contraceptive use, concurrent sexually transmitted diseases, such as chlamydia, positive family history and coinfection with HIV are factors responsible for persistence of HPV infection. [28]

Latent infection :- The presence of virus particles at the epithelial surface without infection is called as latent or silent infection. HPV DNA remain in episomal form in host epithelial cells where its replication is tightly coupled with the replication of host DNA which in turn couple with division of host epithelial cells. No morphological changes are observed in epithelial cells in latent infection. [28]

Productive infection :- In productive infection, viral DNA replication occur independently to that of replication of host DNA resulting in production of large number of infective virions. Viral replication take place in intermediate and superficial cell layer. [28]

In early part of infection early genes are transcribed which encode for proteins required for viral replication and comprises of E1, E2, E4, E5, E6, E7. [28]

In later part of infection replication of late genes occur which include L1, L2 which produces capsid proteins. [28]

Virus-associated changes in form of acanthosis, cytoplasmic vacuolization, koilocytosis, multinucleation, nuclear atypia are seen in productive infection. [28]

Subsequently unregulated gene expression and abnormal proliferation leading to development of precursor lesions. [28]

Virus clearance :- In most cases, HPV infections are resolved as a result of a cell-mediated immune response. Persistent deregulated gene expression seen after integration of viral genome lead to the accumulation of secondary genetic changes in the infected host cell and development of cancer. Over-expression of the high-risk E6 and E7 proteins facilitate development of cervical cancer. [28]

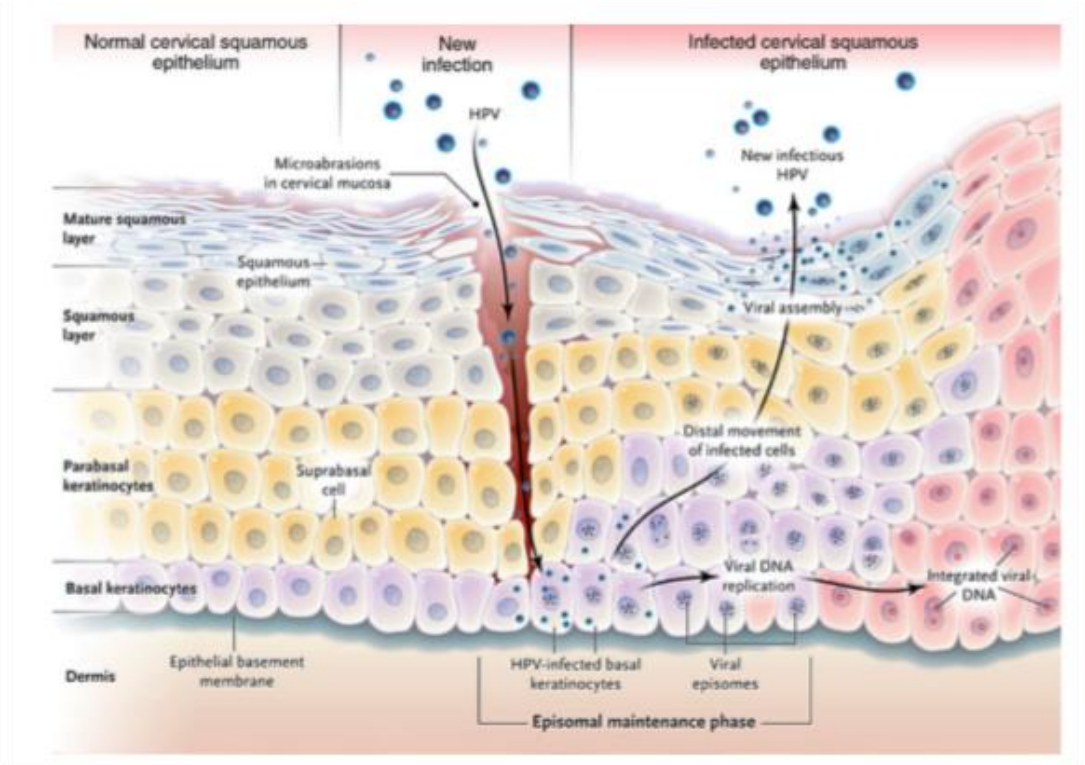


Figure 9. Role of HPV in cervical carcinogenesis[28]

Molecular mechanism of pathogenesis of cervical cancer :-

Normally mature squamous cells of cervix are found to be in arrest of G1 phase of cell cycle but after HPV infection these cells cross the cell cycle because of use of host's DNA synthesis machinery by HPV.

E6, E7 proteins are implicated in the molecular pathogenesis of cervical cancer. Over-expression of the high-risk E6 and E7 proteins facilitate development of cervical cancer.

E6, E7 are zinc binding proteins which exert their effect by binding to cell cycle regulatory proteins. E6 binds to p53 tumour suppressor gene and result in rapid proteolytic degradation of p53 gene through ubiquitin dependent pathway which eventually leads to inhibition of apoptosis. E6 also upregulates expression of telomerase, which leads to immortalization of cells. E7 binds to hypo phosphorylated (active) form of Retinoblastoma (Rb) gene and leads

to its degradation by proteasome pathway. E7 also binds and inhibit p21 and p27 cyclin-dependent kinase inhibitors. The net effect is increased proliferation of cells that are prone to to acquire additional mutations and eventually cancer development. [29]

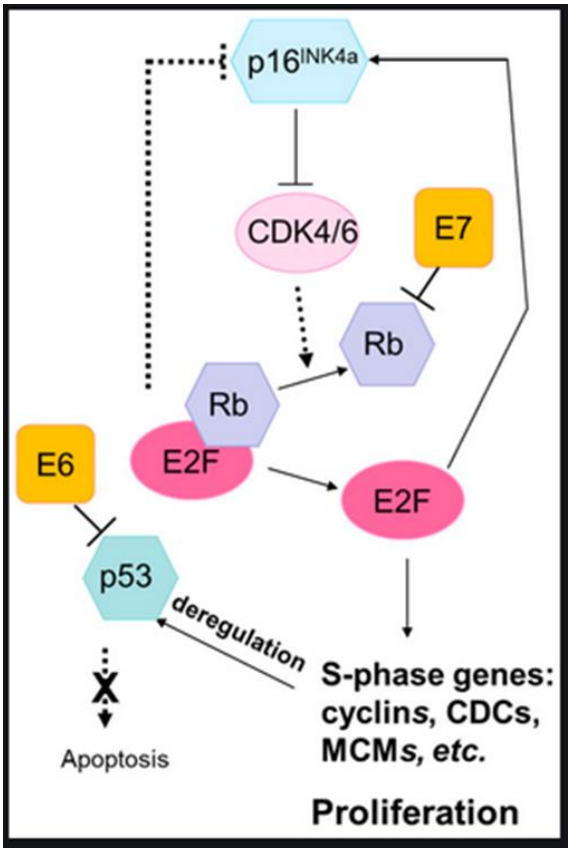


Figure 10. Molecular Pathogenesis of HPV induced Cervical Carcinogenesis[29]

Morphology of Squamous Cell Carcinoma of uterine cervix

Gross :-

Cervical cancer appear as a red, friable, exophytic or ulcerated lesion on visual inspection. Induration or nodularity of the cervix can also be present. Lesions can also be presented as exophytic, papillary, polypoid and endophytic lesions. Endophytic tumours are covered by

normal epithelium. Tumors arising from endocervical canal may remain occult initially and present in late stage of disease. [17]

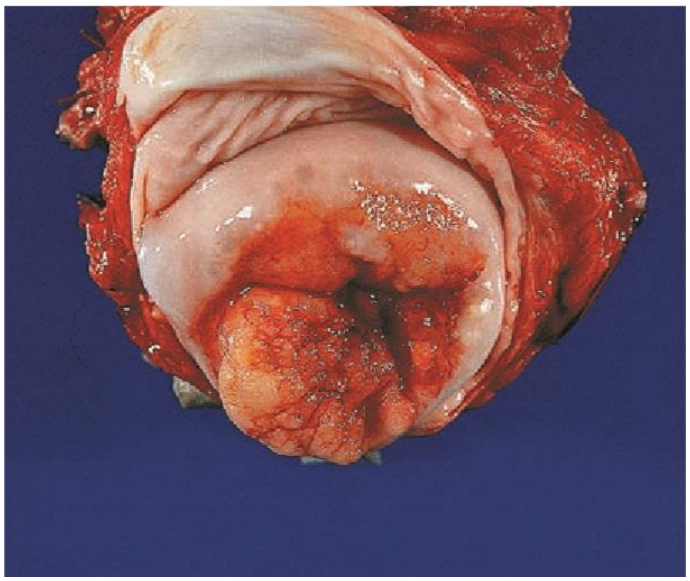


Figure 11. Gross appearance of cervical cancer showing ulcero proliferative growth at ectocervix[17]

Microscopy :-

Invasive squamous cell carcinomas of the cervix vary in their pattern of growth, cell type and degree of differentiation. Most carcinomas have sheet-like growth pattern and infiltrate as networks of anastomosing bands or single cells with an intervening desmoplastic or inflammatory stroma. Superficial stromal invasion may be associated with stromal loosening, desmoplasia and/or increased epithelial cell cytoplasmic eosinophilia. [30]

Grading based on the degree of nuclear pleomorphism, size of nucleoli, mitotic frequency and necrosis. Based on the extent of squamous differentiation, tumours may be graded as well, moderately or poorly differentiated. [31]

Variants of Squamous Cell Carcinoma of uterine cervix

Keratinizing variant :-

They contain keratin pearls, abundant keratohyaline granules or display dense cytoplasmic keratinization, and may be of any grade. The nuclei are usually large and hyperchromatic with coarse chromatin and may appear more smudgy and lack nucleoli. They have got some correlation with keratinizing squamous Intraepithelial Lesion as precursor and in early stages they are mostly ectocervical in location. [32]

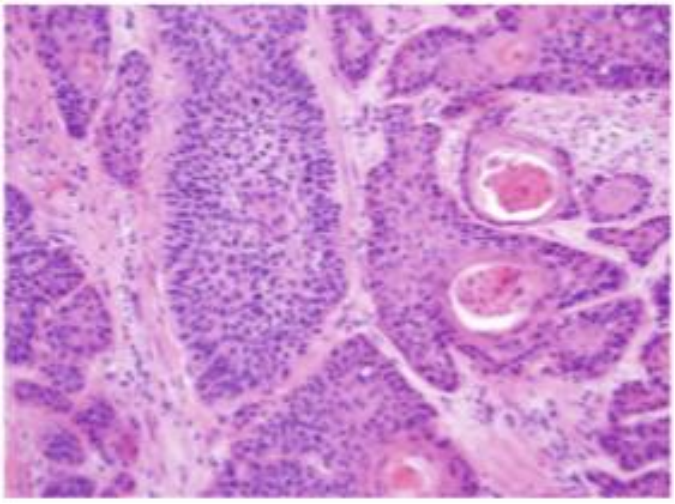


Figure 12. Histomorphology of Keratinizing variant of Squamous Cell Carcinoma of uterine cervix[32]

Non-keratinizing variant:-

This variant is consist of polygonal squamous cells in sheets or nests pattern and may have intercellular bridges, but keratin pearls are not present. Cellular and nuclear pleomorphism are more present in higher grade tumours and contain numerous mitotic figures. The nuclei are relatively large with unevenly distributed, coarsely granular chromatin and distinct nucleoli which may be irregular or multiple. [33]

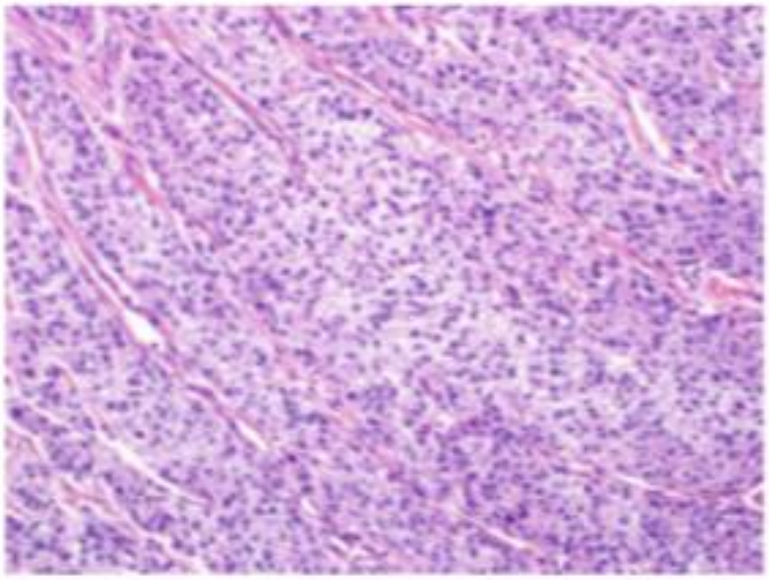


Figure 13. Histomorphology of Non-keratinizing variant of Squamous Cell Carcinoma of uterine cervix[33]

Papillary variant :-

This variant is consist of thin or broad papillae with connective tissue stroma and lining epithelium showing the features of HSIL. Superficial biopsy may not reveal evidence of invasion but complete excision of the clinically visible lesion reveals an underlying invasive tumour of the usual type. [34]

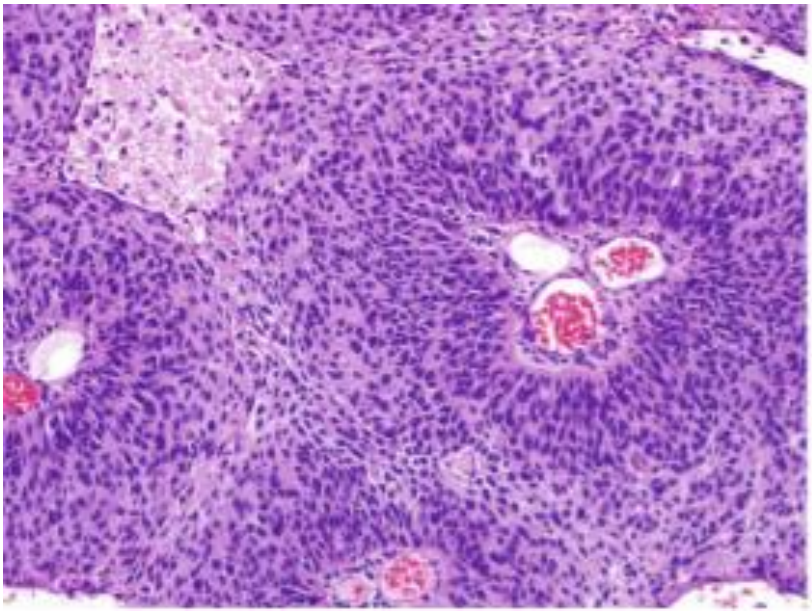


Figure 14. Histomorphology of papillary variant of Squamous Cell Carcinoma of uterine cervix[34]

Basaloid variant :-

They are high grade aggressive tumors composed of nests of immature, basal-type squamous cells with scanty cytoplasm that resemble closely the cells of HSIL (CIN 3) of the cervix. Some individual cell keratinization may be present but keratin pearls are rarely seen. The nuclei are pleomorphic and has high mitotic counts. Sometimes the tumour shows “geographical” or “comedo-like” necrosis. [35]

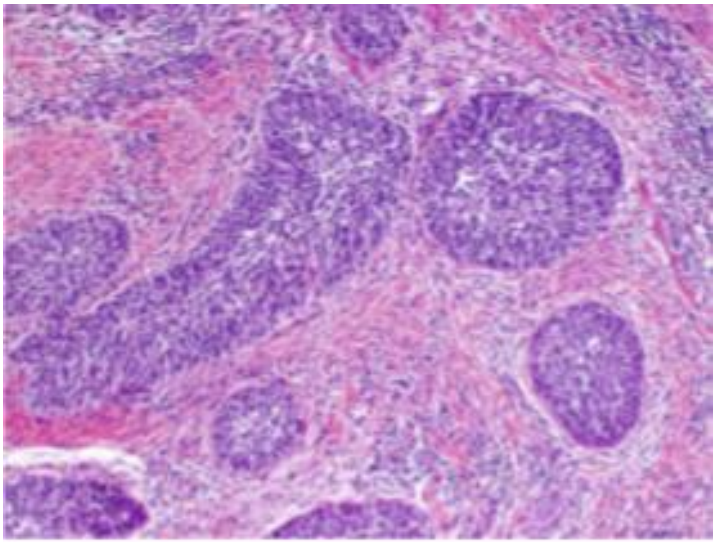


Figure 15. Histomorphology of basaloid variant of Squamous Cell Carcinoma of uterine cervix[35]

Verrucous carcinoma :-

Verrucous carcinoma are highly differentiated squamous cell carcinoma having hyperkeratotic, undulating, warty surface and invades the underlying stroma in the form of bulbous epithelial pegs with a pushing border. The tumour cells have abundant cytoplasm, and their nuclei show minimal atypia. HPV cytopathic effect (koilocytosis) are not found.

Verrucous carcinomas have a tendency to recur locally after excision but do not metastasize. They are distinguished from condylomata acuminata by their broad papillae that lack fibrovascular cores and the absence of koilocytosis.Verrucous carcinoma is distinguished from the more common types of squamous cell carcinoma by presence of minimal nuclear atypia and absence of infiltration. [36]

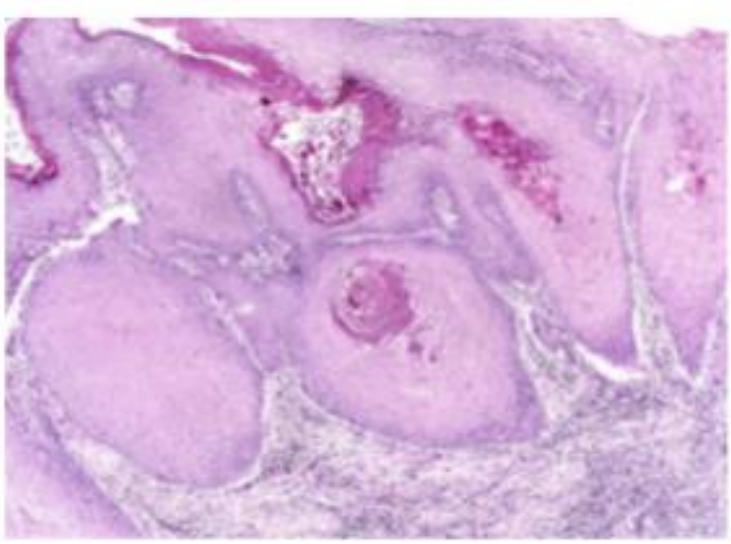


Figure 16. Histomorphology of Verroucous Carcinoma of uterine cervix[36]

Warty/condylomatous squamous cell carcinoma :-

Squamous cell carcinoma with a warty surface and low-power architecture analogous to a condyloma or Bowenoid lesion of the vulva is defined as warty/condylomatous SCC. In early invasive lesions the epithelium may be keratinizing and cells show changes similar to koilocytic atypia. [37]

Squamotransitional carcinoma :-

Transitional cell carcinomas of the cervix are rare and are indistinguishable from their counterparts in the urinary bladder. They may occur in a pure form or may contain malignant squamous elements. Tumours show papillary architecture with fibrovascular cores, lined by a multilayered, atypical epithelium resembling HSIL (CIN 3). No evidence of relation to transitional cell metaplasia seen. [38]

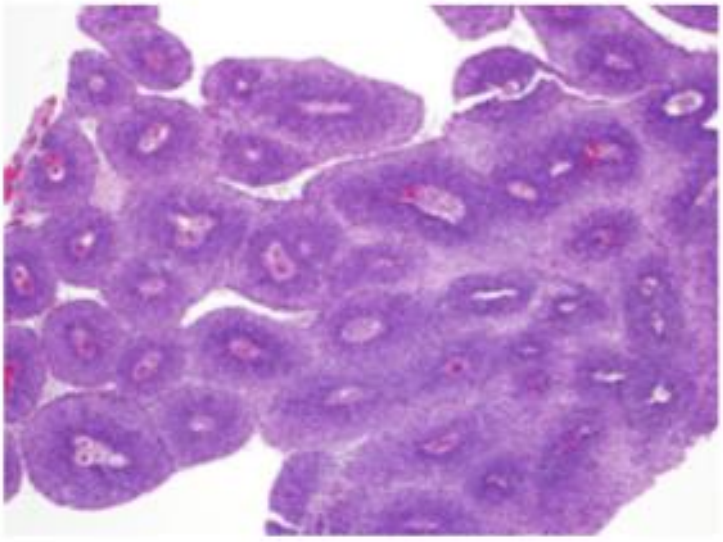


Figure 17. Histomorphology of Squamotransitional Carcinoma of uterine cervix[38]

Lymphoepithelioma- like carcinoma :-

This is a rare tumour in the cervix. Histologically appear similar to Lymphoepithelioma- like carcinoma of nasopharynx . It is composed of poorly defined islands of undifferentiated squamous cells in a background of numerous lymphocytes. The tumour cells have uniform, vesicular nuclei with prominent nucleoli and moderate amounts of slightly eosinophilic cytoplasm with distinct cell borders. The tumours of the cervix are p16 positive and show relation with HPV. [39]

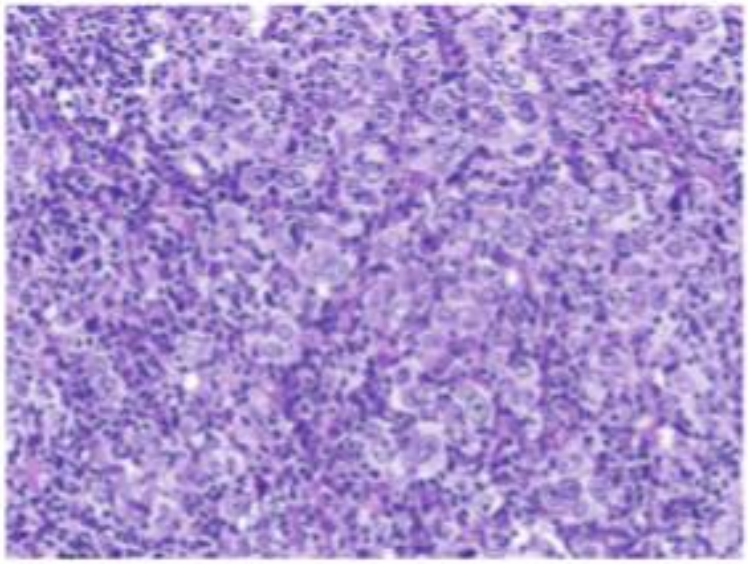


Figure 18. Histomorphology of Lymphoepithelioma-like Carcinoma of uterine cervix[39]

Role of Radiology in cervical cancer :-

Diagnostic imaging during the primary diagnostic work-up is recommended to better assess tumor extent and metastatic disease. For pretreatment local staging, imaging by transvaginal/transrectal ultrasound and/or magnetic resonance imaging (MRI) is used. MRI is used to find extension of pelvic tumor. MRI should include at least two T2-weighed sequences in saggital, axial oblique, or coronal oblique orientation in relation to the long and short axis of the uterine cervix. MRI provide accurate assessment of tumor size, depth of stromal invasion and parametrial invasion. In locally advanced cervical cancer, positron emission topography (PET-CT) or computed tomography (CT scan) is recommended for detection of lymph node metastases and distant spread. Novel imaging techniques like novel positron emission tomography (PET) radiotracers specific for hypoxia provide visualization of microstructural and functional characteristics. [40]

Table 3. The International Federation of Gynecology and Obstetrics (FIGO) Staging of Carcinoma of uterine cervix (FIGO) staging,2018[40]

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 minimum depth of invasion <5mm
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.
IB1	Invasive carcinoma ≥ 5 mm depth of stromal invasion, and < 2cm in greatest dimension.
IB2	Invasive carcinoma ≥ 2 cm and < 4 cm in greatest dimension.
IB3	Invasive carcinoma ≥ 4 cm in greatest dimension.
II	The carcinoma invades beyond the uterus, but has not extended onto lower third of vagina or to pelvic wall.
IIA	Involvement limited to the upper two-third of the vagina without parametrial involvement.
IIA1	Invasive carcinoma < 4 cm in greatest dimension.
IIA2	Invasive carcinoma ≥ 4 cm in greatest dimension.
IIB	With parametrial involvement but not up 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 non-functioning kidney and/or involves pelvic and/or para-aortic lymph nodes.

IIIA	The carcinoma involves 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.
IIIC1	Pelvic lymph node metastasis only
IIIC2	Para-aortic lymph node metastasis
IV	The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. (A bullous edema, as such, does not permit a case to be allotted to StageIV)
IVA	Spread to adjacent pelvic organs.
IV B	Spread to distant organs.

Prognostic factors :-

The prognosis of cervical carcinoma is related to the following parameters:

1. **Clinical stage :-** Stage is the most important prognostic determinator. It should be noted that clinical staging is used for cervical cancer, unlike for other organs. The International Federation of Gynecology and Obstetrics (FIGO) staging is used for cervical cancer. Tumor Node Metastasis (TNM) staging is important only for patients treated surgically. Dual pretreatment staging by TNM and FIGO was recommended. Early stage cancer has better prognosis than late stage cancer. [40]

2. Nodal status. [41,42]

3. Size of the largest involved node and number of positive nodes[41,42]

4. Size of the primary tumor[41]

5. Depth of invasion [41]

6. Endometrial extension :- The presence endometrial extension decreases the survival rate by 10%–20%.[43]

7. Parametrial involvement[44]

8. Blood vessel invasion[41]

9. Microscopic grade is not of prognostic significance[45]

10. Histomorphology has no prognostic significant. [46]

Markers in cervical cancer

Cervical cancer has evolved as public health problem over the years in developing countries like India. Role of HPV is well established in pathogenesis of cervical cancer. Early detection of HPV infection and risk stratification are helpful in primary prevention of cervical cancer. In secondary prevention of cervical cancer early detection of cases in early stages and early initiation of treatment plays central role. Universal pap smear screening was recommended by WHO to curb diagnosis in late stages. But sensitivity and specificity of pap smear are 60-80% and 70-95%. There are still undetected cases by pap smear. Various markers have been identified and some are under research to increase the sensitivity and specificity. [47]

The markers can be broadly divided into :-

1. HPV testing
2. Products of HPVs : E6/E7 and L1 proteins
3. Growth factor receptors : EGFR,C-erb-2
4. Signal transduction proteins : RAS, β-Catenin, E-cadherin, cytokeratins
5. Nuclear regulatory proteins : C-myc

6. Cell cycle regulators : p14, p16, p53, RB, p63, Cyclins/ cyclin inhibitors, Ki67, MN antigen
7. Regulators of metastasis : Metalloproteinases, cathepsin

HPV DNA Based testing :-

Indications for HPV DNA Tesing:-

1. Triage of women with equivocal or low grade cytological abnormalities. [48]
2. Follow up of women with abnormal screening results who are negative at colposcopy/biopsy. [48]
3. Prediction of therapeutic outcome after treatment of cervical intraepithelial neoplasia(CIN) .[48]
4. Primary screening[48]
5. Investigation for prevalence of type-specific HPV. [48]

Four tests have been approved by Federation of Drug Administration (FDA) for HPV testing based on identification of DNA :-

A. **Hybrid capture 2 (Qiagen) :-** Detect 13 high risk HPVs. Hybrid Capture 2 is based on solution-based hybridization assay and signal amplification technology which will be detected by chemiliminescence by using specific HPV (13) probes. [49] It helps in distinguishing between high risk HPV and low risk HPV but it cannot genotype single HPV. It can show cross reactivity to some low risk HPVs. 5 – 12 % of false negative rates are documented in literature. This test does not have any internal control. So, determination of adequacy of specimen or presence of any interfering substances are not possible.[50]

B. **Cervista HPV HR (Hologic) :-** Target 14 high risk HPVs. It is based on signal amplification method for detecting specific nucleic acids. Individual HPV types are not identified by this test. [49] Like other signal amplification technology (Hybrid capture 2), it also show cross reactivity with other low risk HPVs. But it has got a lower cross reactivity as compared to hybrid capture 2 test. Presence of internal control is other advantage of cervista HPV HR test over hybrid capture test. [51]

C. **Cervista HPV 16/18 (Hologic) :-** Identify HPV 16,18. [49,51]

D. **Cobas 4800 HPV :-** Target 14 high risk HPVs. It is based on real time Polymerase Chain Reaction (PCR) method to detect 14 HR-HPV. Individual genotyping of HPV can be done by cobas 4800 HPV test because it is based on PCR. It has got comparable sensitivity and improved specificity as compared to hybrid capture 2 tests. It shows very low level of cross reactivity with other low risk HPVs as compared to hybrid capture 2 test. It is clinically validated for triage of ASC-US cases. False negative results may be seen in setting of multiple infections due to competition for reagents.[52,53]

E6/E7 mRNA reverse transcription Polymerase Chain Reaction :-

Overexpression of E6/E7 mRNA by reverse transcription PCR in setting of persistant infection may show disease progression. APTIMA assay detect E6/E7 mRNA from 14 high risk HPVs and is only FDA approved test for triage of women more than 21 year of age with ASCUS cytology and for screening of women of age > 30 years. APTIMA test show similar sensitivity for CIN 2, CIN 3 but higher specificity for high grade lesions as compare with HPV DNA based tests. [54]

HPV Integration :-

It indicate severe chromosomal instability and advanced stage of transformation process. E2 is frequently lost after integration. Real time PCR based quantification of E2 and E6/E7 genes are commonly employed for studying viral integration. The ratio of E2:E6/E7 is 1:1 before integration but after integration the ratio will shift to E6/E7. [55,56]

p16INK4a :-

It belongs to the category of cyclin dependant kinase inhibitors having role in cell cycle regulation where it act as tumor suppressor protein. In normal cell cycle, p16 inhibit cyclin dependent kinase (CDK)-4/6 which phosphorylate pRB gene leading to splitting of elongation factor E2F. During carcinogenesis viral protein E7 degrade RB protein leading to unchecked expression of E2F. Overexpression of p16 leads to suppression of CDK4/6. Overexpression of p16 can be studied by immunohistochemistry and immunocytochemistry. Overexpression of p16 is seen in HSIL, SCC cases. Greater risk of progression is seen in p16 positive LSIL lesion than p16 negative LSIL lesion. It act as surrogate biomarker of deregulated HPV oncogene expression. It has role in triage of ASCUS and LSIL. [57]

p53 :-

Activation of normal p53 by DNA-damage leads to cell-cycle arrest in G1 and induction of DNA repair, by transcriptional up-regulation of the cyclin-dependent kinase inhibitor p21, and the GADD45 genes, respectively. Successful repair of DNA allows cells to proceed with the cell cycle; if DNA repair fails, the cell gets apoptised. So, it has a role of tumor suppressor gene. Point mutation, loss of heterozygocity and binding of E6 oncoprotein leads to inactivation of p53 gene. Pont mutation at 72 codon lead to substitution of proline in place of arginine. E6 efficiently binds to p53arg as compare to p53pro. Women with p53arg are at higher risk of HPV dependent cervical cancer. [58] Immunohistochemistry can be used to determine expression

of p53. Expression of p53 increases from LSIL to HSIL to SCC of cervical cancer as compared with normal cervix. Conflicting results are also found due to degradation of p53. It has no prognostic significance. [59]

P63/73 :-

They are homoloque of p53. Basal cells and immature squamous cells normally show expression of p63/73. But their expression progressively increases in LSIL, HSIL and SCC of cervix. They are used as markers of intraepithelial lesions. [60]

pRb :-

Product of Retinoblastoma gene (Rb) and has role in regulation of cell cycle in S phase. The vial oncoprotein E7 protein binds to the RB protein and displaces the E2F transcription factors which promote cell cycle progression. Immunohistochemistry, southern blotting are used for detection of pRb expression. Premalignant and malignant lesions show increased expression of pRb. Inverse correlation is seen between expression of pRb and E7 oncoprotein because of degradation of pRb after binding to E7. [61]

C-myc –

Overexpression of C-myc oncogene is seen in LSIL, HSIL and invasive carcinoma. However it show variable expression depending on extent of disease. [62]

p14 Alternate Reading Frame protein/ p14ARF :-

Normally p14ARF activates the p53 pathway by inhibiting MDM2 and preventing destruction of p53.Thus acting as a tumor suppressor. Overexpression of p14 is due to destruction of p53 by E6 leading to loss of negative feedback regulation. Increase expression of p14 in basal,

parabasal and superficial cells indicates advanced lesion. HPV negative cervical cancer cases also show increase expression of p14. Increase p14 expression predict disease progression. [63]

Cyclins :-

Cyclins are group of cell cycle regulators having positive effect on cell cycle progression. They act together with Cyclin Dependent Kinases (CDK). Three types of cyclins are implicated in cell cycle progression :- Cyclin A,D and B. Immunohistochemistry helps in detection of expression of cyclins. In normal cervix, only basal and parabasal cells show expression by cyclins. Cyclin-D1 show progressive expressions in between LSIL, HSIL and cervical carcinoma. It also correlates wth behaviour of cancer. [64]

Epidermal Growth Factor Receptor (EGFR):-

EGFR is a 170KDa transmembrane glycoprotein receptor which has role in regulation of intracellular signal transduction required for cell growth. Deregulation of EGFR leads to malignant transformation. Normal expression is seen in ectocervical basal cell Membrane. Cytoplasmic expression is seen as differentiation progresses. Increase in expression varies with the grade of lesion. It is found to be associated with HPV infection. More expression is seen in recurrent/metastatic site as compare to primary site. [65]

Ki67 :-

It is a proliferation marker and is expressed in cells in all phases except G0 phase of cell cycle. Maximum intensity is seen in mitosis. Ki67 expression is demonstrated by IHC where it take nuclear staining. Only basal and parabasal epithelial layer take positive staining in normal cervix. Increase expression is seen in LSIL, HSIL, Cervical carcinoma. In cervical carcinoma

expression of Ki67 correlates with histological grades. It has a prognostic role in cervical carcinoma. [66]

p16 and Ki67 dual staining :-

Dual expression of p16 and Ki67 indicate E7 mediated inactivation of Rb gene and malignant transformation. Dual staining is present in HSIL and absent in LSIL. It is utilize to detect HSIL in cervical cytology. It is 44orphology independent and has high sensitivity and specificity in identification of HSIL. It can also be used in triage of women with ASCUS/LSIL cytology where it decreases unnecessary follow up diagnostic procedure. [67]

MN antigen :-

It is a membrane associated antigen identical to carbonic anhydrase. Low expression is seen in poorly differentiated carcinomas, stromal invasion, regional lymph node metastasis and HPV negativity. Thus it has a prognostic significance in cervical carcinomas. [68]

Metalloproteinase (MP):-

It causes degradation of basement membrane type IV collagen and thus play role in tumor invasion and metastasis. Increased expression is seen in microinvasive SCC than CIN lesions. It has got prognostic significant as overexpression of MP correlates with presence of nodal metastasis, number of positive lymph nodes and recurrence. [69]

Role of Epithelial Mesenchymal Transition in cervical cancer

Epithelial cells have apical-basal polarity and are connected by specialized adhesion complex, while mesenchymal cells are spindle shaped, more motile cells with front-back cell polarity

and lack adhesion complexes. Epithelial cells can converts into mesenchymal cells by a process called epithelial mesenchymal transition. During epithelial mesenchymal transition, there is suppression of epithelial adhesion junctions, gain of mesenchymal markers, cytoskeleton reorganization, anoikis resistance and increased cellular migration and invasiveness. [70]

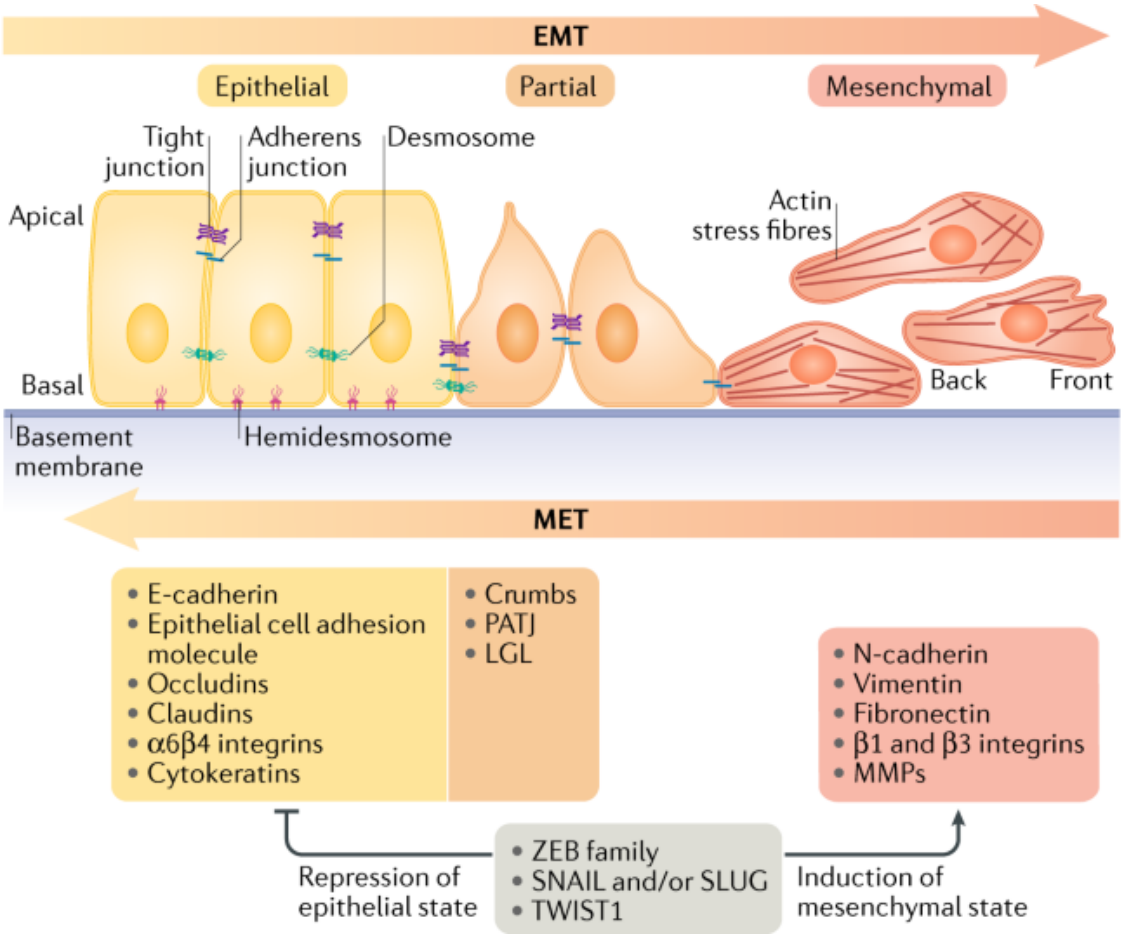


Figure 19. Role of EMT and MET in carcinogenesis and phenotypic and molecular changes occur during transition[70]

Types of Epithelial mesenchymal transition :-

There are three types of EMT :-

1. **Type 1 EMT:-** It is seen during embryonal developmental as part of gastrulation and neural crest migration where primitive epithelial cells converts into mesenchymal cells.

Some of these mesenchymal cells get converted into secondary epithelial cells by mesenchymal-epithelial transition (MET). [70]

2. **Type 2 EMT:-** It is inflammation-induced in response to persistent injury where epithelial/endothelial cells converts into fibroblasts. [70]
3. **Type 3 EMT:-** It is seen in cancer where malignant epithelial cells converts into mesenchymal cells in the primary nodules and migrate through blood vessels to distant metstatic site where it forms secondary nodule through MET. [70]

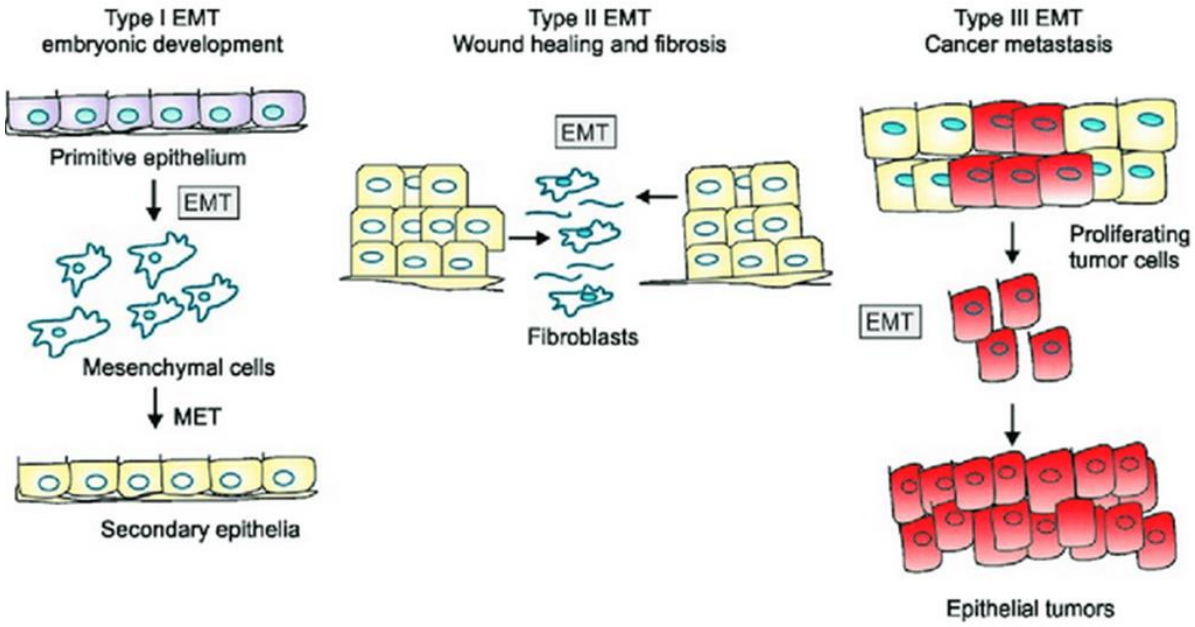


Figure 20. Types of EMT[70]

Regulators of EMT

EMT is a highly regulated mechanism.

Several regulators are implicated in EMT regulation:- [5]

1. **Oncoproteins :-** HPV16E**7.** [5]
2. **EMT Activators :-** Soluble factors like EGF, Ion transport system, Cytoskeletal modulators, Transcription factors like Snail, Twist 1, Twist 2. [5]
3. **EMT suppressor :-** Secreted factors, Transcription factors. [5]

Table 4. Regulators of Epithelial Mesenchymal Transition[5]

EMT Regulators[5]	**Categories[5]**	**Examples[5]**
Oncoproteins	**HPV Viral protein**	HPV16E7
EMT activators	**Soluble factors**	Transforming growth factor (TGF-β) Epidermal growth factor (EGF) Jagged 1
	Ion transport system	KCl cotransporter-3
	Cytoskeletal modulators	Ras homolog guanosine triphosphatase(Rho-GTPase) Rho C Gelsolin
	Transcription factors	Snail Twist1 Twist2 Zinc finger E-box binding homeobox 1 (ZEB1) Notch 1 Matrix Metalloproteinases
EMT suppressors	**Secreted factors**	Secreted frizzled-related protein (SFRP1/2)

	Transcription factors	Lim homeobox transcription factor 1 alpha (LMX-1A) homeobox protein

HPV Viral proteins :-

It is postulated that HPV6E7 can mediate EMT in the early stage of carcinogensis by inducing molecular changes in the epithelial cells. There is loss in epithelial characeristics and acquisition of mesenchymal characteristics in keratinocytes.[71]

Soluble factors:-

Soluble factors are one of the components of tumor microenvironment. TGF-β1 has role in EMT as studied in cervical cancer cell line. EGF modulate extracellular fibronectin and integrins and thus take part in EMT. Jagged 1 is a ligand of Notch1 receptor which regulate EMT through phosphatidylinositol 3- kinase dependent signalling. Interplay of soluble factors with their receptors modulates extracellular matrix and helps in EMT.[72,73]

Ion transport system :-

Ion transport system maintain intracellular ion homeostasis. They have role in initiaition of EMT process. Overexpression of Potassium chloride cotransporter-3 (KCC3) by oncogenic growth factor receptor leads to increase in KCl cotransport which further inhibits E-cadherin/β-catenin complex formation and eventually leading to weakening of cell junction and promotion of EMT. KCC3 inhibit promoter activity of E-Cadherin and increase proteasome-dependent degradation of β-catenin.[74]

Rho GTPase:-

They belongs to category of cytoskeletal modulators having role in EMT, migration and invasion. Inactive GDP- bound form and active GTP-bound forms exists. Guanine nucleotide exchange factor (GEFs) and GTPase activating proteins (GAPs) tightly regulates activity of RhoGTPase. Active RhoGTPase interact with cell membrane which leads to activation of downstream effectors which brings biological changes like reorganisation of actin cytoskeletal, cell motility and polarity.[75] Abberant RhoGTPase signalling is seen after activation of carbonic anhydrase IX which leads to cytoskeletal remodelling, increase motility and invasion.[76]

RhoC:-

RhoC stand for Ras homolog gene family member C belongs to RhoGTPase family. It is effector of transcription modulator Notch1 through phosphatidyl inositol-3 kinase pathway in cervical cancer.[77] It regulate actin organisation in tumors resulting in enhanced migration, invasion and metastasis. RhoC mediate EMT which is stimulated by growth factors like TGF-β1. It regulates Mitogen Activated Protein Kinase (MAPK) and Phosphatidylinositide 3 kinase/AKT Serine Threonine Kinase (PI3K/AKT) pathways which have role in cancer progression and maintenance. RhoC also has role in angiogenesis by modulating expressions of growth factors required in angiogenesis like Vascular Endothelial Growth Factor (VEGF), fibroblast growth factor (FGF), interleukin 6 and interleukin 8. Role of RhoC in increasing stemness among cancer cells is also recently implicated.[78]

RhoC is strongly expressed in squamous cell carcinoma. Across all grades of squamous cell carcinoma, the positivity for RhoC increases. Percentages of positive cases for cytoplasmic expression for RhoC increases from 41% of normal cervix to 87% of squamous cell carcinoma of cervix (n=36) in a study done by Srivastava et al in 2012. The significant differences in

cellular positivity and intensity of RhoC expression is reported across the grades ($p<0.007$) with increase in the percentages of positive cases across the grades.[77]

Recently targeted therapy against RhoC is developed by targeting an HLA restricted epitope of RhoC. Apart from targeted therapy and immunotherapy against RhoC, it is seen that statins have role in reversal of RhoC induced tumor phenotypes. Statins are inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG- CoA reductase). HMG CoA reductase play role in HMG-CoA reductase pathway. This pathway produces intermediate products like geranylgeranyl pyrophosphate (GGPP) and Farnesyl pyrophosphate (FPP) which have role in activation of RhoGTPases.[79,80,81]

Transcription factors :-

Several transcription factors like Snail, Twist 1, Twist 2, Six1 homeoprotein are implicated in the regulation of gene expression related to EMT.

Snail is a zinc-finger transpription regulator. EGF promotes expression of snail. There are three members of snail family :- Snail 1, Snail 2 (Slug) and Snail 3 which are involve in EMT. They are common downstream target of various pathways involved in EMT. Snail activation leads to suppression of E-Cadherin, increased expression of fibronectin and fibrosis.[82]

Twist is a basic helix-loop-helix protein involve in regulation of lineage determination and cell differentiation. Its upregulation is seen during early embryonic development, tissue fibrosis and cancer metastasis. It can independently suppress E-Cadherin and upregulate fibronectin.[83]

Zinc finger E-box binding homeobox 1 (ZEB 1) is a transcription factor contain a conserved central homeobox region and two zinc finger domains at the N and C-terminals. These zinc binding domains help in binding to target DNA sequence. Normally it is not expressed.

Aberrant overexpression is seen in cancers which leads to induction of EMT. It is postulated that snail indirectly induces ZEB1 expression.[84]

Notch 1 regulate EMT via notch- PI3K -dependant pathway. RhoC is downstream effector of notch- PI3K -dependant pathway leading to inhibition of β-catenin.[77]

Matrix Metalloproteinases (MMPs) are proteolytic enzymes associated with tumorogenesis. They degrades extracellular matrix and enables tumor cells to migrate, invades and spread to secondary sites. MMPs can stimulate EMT process by enhancing invasion and metastatic potential.[85]

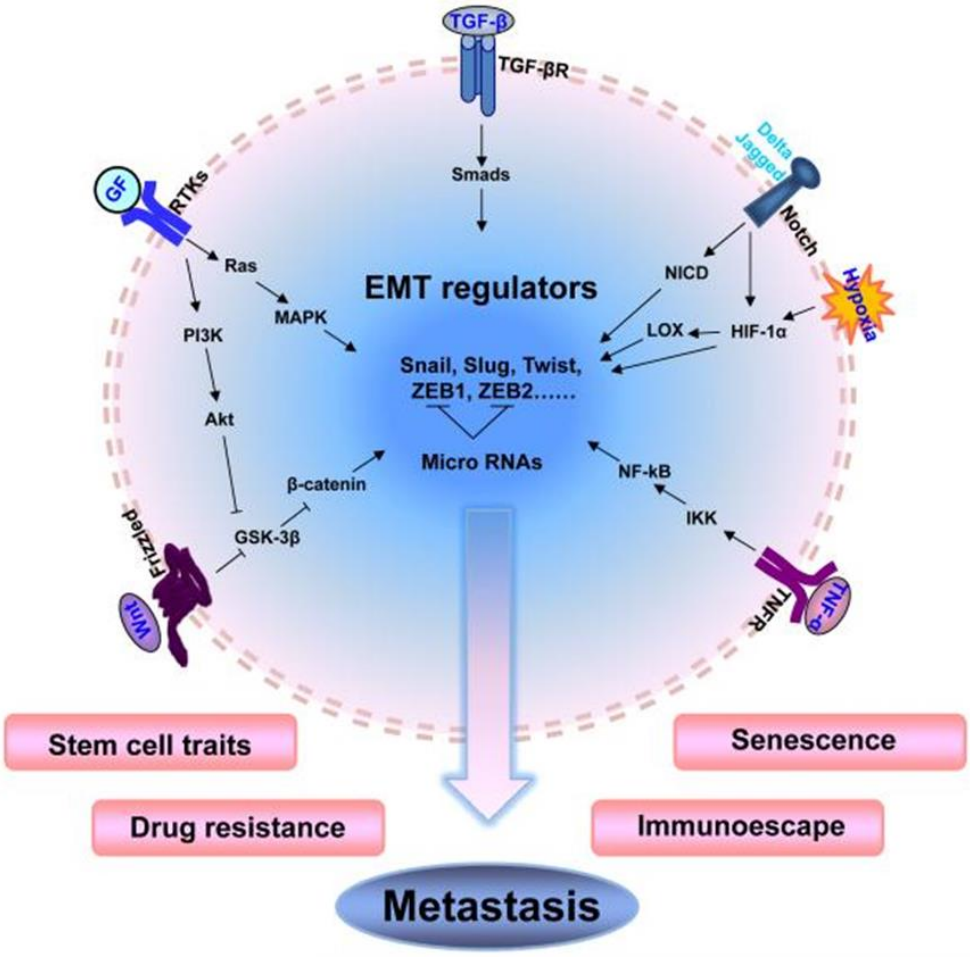


Figure 21. Regulators of EMT[5]

EMT Supressors :-

Secreted frizzled-related protein 1/2 (SFRP1/2) and Lim homeobox transcription factor 1 alpha (LMX-1A) are two tumor suppressor genes which inhibit invasion and metastasis through incomplete EMT. SFRP1/2 act as antagonist of Wnt pathway leading to decrease Wnt signalling and suppression of tumorogenicity. Epigenetic silencing of SFRP1/2 genes by promoter hypermethylation leads to hyperactivation of Wnt signalling and EMT.[86] LMX-1A suppress cancer metastasis by inhibiting BMP 4/6.[87]

Markers of EMT

Several markers are used to demonstrate the process of EMT. Some of these markers can be used for development of targeted therapy. The EMT markers can be broadly divided into cell surface proteins, Cytoskeletal markers, Extracellular matrix (ECM) proteins, transcription factors, MicroRNAs.[6]

Table 5. Markers of Epithelial Mesenchymal Transition[6]

Name[6]	**Acquired markers**[6]	**Attenuated markers**[6]
Cell Surface Proteins	N- Cadherin OB- Cadherin α5β1 Integrin α5β6 Integrin Syndecan-1	E-Cadherin Zona Occludens (Z0)-1

Cytoskeletal markers	Fibroblast Secretory Protein 1 (FSP-1) α-Smooth Muscle Actin (α-SMA) Vimentin β- Catenin	Cytokeratins
ECM Proteins	α1(I) Collagen α1(III) Collagen Fibronectin Laminin 5	α1(IV) Collagen Laminin 2
Transcription Factors	Snail1 Snail2 ZEB1 Twist FOXC2 Goosecoid	
MicroRNAs (miR)	miR10b	miR-200 family

	miR-21	

Cadherins :-

Cadherins are transmembrane glycoproteins involve in calcium dependant cell-cell adhesion. They maintain tissue morphology, integrity and homeostasis. Classical cadherins include E-Cadherin, N-Cadherin, P-Cadherin,VE- Cadherin and OB-Cadherin. Altered expression of cadherins play role in tumorogenesis, tumor progression, angiogenesis and tumor immune response.[88]

Cadherins play role in EMT by two processes:- 1. Loss of E-Cadherin and 2. Cadherin switch. Loss of E-Cadherin lies at the centre of E-Cadherin. Cadherin switch from E-Cadherin to N-Cadherin is also discovered. N-Cadherin is expressed by mesenchymal cells, fibroblasts, cancer cells and neural tissue. N-Cadherins have role in embryonic development and cancer progression. OB-Cadherins are expressed in activated fibroblasts and is consider as definite marker for activated fibroblasts. It has role in type 2 EMT associated with fibrogenesis.[89]

β- Catenin:-

It is a core component of cadherin protein complex. It take part in Wnt signalling pathway which regulates cellular proliferation, differentiation, migration, genetic stability, apoptosis and stem cell renewal. The lack of β- Catenin promotes EMT process in cervical carcinogenesis. It show decreased expression in SCC as compare to normal cervix.[90]

Integrins :-

Integrins are transmembrane receptors which facilitate cell-ECM adhesion by activating signal transduction pathways mediating cytoskeletal organisation. They have two subunits :- 1. α-subunit and 2. β-subunit. They are diverge in binding to varieties of matrix proteins. Role of α5 integrin is seen in EMT process. They mediate EMT during embryonal development, fibrosis and cancer progression.[91]

Syndecans :-

They are transmembrane proteins belonging to family of cell surface heparan sulfate proteoglycans. They binds with various growth factors and modulate their downstream signalling. Syndecan 1 promotes epithelial differentiation. Decreased expression of Syndecan 1 is seen as mesenchymal differentiation proceed.[92]

Cytokeratins:-

Cytokeratins are intermediate filaments containing keratin and they are found in the intracytoplasmic cytoskeletal of epithelial tissue. They are divided into 2 types :- 1. Type I/Acidic and 2. Type II/Basic. CK9-20 are examples of type I cytokeratins while CK1-8 belongs to type II cytokeratins. They can also be divided into 2 types based on their molecular :- 1. High Molecular Weight Cytokeratins which comprise of CK1-6, CK7-8 and 2. Low Molecular Weight Cytokeratin consisting of CK9-17 AND CK18-20. Cytokeratins are universal epithelial markers. Functional filaments are heteropolymers consist of equimolar amount of both type I and type II CKs.[93]

Normal cervix show different immunoreactivity to different types of CKs based on epithelial layers and sites (ectocervix/endocervix). Ectocervical squamous epithelium and mature squamous metaplastic epithelium stain for CK4,5,13,14 while basal layer of stratified squamous epithelium stain for CK 5,14,19. CK 16,18 stain endocervical columnar cells. Diverse and inconclusive results are observed in various studies done to evaluate cytokeratin expressions in cervical dysplasia and cervical cancer cases. Histogenesis of tumour dictates expressions of different cytokeratins in cervical neoplasms. SCC show positive staining for basal layer keratins like CK5,14,19,17. Circulating CK19 cells can be detected and has got prognostic implication.[94]

Cytokeratin 19 is simple form of keratin expressed in basal layer and is used as a stem cell marker.[95] Cytokeratin 19 expression is reported as 100 % (n=30) in squamous cell carcinoma of cervix by Lee H et al. Weak staining is reported in columnar cells at the squamocolumnar junction, squamous cells at the transformation zone and basal layer cells at the ectocervix in non- neoplastic cervix. Patchy CK19 expression is noted in the basal layer in Cervical Intraepithelial Neoplasia(CIN) 2. 60% of CIN3 cases show homogeneous expression in entire layer at the transformation zone and at the squamocolumnar junction.[96]

Vimentin :-

Vimentin is a structural protein belonging to type III of intermediate filament proteins expressed in mesenchymal cells and has role in cytoskeletal formation and thus provide mechanical resistance to the cells. Vimentin has role in organelle positioning, cell migration and cell adhesion and cell signaling.[97]

Vimentin expression increases significantly within normal cervix, LSIL, HSIL and SCC of cervix. Vimentin also show significant expression among grades of cervical cancer.[98]

A study done by Myong NH showed that squamous cell carcinoma cells show dense cytoplasmic expression for vimentin and no much difference is noted in the staining pattern

between non-invasive and invasive foci of CIS and microinvasive carcinoma groups. About 66.7% of invasive squamous cell carcinoma showed dense cytoplasmic expression for vimentin as compared to 16.7% of CIS and microinvasive Squamous cell carcinoma (n=119) with a p <0.001.[98]

A study done by Jian J et-al in 2019 showed that 12.5% of normal human cervix (n=40), 18.2% of CIN-1 (n=22), 45% of CIN-2,3 (n=60), 72.1 % of SCC cases(n=86) shows significant difference in expression of Vimentin (p<0.01).Normal cervix, CIN-1 showed weak cytoplasmic staining, CIN-2,3 show moderate cytoplasmic staining and SCC showed strong cytoplasmic staining with Vimentin.[99]

ECM proteins :-

ECM is the non cellular component present within tissues and provide natural scaffolding to the cellular components. ECM get role in maintaining tissue morphology and homeostasis. Altered ECM structure mediates EMT process. Type I and type X collagen upregulate MMP1 and increases the mobility of cells. Upregulation of fibronectin, laminin 5 and downregulation of laminin 1 and type IV collagen are seen during EMT process.[100]

MATERIAL AND METHODS

STUDY DESIGN: Laboratory Observational Study

STUDY PERIOD :- January 2019 to July 2020

STUDY PLACE :- R.L. Jalappa Hospital, Department of pathology with Department of Obstetrics and Gynaecology, Kolar, a tertiary care hospital.

STUDY DURATION :- 1 year 6 months

SAMPLE SIZE :-

Sample size (n) was calculated based on the expected proportions/prevalence of the expressions of cytokeratin19 (100%),Vimentin(66.7%), RhoC (87%)according to different studies within confidence interval of 95% and absolute error of 12% (0.12) by using following formula. [77,96,98]

$$n = \frac{z^2(pq)}{e^2}$$

where

n = the sample size
z = standard error associated with the chosen level of confidence (typically, 1.96)
p = estimated percent in the population
q = $100 - p$
e = acceptable sample error

For Vimentin, sample size= 60+6 (10 % sample size for non- responding cases)=66

For RhoC, sample size=30+3 (10 % sample size for non- responding cases)=33

Since cytokeratin 19 show 100% positivity, so sample size for cytokeratin could not be calculated as all samples would show positivitivity. Atleast 66 samples should be taken for the study. We took 70 samples for our study.

INCLUSION CRITERIA :-

Normal cervix with squamo-columnar junction from hysterectomy specimen or from cervical biopsies with non neoplastic diagnosis.

Cervical biopsies with HSIL(CIN II AND CIN III)

Cervical biopsies with squamous cell carcinoma cervix

EXCLUSION CRITERIA :-

Cases that have undergone chemotherapy or radiotherapy.

Cases with malignancies other than squamous cell carcinoma cervix.

Recurrent cases of squamous cell carcinoma cervix.

Metastatic deposits in cervix.

SOURCE OF DATA:

Cervical biopsy cases of normal cervix, HSIL and cervical cancer was retrieved from the records of department of Pathology, Sri Devaraj Urs Medical College. Normal cervix was obtained from hysterectomy specimen and cervical biopsies with non neoplastic diagnosis.

10 cases of normal cervix, 30 cases of HSIL and 30 cases of squamous cell carcinoma was studied. The case details were collected from hospital record section, RL Jalappa, Hospital and Research Centre.

The sections were stained with 3 markers i.e. RhoC, Vimentin and cytokeratin 19 by immunohistochemistry.

METHOD OF COLLECTING DATA:

Cervical biopsy specimens with histomorphological features of normal cervix, HSIL and cervical cancer was considered for the study. Normal cervix was obtained from hysterectomy specimen and cervical biopsies having non-neoplasic diagnosis. 10 samples of normal cervix, 30 samples each of HSIL and squamous cell carcinoma of cervix each was included in the study.

Clinical details of the patient such as age, marital status, parity, presenting symptoms, signs as per abdomen, per vagina and per speculum findings was collected from medical record section. Biopsy findings, histological grading and stage of carcinoma was studied and noted. FIGO staging was done by MRI findings. MRI reports were collected from records of Department of Radiodiagnosis.

Immunostaining for Vimentin, Cytokeratin 19 and RhoC was performed on all cases of cervical biopsy using appropriate positive and negative control as per manufacturer's instruction. There expression was studied by using appropriate grading and scoring system. The findings was compared between normal, HSIL and squamous carcinoma of cervix and also with histological grade and clinical /radiological stage of the disease.

IMMUNOHISTOCHEMISTRY PROTOCOL :

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue section. 3-4mm thick sections were cut and floated on coated slide and incubated overnight on hot plate at 60^0C.

The section was deparaffinized with 2 cycles of standard xylene-I, II for 15 minutes each cycle and then dexylenised by using graded alcohol and then the section was rinsed by water.

Antigen retrieval was done by using 4 cycles of heating in microwave in Tris-EDTA buffer at pH= 9 for 20 minutes and later treated with 3% hydrogen peroxide to reduce endogenous peroxidase activity. Sections were washed by Trish Buffer Saline (TBS) at pH 6.

The sections were treated with power block for 30 minutes to block nonspecific reactions with other tissue antigen.

The tissue sections were separately subjected to primary antibodies:- 1. Vimentin (Biogenex), 2. CK 19 (Biogenex) and 3. RhoC (Immunotag, dilution:- 1:100) and left at room temperature for 2 hours.

After 2 hours sections were washed by TBS wash buffer.

The sections were then incubated with superenhancer for 30 minutes.

After 30 minutes, sections were washed by TBS wash buffer.

The sections was then incubated with secondary antibody for 90 minutes.

After 90 minutes, sections were washed by TBS wash buffer.

Sections were then treated by supersensitive polymer for 30 minutes at room temperature.

After 30 minutes, sections were washed by TBS wash buffer.

3,3- diaminobenzidine tetrahydrochloride (DAB solution) was then be added and left for 15 minutes at room temperature.

After 15 minutes sections were washed by TBS wash buffer.

Sections were kept in tap water for 5 minutes to wash excess stain.

Counter staining was done by keeping sections in Hematoxylin for 1 minutes.

Sections were kept in tap water to wash excess stains.

Sections were left for drying and then sections were mounted.

IMMUNOHISTOCHEMISTRY INTERPRETATION:-

Immunohistochemistry slides were interpreted by taking in consideration of percentage of cells showing positive immunoreactivity and intensity of the reaction. A semiquantitative immunoreactivity score was given based on existing literature. However interpretation of each markers were different based on existing literature.

Interpretation of Cytokeratin 19 immunoreactivity :-

The cells were regarded as positive when immunoreactivity was clearly observed in the cytoplasm and/or membrane. The percentage of cells staining positively with CK19 was scored (proportion score) as :- 0 if unstained, 1 if 1% to 5% of cells show positivity, 2 if 6% to 25% of cells show positivity, 3 if 26 to 75% of cells show positivity and 4 if 76 to 100% of cells show positivity. The staining intensity (intensity score) was graded as 0 if none of cells are positive/ show weak positivity, 1 for moderate intensity, 2 for strong intensity. The final immunoreactivity response score was calculated by multiplying proportion score and intensity

score. The final immunoreactivity score was graded as 0 if negative, 1+ if focally positive, 2+ positive and 3+ if diffusely positive.[101]

Interpretation of Vimentin immunoreactivity :-

Vimentin show cytoplasmic staining. Proportion score was given as 0 if no cells showed positivity, 1 if 1% to 10% of cells show positivity, 2 if 11% to 40% of cells show positivity, 3 if 41% to 75% of cells show positivity and 4 if 76% to 100% of cells show positivity. Intensity score was given as 0 if cells remain colourless, 1 if cells took light yellow colour, 2 if cells took brown yellow colour and 3 if cells took dark brown colour. The final immunoreactivity response score was calculated by multiplying proportion score and intensity score. The final immunoreactivity score was graded as 0-1 as negative, 2-3 as weakly positive, 4-7 as positive and 8-12 as strongly positive .[102]

Interpretation of RhoC immunoreactivity :-

RhoC take cytoplasmic staining. Proportion score was given as 0 if no cells showed positivity, 1 if 0% to 25% of cells show positivity, 2 if 26% to 50% of cells show positivity, 3 if 51% to 75% of cells show positivity and 4 if 76% to 100% of cells show positivity. Intensity score was given as 0 if cells remain colourless, 1 if cells show faint intensity , 2 if cells show moderate intensity and 3 if cells show strong intensity. The final immunoreactivity response score was calculated by multiplying proportion score and intensity score. The cells were considered positive for RhoC with final immunoreactivity score of 6 or higher. The final immunoreactivity score was graded as 0 if negative, 1+ if equivocal , 2+ moderately positive and 3+ strongly positive. Equivocal reaction was considered as negative for RhoC expression.[103]

STATISTICAL ANALYSIS

Data was entered into Microsoft excel data sheet and was analysed using SPSS 22 version software. Continuous variables were represented as frequencies, percentages, mean and standard deviation. Independent t test or Mann Whitney U test was used as test of significance to identify the mean difference between two quantitative and qualitative variables respectively. ANOVA (Analysis of Variance) or Kruskal Wallis test was the test of significance to identify the mean difference between between more than two groups for quantitative and qualitative data respectively. Chi-square test or Fischer's exact test was used as test of significance for qualitative data. p value (Probability that the result is true) < 0.005 was considered statistically significant. Microsoft excel and Microsoft word was used to obtain various types of graphs like bar diagram and pie chart. Epi Info (Centre for Disease Control, Atlanta) was used to estimate sample size.

RESULTS

70 specimens which consisted of 10 specimens of normal cervix, 30 specimens of High Grade Squamous Intraepithelial Lesions (HSIL) and 30 specimens of squamous cell carcinoma of cervix were studied during the period of January 2019 to July 2020 in the Department of Pathology in Sri Devaraj Urs Medical College and RL Jalappa Hospital and Research Centre, Tamaka, Kolar.

Immunohistochemistry using CK19, Vimentin and RhoC markers were performed in each of 70 selected specimens.

Data regarding Age distribution, chief complaints, per speculum findings, FIGO staging, Histological grading, CK 19 expression, Vimentin expression and RhoC expression was recorded and analysed.

Table 6 : Age distribution among normal cervix cases

Age distribution (Years)	**No of cases**
20-30	02
31-40	04
41-50	00
51-60	1

61-70	3

Maximum females with normal cervix belonged to 31-40 years of age group.

Table 7 : Age distribution among HSIL cases

Age distribution (Years)	**No of cases**
20-30	01
31-40	05
41-50	11
51-60	09
61-70	03
>70	01

Among HSIL cases maximum women belonged to age group of 41-50 years.

Table 8 : Age distribution among SCC cases

Age distribution (Years)	**No of cases**
20-30	00
31-40	01
41-50	09
51-60	09

61-70	10
>70	01

Among SCC of cervix cases maximum women belonged to 61-70 years of age group.

Chart 1 : Age distribution among normal, HSIL,SCC cases.

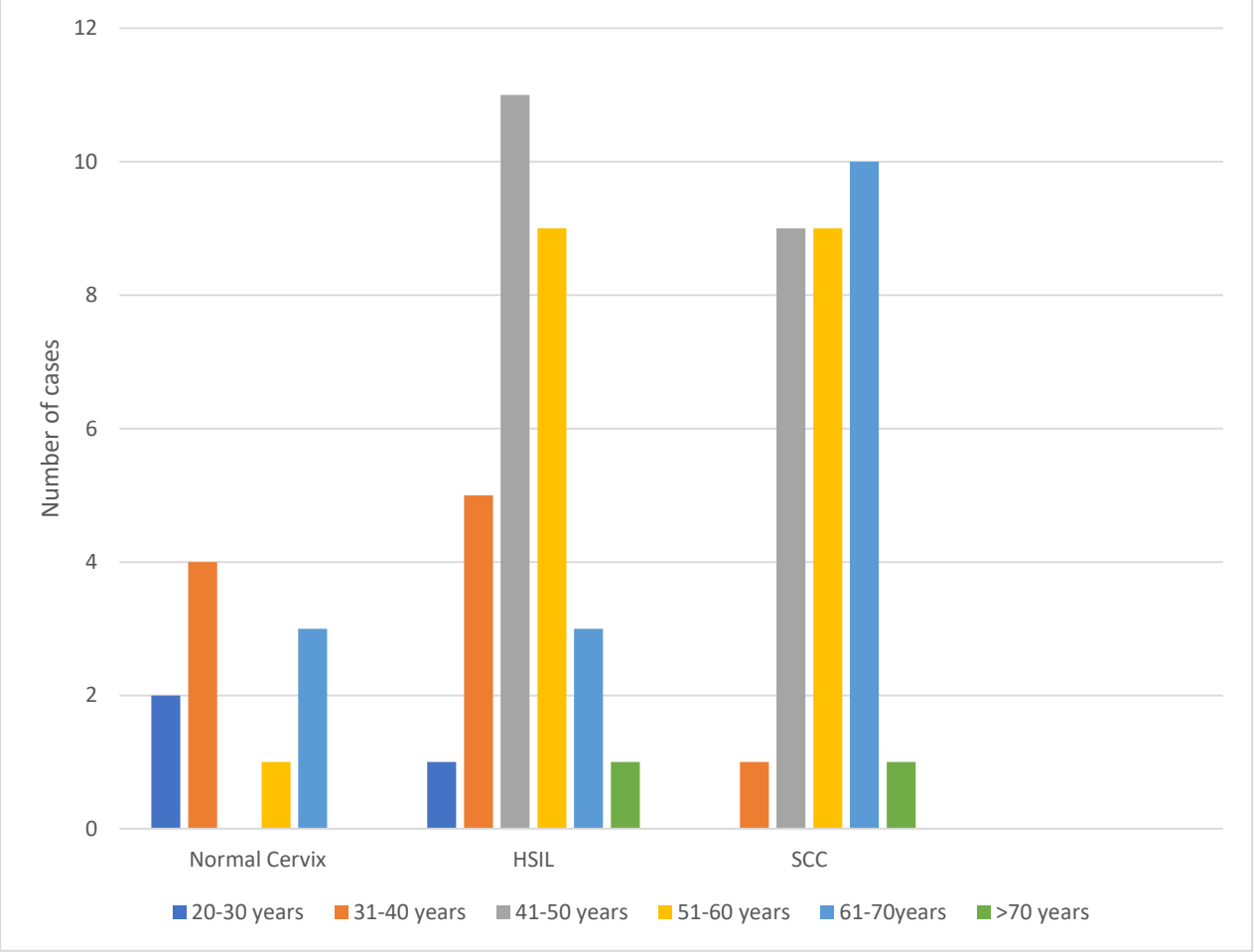


Table 9: Comparison between age groups

Types of lesions	Sample Size (n)	Mean Age (years) ± Standard Deviation (Years)	p value*
Normal	10	46.20 ± 16.12	**0.01**
HSIL	30	49.10 ± 10.13	
SCC	30	56.27 ± 9.29	

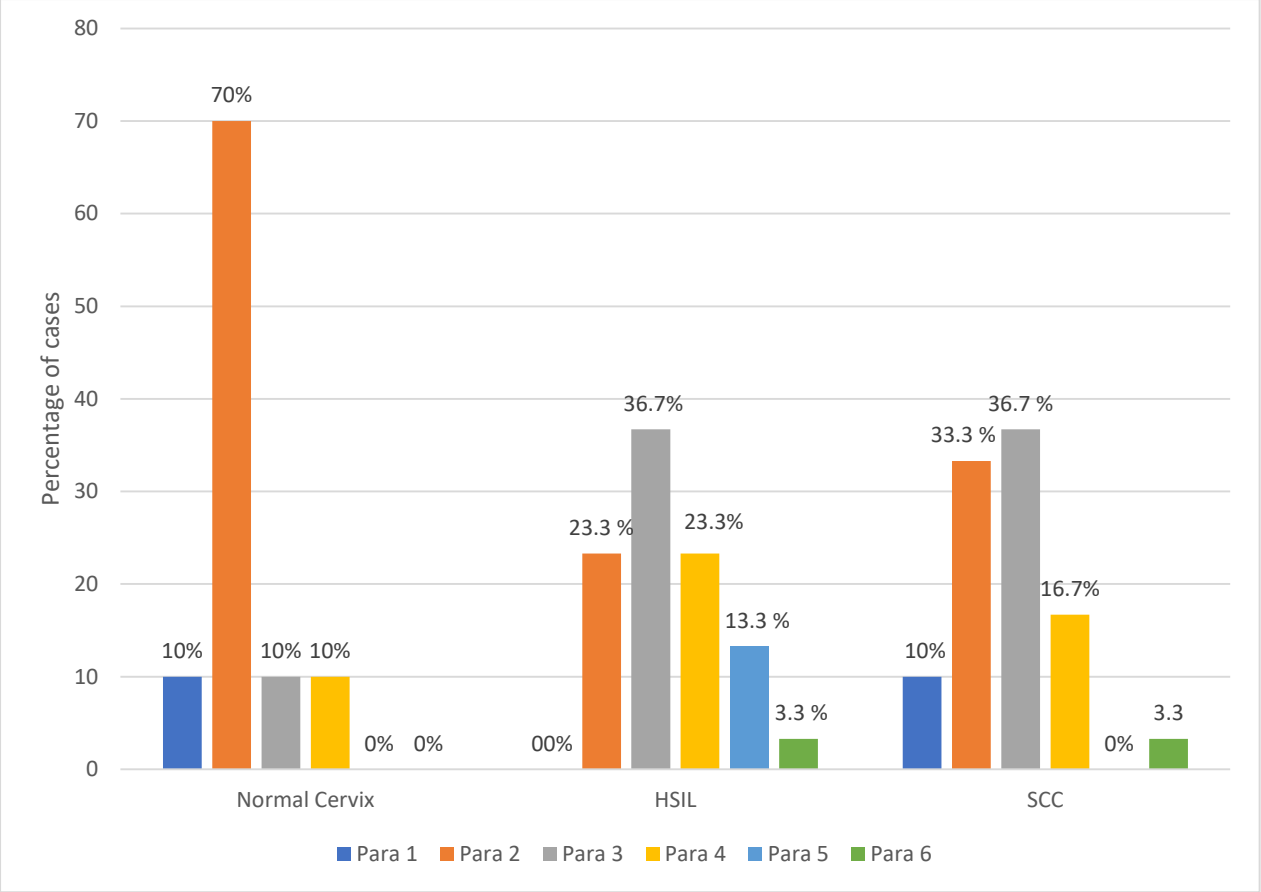
***ANOVA**

Mean age in normal, HSIL and SCC cases were 46.2 ± 16.12 years, 49.10 ± 10.13 years and 56.27 ± 9.29 years respectively. Statistically significance difference in age among 3 groups was found.

Parity:-

About 36.7% (n= 11) of HSIL cases had parity 3. 23.3% (n=07) , 23.3% (n=07) ,13.3% (n=04) and 3.3% (n=01) of HSIL cases had parity 2, 4, 5 and 6 respectively. About 36.7% (n=11) SCC cases had parity 3. 10 % (n=01) , 33.3% (n=10) ,16.7% (n=05) and 3.3 % (n=01) of cases with SCC had parity 1, 2, 4 and 6 respectively.

Chart 2 : Distribution of cases according to parity



Chief Complaints of cases with HSIL

About 56% of females with HSIL presented with bleeding per vaginum. Other chief complaints were discharge per vaginum (23.3%), post coital bleed (6.7 %), postmenopausal bleed (3.3%) and white discharge per vaginum (10%).

Chief Complaints of cases with SCC

About 53.3% of females with SCC presented with white discharge per vaginum. Other chief complaints were bleeding per vaginum (36.7%), postmenopausal bleed (6.7 %), lower abdominal pain (3.3%).

Per Speculum findings

Per speculum findings of females with HSIL

About 60% of females with HSIL showed erosion on per speculum examinations.

Per speculum findings of females with SCC

About 53.3 % of females with SCC showed growth on per speculum examinations. Other findings were bleed on touch (26.7%), discharge (13.3%), ulcer (3.3 %) and bulky cervix (3.3%).

Table 10 : Distribution of SCC cases according to grades

Grade of SCC of cervix	**Number of cases**	**Percentage**
Well Differentiated SCC	08	26.67%
Moderately Differentiated SCC	14	46.66%
Poorly Differentiated SCC	08	26.67%

About 46.66% of SCC of cervix showed features of Moderately Differentiated Squamous Cell Carcinoma. Features of Well Differentiated SCC and Poorly Differentiated SCC were seen in 26.67% and 26.67% of cases with SCC of cervix respectively.

Chart 3 : Distribution of SCC cases according to grades

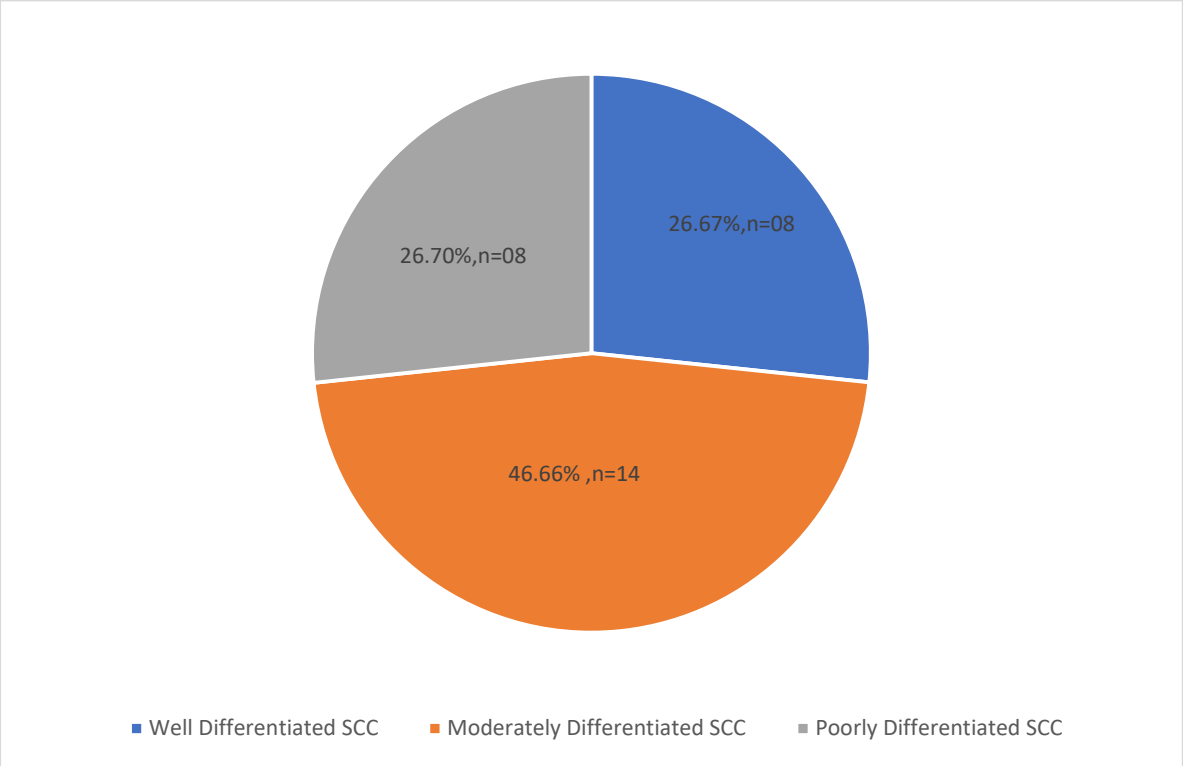


Table 11:- Cases of SCC of uterine cervix stages according to The International Federation of Gynecology and Obstetrics (FIGO) Staging of Carcinoma of uterine cervix (FIGO) staging,2018

FIGO Stage	Number of cases	Percentage
II	21	70%
III	09	30%

Maximum case (70%) had stage II cancer while remaining 30% had Stage III cancer.

Chart 4 :- Case distribution according to FIGO stage,2018

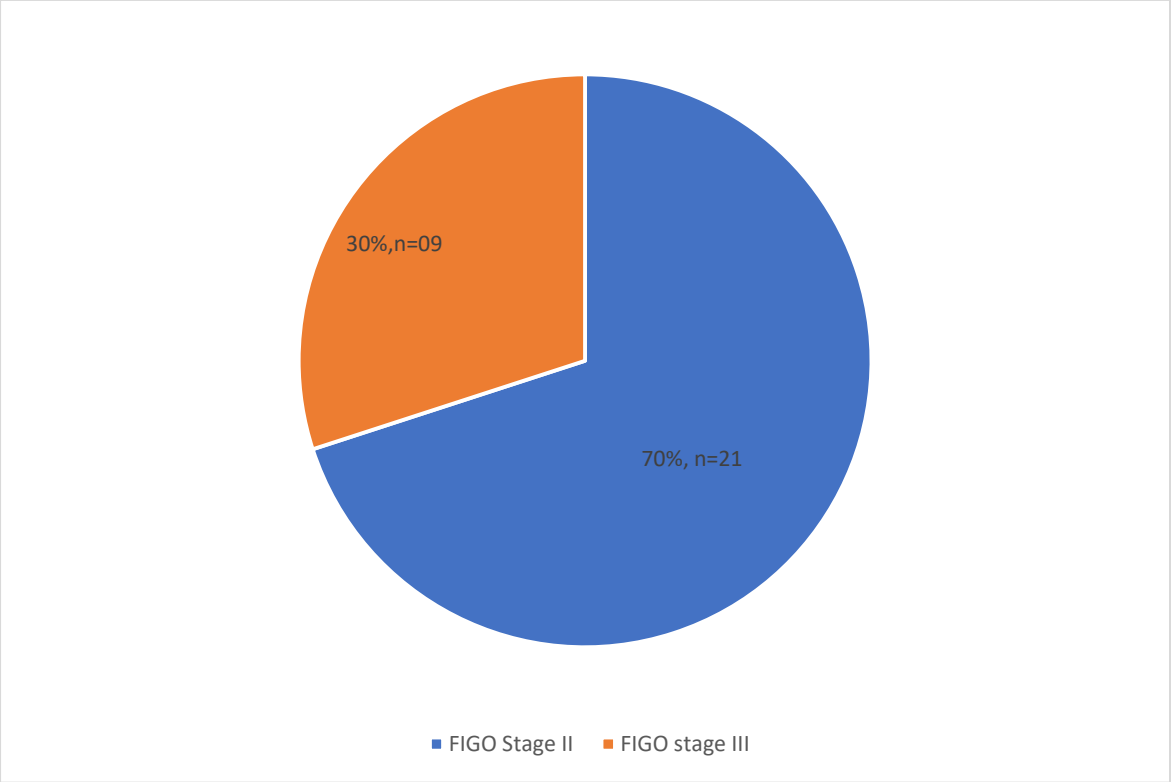


Table 12 : Expression of CK19 in normal, HSIL and SCC

Types of Lesions	CK 19 Expression			
	Negative	**Weak Positive**	**Moderate Positive**	**Strong Positive**
Normal	02(20%)	08(80%)	00(0%)	00(0%)
HSIL	02(6.7%)	24(80%)	03(10%)	01(3.3%)
SCC	00(0%)	03(10%)	10(33.3%)	17(56.7)

Normal cervix showed a weak basal positivity in 80% (n=8) of cases while 20% (n=2) were negative for CK19 expression.

About 93.3 % (n=28) of HSIL cases showed positivity for CK19 and 6.7% (n=2) were negative. Out of 93.3 % (n=28) of CK 19 positive HSIL cases, 80% (n=24) were weak positive and 10% (n=10) were moderate positive and remaining 3.3% (n=10) showed strong positivity for CK19.

All the cases of SCC were positive for CK19 where majority of cases (56.7%, n=17) showed strong positivity for CK19 while 33.3% (n=10) were moderate positive and remaining 10% (n=3) were weak positive for CK19.

Chart 5 : Expression of CK19 among normal, HSIL and SCC

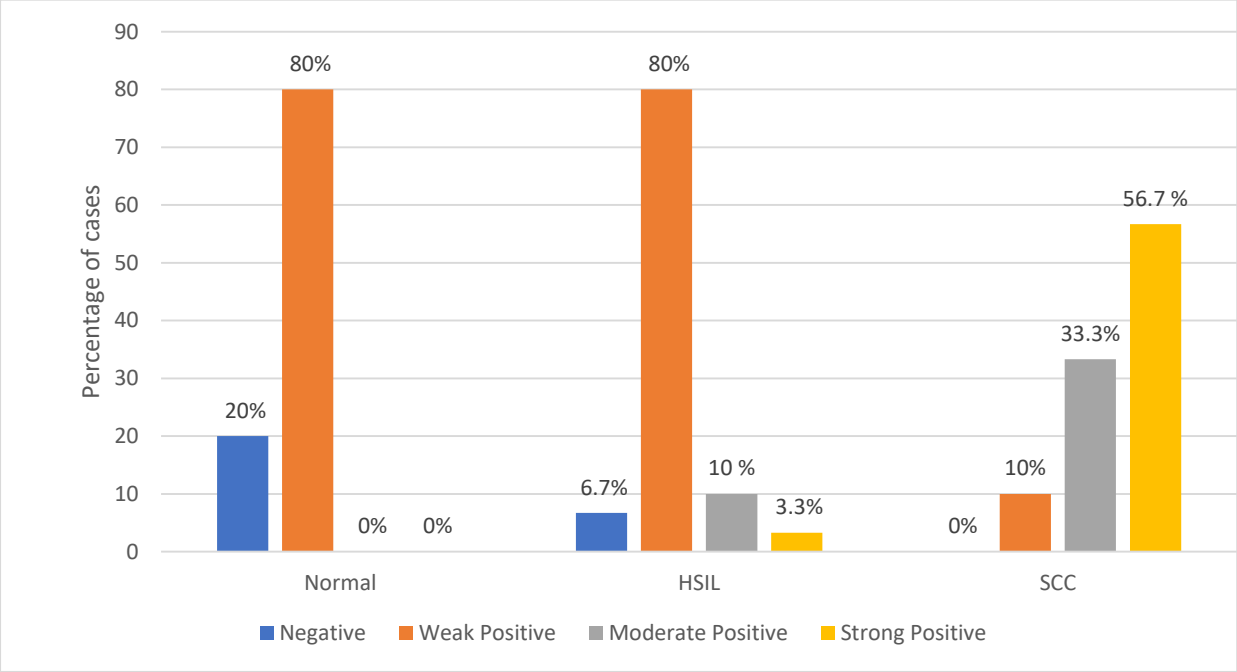


Table 13 :- Expression of CK19 among Normal, HSIL and SCC

Types of Lesions	Expression of CK19		p Value
	Negative	Positive	
Normal	O2 (10%)	08 (80%,Basal cells)	

HSIL	02(6.7%)	28 (93.3%)	0.492
SCC	00 (00%)	30 (100%)	

Fischer's exact test was done to determine level of statistical significance in between expression of CK19 and HSIL and SCC. No statistical significance was found between expression of CK19 among HSIL and SCC with p value of 0.492.

Table 14 : Expression of Vimentin in normal, HSIL, SCC

Types of Lesions	**Expression of Vimentin**			
	Negative	**Weak Positive**	**Moderate Positive**	**Strong Positive**
Normal	10 (100%)	00(00%)	00(00%)	00(00%)
HSIL	20(66.7%)	04(13.3)	06(20%)	00(00%)
SCC	08(26.7%)	00(00%)	08(26.7%)	14(46.7%)

All the cases (n=10) with normal cervix were negative for the expression of vimentin. About 66.7% (n=20) of cases with HSIL showed negative expression for vimentin. 13.3% (n= 04) of HSIL cases showed weak positivity for vimentin and rest 20% (n=06) of cases showed moderate positivity for vimentin. In SCC cases about 73.3% (n=22) of cases were positive for vimentin. Out of 73.3% (n=22) of cases, 46.6% (n=14) of cases showed strong immunopositivity for vimentin while rest 26.7% (n=08) were moderately positive for vimentin.

Chart 6: Comparison of expression of Vimentin among normal, HSIL, SCC

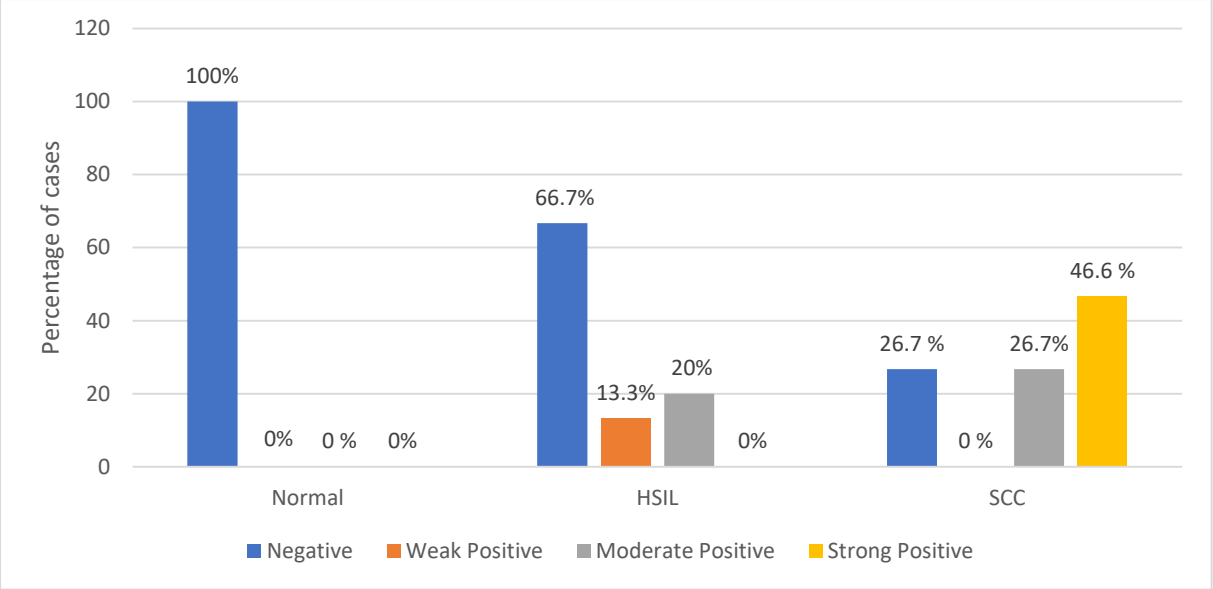


Table 15 : Expression of Vimentin in normal, HSIL, SCC

Types of Lesions	Expression of Vimentin		p Value
	Negative	Positive	
Normal	10(100%)	00(00%)	**<0.001 (Significant)**
HSIL	20(66.7%)	10(33.3)	
SCC	08(26.7%)	22(73.3)	

Chi-Square Test = χ^2 = 19.496, degree of freedom=df = 2, p Value < 0.001

The differences in expression of vimentin among normal, HSIL and SCC was found to be statistically significance with a p value < 0.001.

Chart 7 : Comparison of expression of Vimentin among normal, HSIL and SCC

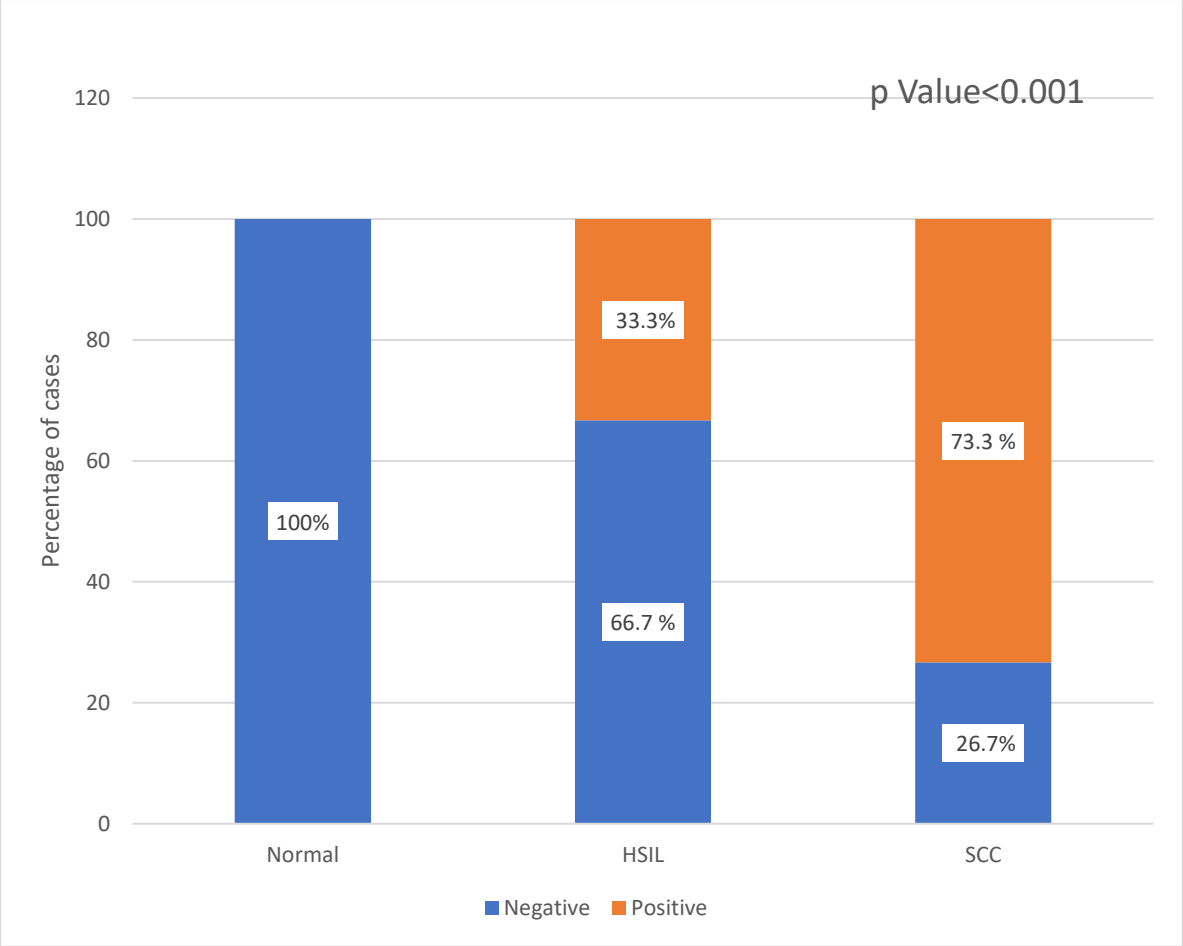


Table 16 : Expression of RhoC in normal, HSIL,SCC

Types of Lesions	Expression of RhoC			
	Negative	**Equivocal**	**Moderate Positive**	**Strong Positive**
Normal	09(90%)	00(00%)	00(00%)	01 (10%)
HSIL	22 (73.3)	02 (6.7%)	02 (6.7%)	04 (13.3%)
SCC	04 (13.3%)	01(3.3%)	10 (33.3)	15 (50%)

About 90% (n=09) of cases with normal cervix were negative for RhoC expression. 1 case with normal cervix showed strong positivity for RhoC. 73.3% (n=22) of HSIL cases were negative for RhoC expression while 6.7% (n=02) of HSIL cases showed an equivocal

immunopositivity. Equivocal immunopositivity was considered as negative for RhoC expression. [103] 20 % (n=06) of HSIL cases were positive for RhoC expression. Out of 20% (n=06) , 13.3% (n=04) of cases showed strong positive staining while rest 6.7% (n=02) of cases were moderately positive for RhoC expression. 83.33% (n=25) of SCC cases were positive for RhoC expression. Out of 83.33% (n= 25), 50% cases (n=15) were strongly positive for RhoC expression while remaining 33.33% SCC cases (n=10) showed moderate positivity for RhoC.

Chart 8: Expression of RhoC in normal, HSIL,SCC

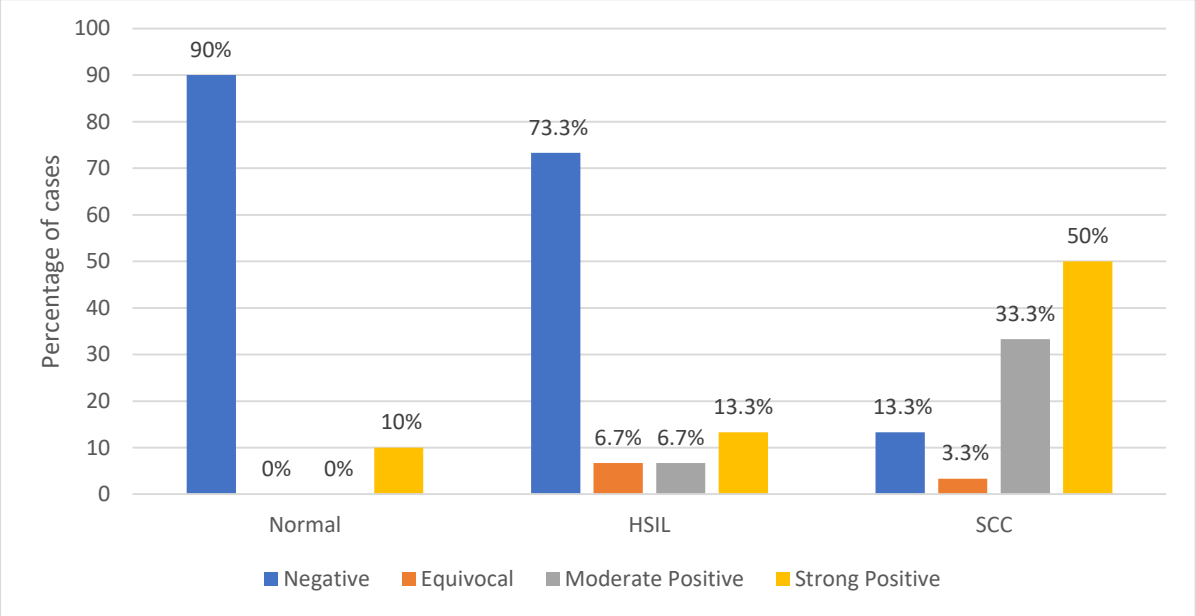


Table 17 : Expression of RhoC in normal, HSIL,SCC.

Types of Lesions	Expression of RhoC		p Value
	Negative	Positive	
Normal	09 (90%)	01(10%)	
HSIL	24 (80%)	06 (20%)	**<0.001**
SCC	05 (16.67%)	25 (83.33)	**(Significant)**

Chi-Square Test = χ^2 = 30.241, degree of freedom= df = 2, p Value < 0.001

The difference between expression of RhoC among normal cervix, HSIL and SCC was found to be statistically significant. Maximum expression was seen in SCC group while majority of cases with normal cervix were negative for the expression of RhoC.

Chart 9: Comparison of expression of RhoC in normal, HSIL, SCC

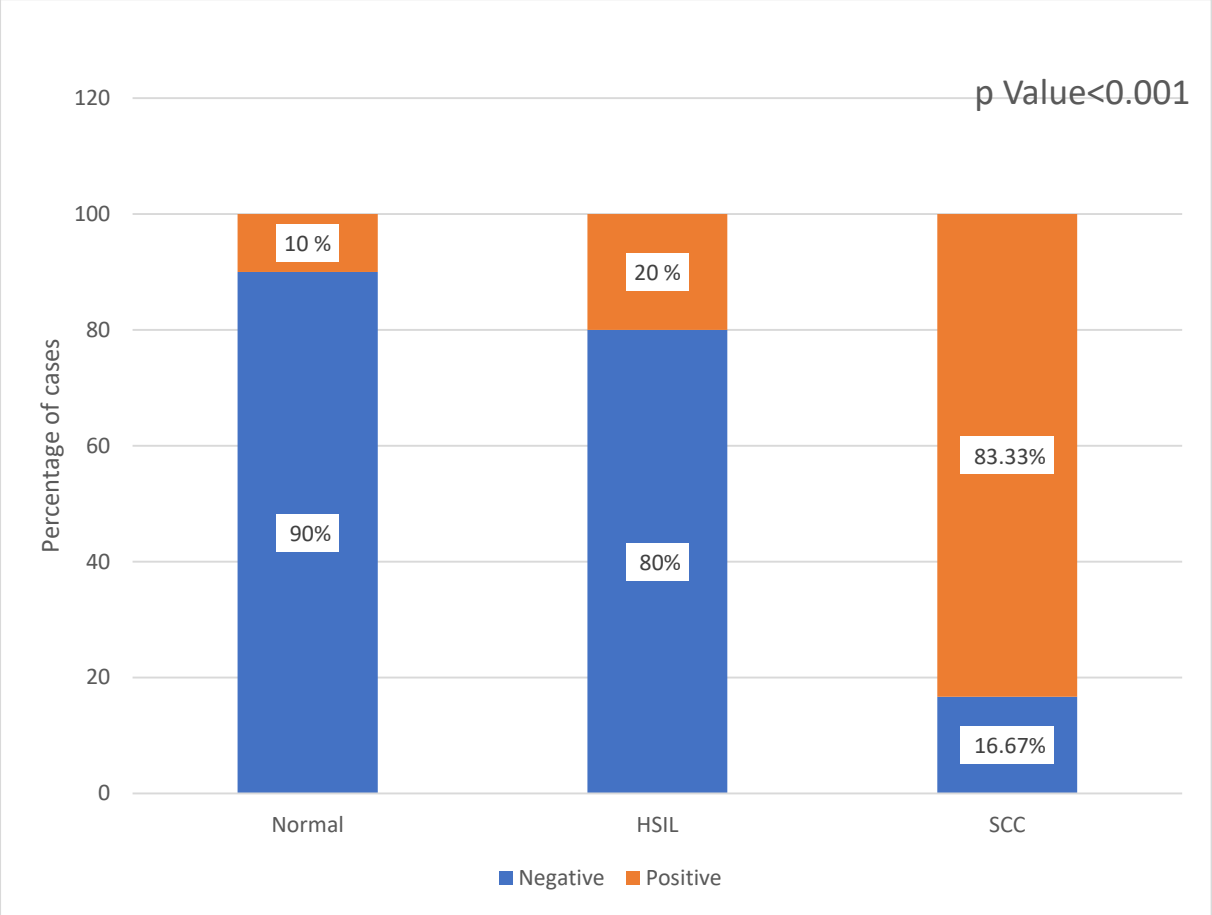


Table 18: Correlation of Vimentin with grade of tumour

Histological grade	Expression of Vimentin		p Value
	Negative	Positive	
Well Differentiated SCC	03 (37.5%)	05 (62.5%)	0.515
Moderately Differentiated SCC	04 (28.57)	10 (71.43%)	
Poorly Differentiated SCC	01 (12.5%)	07 (87.5%)	

Chi-Square Test = χ^2 = 1.327, degree of freedom= df = 2, p Value = 0.515

37.5% (n=03) cases of well differentiated SCC were negative for vimentin expression while the rest 62.5% (n=5) cases of well differentiated SCC were positive for the expression of vimentin. 28.57% (n=4) of moderately differentiated SCC were negative for expression of vimentin while rest of the 71.43% (n=10) of cases with moderately differentiated SCC was immunopositive for vimentin. 12.5% (n=01) of cases with poorly differentiated SCC were negative for expression of vimentin. Rest of 87.5% (n=07) of cases of poorly differentiated showed immunopositivity for vimentin. Percentage positivity for vimentin increases as grade increases. The difference between expression of vimentin among different grades of SCC of cervix was found to be statistically insignificant**.**

Table 19 : Correlation of Vimentin with FIGO staging

FIGO Stage	**Expression of Vimentin**		**p Value**
	Negative	**Positive**	
II	05 (23.8%)	16 (76.2%)	0.589
III	03 (33.33%)	06 (66.67%)	

Chi-Square Test = χ^2 = 0.292, degree of freedom= df = 1, p Value = 0.589

23.8% (n=05) of cancer patients with FIGO stage II showed negative expression for vimentin, while rest of the 76.2% (n=16) cases with FIGO stage II showed positive expression for vimentin. 33.33% (n=03) of cases with FIGO stage III were negative for expression of vimentin and 66.67% (n=06) of cases with FIGO Stage III showed positive expression for vimentin. The difference between the expression of vimentin and the FIGO stage was found to be statistically insignificant.

Table 20 : Correlation of RhoC with grade of tumour

Histological grade	Expression of RhoC		p Value
	Negative	Positive	
Well Differentiated SCC	01 (12.5%)	07 (87.5)	
Moderately Differentiated SCC	03 (21.43)	11 (78.57)	
Poorly Differentiated SCC	01 (12.3%)	07 (87.5%)	0.827

Chi-Square Test = χ^2 = 0.382, degree of freedom= df = 2, p Value = 0.827

12.5% (n=01) cases of well differentiated SCC were negative for RhoC expression while the rest 87.5% (n=07) cases of well differentiated SCC were positive for the expression of RhoC. 21.43% (n=03) of cases with moderately differentiated SCC were negative for expression of RhoC while rest of the 78.57% (n=11) of cases with moderately differentiated SCC were immunopositive for RhoC. 87.5% (n=07) cases of poorly differentiated showed immunopositivity for RhoC. Other 12.5% of cases (n=01) was negative for the expression of RhoC. The difference between expression of RhoC among different grades of SCC of cervix was found to be statistically insignificant (p=0.827)**.**

Table 21 : Correlation of RhoC with FIGO staging

FIGO Stage	Expression of RhoC		p Value
	Negative	Positive	
II	04 (19.05%)	17 (80.95%)	0.593
III	01 (11.11%)	08 (88.89%)	

Chi-Square Test = χ^2 = 0.286, degree of freedom= df = 1, p Value = 0.593

80.95 % (n=17) of cancer patients with FIGO stage II showed positive expression for RhoC, while rest of the 19.05% (n=04) cases with FIGO stage II showed negative expression for RhoC. 11.11% (n=01) of cases with FIGO stage III were negative for expression of RhoC and 88.89% (n=08) of cases with FIGO Stage III showed positive expression for RhoC. The

difference between the expression of RhoC and the FIGO stage was found to be statistically insignificant (p = 0.593).

Correlation between CK19 expression and histological grade and the FIGO staging

Because CK 19 showed 100% positivity in SCC cervix, comparison between expression of CK 19 with the histological grade and with the FIGO staging was not possible as every cases were positive.

DISCUSSION

Cervical cancer is the fourth most common cancer among females worldwide. In, India, cervical cancer constitute about 16.5% of all cancers in females and is the second most common cause of death due to cancer in females of India. [1]

Increase age, low socioeconomic status, high parity, improper hygiene, multiple partners, HPV infections are common risk factors.

Cervical cancer occur in a step wise pattern from normal to HSIL and finally cervical cancer.[4]

Table 22: Mean Age in HSIL cases in the present study compared with other similar studies

Studies	**No of HSIL cases**	**Mean age (Years) ± Standard Deviation (Years)**
Alexander M et al, 2015[104]	20	45.69 ± 10.29
Meng JW et al, 2019[105]	236	44.22 ± 9.13
Present Study	**30**	**49.10 ± 10.128**

Mean age was 49.10 years in our study which is comparable to the findings of Alexander M et al and Meng JW et al. Mean age were 45.69 years and 44.22 years in a study done by Alexander et al and Meng JW et al respectively. [104,105]

Table 23: Mean Age in SCC cases in the present study compared with other similar studies

Studies	**No of SCC cases**	**Mean age(Years)±Standard Deviation (Years)**
Dahiya N, et al, 2016[106]	67	52.28 ± 11.29
Raju K et al, 2019[107]	75	54.2 ± 12
Present Study	**30**	**56.27 ± 9.29**

In our study, mean age of SCC cases was 56.27 years with a standard deviation of 9.29 years which can be compared by findings of Dahiya N et al and Raju K et al. [106,107]

Clinical features of HSIL.

In our study about 56% females with HSIL presented with chief complain of bleeding per vaginum and about 60% females with HSIL had erosion on per speculum examination. In a study done by Aloan HH et al Per Vaginum bleeding was complained by about 11.11% of female with HSIL (n=09) while 55.55% females with HSIL complained of Post Coital Bleeding and 88.88% of HSIL (n-09) cases had cervical erosion on examination. [118] In a study done by Kaveri S et al about 50% (n=16) female with HSIL showed cervical erosion on examination. However both the studies had less sample size. [119]

Table 24: Chief complaint of SCC cases in the present study compared with other similar studies.

Chief Complaint	**Patil et al,2019**[108] **(n=150)**	**Raju K et al,2019**[107] **(n=75)**	**Present Study (n=30)**
Bleeding per vagina	45.3%	80%	**36.7%**
White Discharge Per Vagina	64%	68%	**53.3%**
Lower Abdomen Pain	_	53.3%	**3.3%**
Post Menopausal Bleeding	16%	85.7%	**6.7%**

The most common complaint of SCC patient was white discharge per vagina which constituted about 53.3% of cases. In a study done by Patil et al in 2019, 64 % (n=96) of cases had complaint of white discharge per vagina. [108] White discharge per vagina was seen in 68% (n=51) of the cases in a study done by Raju K et al in 2019. [107]This difference may be due to difference in study population. Bleeding per vagina was complaint by 36.7% of cases in our study. This finding is comparable to the finding of Patil et al, where 45.3% (n=68)of cervical cancer cases. [108] Raju K et al showed a higher percentage (80%,n=60) of cases with bleeding per vagina. [107] 6.7 % of cases had features of post menopausal bleed in our study but it was present in higher percentages in the study done by Raju K et al (85.7%,n=64) and by Patil et al (16%, n=24). [107,108] This difference may be because of different study population. 3.3 % of cases had lower abdominal pain. While lower abdominal pain was present in 53.3% (n=40) of the cases in the study by Raju K et al. [107]

Table 25: Per Speculum findings in SCC in the present study compared with other similar studies

Per Speculum	Hazra SK et al, 2019[114] (n=300)	Raju K et al, 2019[107] (n=75)	Present Study (n=30)
Growth	51.3%	65.3%	**53.3%**
Bleed on touch	37.3%	_	**26.7%**
Discharge	_	_	**13.3%**
Ulcer	_	6.6%	**3.3%**
Bulky Cervix	_	_	**3.3%**

Most common per speculum finding was presence of growth which was present in more than half of the cases. This finding is comparable to the finding of Raju K et al (65.3%, n=49) , and Hazra SK et al (51.3% , n=154). [107,114] In our 26.7% of SCC cases showed bleed on touch which can be compare with the findings of Hazra SK et al where 37.3% (n=112) cases showed bleed on touch. [114] Ulcer was present in 3.3% of SCC cases which can be comparable with the finding of Raju K et al. [107] Bleed on touch, discharge and bulky cervix were other per speculum findings in our study. This difference in the findings may be because of use of less sample size.

Table 26: Comparison of parity of SCC cases in the present study with other similar studies.

Parity	Kaku M et al, 2019[115]	Raju K et al, 2019[107]	Present Study

	(n=349)	(n=75)	(n=30)
Para 1	10.3%	8%	**10%**
Para 2	25.8%	14.6%	**33.3%**
Para 3	28.9%	22.6%	**36.7%**
Para 4	12%	26.6%	**16.7%**
Para ≥ 5	19.8%	28%	**3.3%**

In our study maximum cases (36.7%) of SCC were para 3. In a study done by Kaku M et al, 28.9% (n= 101) cases of cervical cancer had parity 3. Our study is comparable with the findings of Kaku et al. [115] 22.6% (n=17) had parity 3 in a study done by Raju K et al (n=17). 33.3 % of cases had parity 2 in our study. While 25.8% (n= 90) cases had parity 2 in a study done by Kaku M et al. 14.6% (n=11) of cases had parity 2 in the study done by Raju K et al. This differences in findings may be due to small sample size in our study. In our study, 3.3% of cases had parity ≥ 5. Maximum patient were para ≥ 5 in the study done by Raju K et al (28%, n=21) respectively. 19.8% (n=69) cases had parity ≥ 5 in a study done by Kaku M et al[107,115] The differences in the findings may be because of less sample size.

Table 27: Comparison of Histopathological grades in present study with other studies.

Histopathological Grade	Gaikwad et al, 2016[116]	Patil et al, 2019[108]	Present Study (n=30)

	(n=76)	**(n=144)**	
Well Differentiated SCC	11.84%	22.2%	**26.67%**
Moderately Differentiated SCC	71.05%	61.1%	**46.66%**
Poorly Differentiated SCC	17.11%	16.6%	**26.67%**

Maximum patient (46.66%) with SCC had features of moderately differentiated SCC in our study. In a study done by Gaikwad et al and Patil et al ,71.05% (n=54) and 61.1 % (n=88) of SCC patient had features of moderately differentiated SCC respectively. [108,116]

11.84% (n=09) and 22.2% (n=32) of cases had features of well differentiated SCC in the study done by Gaikwad et al and Patil et al respectively. [108,116]

17.84% (n=13) and 16.6 % (n=24) of cases had features of poorly differentiated SCC in the study done by Gaikwad et al and Patil et al respectively. [108,116]

These differences may be because of less sample size in our study or may be because of different population of cases selected in different studies.

Table 28 : FIGO staging in present study comparison with other similar study.

FIGO Stage	**Jain et al, 2017**[117]	**Raju K et al,2019**[107]	**Present Study**

	(n=46)	**(n=75)**	**(n=30)**
I	0	08%	**0**
II	80%	32%	**70%**
III	20%	40%	**30%**
IV	0	20%	**0**

In our study, 70% (n=21) cases of SCC had FIGO stage II, while remaining 30% (n=09) cases had FIGO stage III disease. This finding is comparable with the study done by Jain et al,2017 where 80% (n=37) of cases had FIGO stage II disease and rest of the 20% (n=09) cases had FIGO stage III disease. [107,117]

In a study done by Raju K et al, 40% (n=30) of SCC cases had FIGO Stage III disease while 32% (n=24) of cases had Stage II. 20% (n=15) of cases had FIGO stage IV disease and rest 08% (n=06) of cases had FIGO stage I disease. [107,117] This difference may be because of less sample size in our study.

Table 29 : Expression of CK19 in normal, HSIL and SCC in the present study compared with other studies.

	Lee H et al, 2017[96] (Number of cases =n= 55)			Present Study (Number of cases =n= 70)		
	Negative	**Positive**		**Negative**	**Positive**	
Normal	_	_		**20% (n=02/10)**	**80% (n=08/10)**	**Weak Positive :- 80% (n=8/10)**
						Moderate Positive :-- 00%
						Strong Positive :- 00%
HSIL	00%	100% (n=25)	Patchy :- 40% (n=10/25)	**6.7% (02/30)**	**93.3% (28/30)**	**Weak Positive :- 80% (n=24/30)**
			Diffuse Positive:- 60% (n=15/25)			**Moderate Positive:- 10% (n=03/30)**
						Strong Positive:- 3.3% (n=01/30)
			Patchy:- 63% (n=19/30)			**Weak Positive:- 10% (n=03/30)**

SCC	00%	100% (n=30)		**0%**	**100% (30/30)**	**Moderate Positive :- 33.3% (n=10/30)**
			Diffuse Positive :- 37% (n=19/30)			**Strong Positive :- 56.7% (n=17/30)**

Cytokeratin 19 is basal cell marker. 80% of normal cervical biopsy (n=10) showed a weak basal cell positivity for CK19, while rest 20% were negative for CK 19 expression.

In our study 93.3% (n=28) of cases with HSIL showed immunopositivity for CK 19. Majority were weak positive. During transformation from normal to HSIL, more CK 19 positive cells appear in the intermediate cell layer. This finding is comparable to the study done by Lee H et al (n=25). However in the study by Lee H et al, 60% (n=15) of cases with HSIL showed a diffuse staining with CK19. While in our study 80% (n=24) showed a weak positivity for CK19. [96] This difference may be because of use of different methodology for the interpretation of CK 19 immunohistochemistry.

In our study 100% of SCC (n=30) cases were positive for the expression of CK19 which is comparable to the study done by Lee H et al (n=30). However 37% (n=11) of SCC cases showed diffuse positivity to CK 19 in the study done by Lee H et al. In our study 56.7% (n=17) cases were strongly positive for SCC. During transformation from HSIL to SCC, more CK 19 positive cells migrated to intermediate and superficial layer of cervix. This difference may be because of use of different methodology for the interpretation of CK 19 immunohistochemistry. Heterogeneity in tumor cells might be possible reason for this difference in findings.

Table 30 : Expression of vimentin in normal, HSIL and SCC in the present study compared with other similar studies .

	Expression of Vimentin		
	Normal (Number of cases=n)	**HSIL (Number of cases=n)**	**SCC (Number of cases=n)**
Myong NH et al,2012[98]	–	8.75%(n=7/80)	66.67% (n=14/21)
Jiang J et al,2019[99]	12.5% (n=5/40)	45% (n=27/60)	72.1% (n=62/86)
Present study	**00% (0/10)**	**33.3% (10/30)**	**73.3% (22/30)**

In our study none of the normal cervical biopsy samples showed immunopositivity for vimentin. 33.3% (n=10) of HSIL cases were positive for vimentin expression and 73.3% (n=22) of SCC cases were positive for the expression of vimentin. It was observed that there was increase in the positivity for vimentin expression across normal, HSIL and SCC. This finding is comparable to the findings of Myong NH et al and Jiang J et al.[98,99]

Table 31 : Expression of RhoC in normal, HSIL and SCC in the present study compared with other similar studies.

	Expression of RhoC		
	Normal (Number of cases=n)	**HSIL (Number of cases=n)**	**SCC (Number of cases=n)**
Nai NM et al, 2009[109]	00% (n=0/14)	15.38%(n=2/13)	82.46% (n=47/57)
Srivastava S et al,2010[77]	41% (n=5/12)	_	87% (n=21/24)
Tanaka K et al, 2019[110]	_	_	69.4% (n=34/49)
Present study	**10% (n=01/10)**	**20% (n=6/30)**	**83.3% (n=25/30)**

83.3% (n=25) of SCC cases showed positive expression for RhoC as compared to 10% (n=01) and 20% (n=06) cases of normal cervix and HSIL respectively. Cases with SCC of cervix had higher expression of RhoC. This finding is comparable to the findings of Nai NM et al, Srivastava S et al and Tanaka K et al. [77,109,110] RhoC expression was seen to increase from normal to SCC. This finding is comparable with the finding of Nai NM et al and Srivastava S et al. [77,109]

Table 32 .Correlation of expression of Vimentin with grade of SCC in the present study compared with other similar studies.

	%age of cases positive for Vimentin		
	Well Differentiated SCC (Number of cases=n)	**Moderately Differentiated SCC (Number of cases=n)**	**Poorly Differentiated SCC (Number of cases=n)**
Yu JQ et al,2015[111]	58.6% (n=17/29)		92.8% (n=26/28)
Lin J et al, 2017[112]	25% (n=27/108)		36.3% (n=08/22)
Present Study	**62.5% (n=05/08)**	**71.4% (n=10/14)**	**87.5% (n=07/08)**

In our study, 87.5% (n=07) of poorly differentiated cases of vimentin showed positivity for Vimentin. Increased expression of vimentin was seen in poorly differentiated cancer. The finding is comparable with the finding of Yu JQ et al. [111] However lesser expression of vimentin (36%) was seen in the study done by Lin J et al. [112] Expression of vimentin increased as the grade of tumor increases. However, this difference of the expression of vimentin across the tumor grades was statistically insignificant.

Correlation of expression of RhoC with grade of SCC in present study compared with other similar study.

Expression of RhoC among the grades of cervical cancer was found to be statistically insignificant. This finding is similar to the conclusion of the study done by Nai NM et al in 2009. [109]

Table 33. Correlation of expression of Vimentin with the FIGO staging of SCC in the present study compared with other similar studies.

	FIGO Stage I (Number of cases=n)	**FIGO Stage II (Number of cases=n)**	**FIGO Stage III (Number of cases=n)**	**FIGO Stage IV (Number of cases=n)**
Yu JQ et al, 2015[111]	70.58% (n=24/34)	82.6% (n=19/23)	_	_
Li Q et al, 2016[113]	95% (n=39/41)	81.8% (n=18/22)	_	_
Lin J et al,2017[112]	24.71% (n = 22/89)	31.7% (n=13/41)	_	_
Present Study	**00%**	**76.2% (n=15/21)**	**66.67% (n=06/09)**	**00%**

No statistical significance was found in between expression of vimentin and FIGO staging in our study. This finding is comparable with the findings of Yu JQ et al, Li Q et al and Lin J et al. In all the studies only early stages were taken. [111,112,113]

Table 34. Correlation of expression of RhoC with the FIGO Staging of SCC in present study compared with other similar studies.

	FIGO Stage I (Number of cases=n)	**FIGO Stage II (Number of cases=n)**	**FIGO Stage III (Number of cases=n)**	**FIGO Stage IV (Number of cases=n)**
Tanaka et al, 2020[110]	_	77.78 **(n=07/09)**	67.5% **(n=27/40)**	_
Present Study	**00%**	**85.71% (n=18/21)**	**88.89% (n=08/09)**	**00%**

No statistical significance between the expression of RhoC and the FIGO Staging was found in our study which is similar to the studies of Nai NM et al and Tanaka et al. [109,110]

80% of normal, 93.3% of HSIL cases and 100% of SCC cases showed positivity for CK19.

00% of normal, 33.3% of HSIL and 73.3% of SCC were immunopsoitive for Vimentin.

10% of normal, 20% of HSIL and 83.3% of SCC showed positivity for RhoC expression.

Epithelial Mesenchymal Transition leads to increase in the expressions of Vimentin and RhoC. CK19 is a basal cell marker. CK19 expression highlights the role of basal cells in the cervical carcinogenesis.

Expression for vimentin increased as grading progresses from well differentiated SCC to moderately differentiated SCC to poorly differentiated SCC. However this difference of expression across the grades was statistically insignificant.

Maximum expression for RhoC was seen in well differentiated SCC and poorly differentiated SCC than moderately differentiated SCC. However this difference in the expression of RhoC across grades of tumor was statistically insignificant. 76.2% of FIGO Stage II SCC and 66.67% of FIGO stage III showed positivity for Vimentin. 80.95% of FIGO Stage II and 88.89% of

FIGO Stage III showed positivity for the expression of RhoC. No statistical significance was seen between the expressions of Vimentin and RhoC among FIGO Stages.

Vimentin and RhoC show maximum expression in poorly differentiated SCC. Hence role of Vimentin and RhoC can be considered for adjuvant targeted chemotherapy.

LIMITATIONS OF STUDY

It is a cross-sectional, unicentric study done in a smaller sample size. Due to small sample size and less distribution of cases, we were not able to correlate immunohistochemical grade with the study groups of Normal, HSIL and SCC and also with the histopathological grades and the staging of the disease.

Further multicentric studies with a larger sample size are required. Further studies on role of RhoC in targeted therapy for cervical cancer is recommended.

CONCLUSION

Epithelial mesenchymal transition is an important phenomenon that promotes invasion, migration in cervical cancer. Increase expression of CK 19 in HSIL and SCC of cervix highlights role of basal cells of cervix in cervical carcinogenesis. Vimentin and RhoC in cervical cancer promotes the phenomenon of epithelial mesenchymal transition and eventually leads to malignant transformation, increase motility and increase invasiveness.

SUMMARY

- Total of seventy cases were selected for the study based on the calculation of sample size statistically.
- Out of 70, 10 were cases with normal cervix, 30 had HSIL and 30 had SCC of cervix.
- All the 70 cervical biopsies were subjected for immunohistochemistry for Cytokeratin 19(CK19), Vimentin and RhoC each.
- Mean age among normal, HSIL and SCC cases were 46.2 years, 49.1 years and 56.27 years. This difference in mean age among normal, HSIL and SCC were found to be statistically significant.
- Maximum patients of HSIL and SCC had parity 3. Maximum patients with normal cervix were para2.
- Maximum patients of HSIL and SCC presented with chief complaint of per vaginal bleeding.
- On per speculum examination, maximum patient with normal cervix, HSIL and SCC showed white discharge, erosion and growth respectively.
- Weak basal cell positivity for CK 19 was seen in 80% cases with normal cervix. 93.3% cases of HSIL showed positivity for CK19. 100% cases of SCC showed positivity for CK19. No statistical significance was seen between the expression of CK19 and study groups.
- All the patients with normal cevix were negative for Vimentin expression. 33.3% of HSIL cases were vimentin positive. 73.3% cases of SCC were positive for vimentin. Increase in expression of vimentin from normal to HSIL and to SCC was statistically significancant.

- 10% of SCC cases showed positivity for RhoC expression. 20% cases of HSIL were positive for RhoC and 83.3% cases of SCC showed positive RhoC expression. Differences in expressions of RhoC among the study groups were statistically significant.
- No correlation was seen between expressions of vimentin and RhoC and histopathological grades and the FIGO staging among SCC cases.
- SCC had higher expressions of CK19, Vimentin and RhoC.
- Positive CK 19 expression among HSIL and SCC highlights role of basal cells in cervical carcinogenesis.
- Vimentin and RhoC has role in epithelial mesenchymal transition in evolution of cervical cancer which eventually promotes increase in cellular motility and invasiveness.

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