"ASSOCIATION OF p16, Ki-67 AND CD44 MARKERS IN CERVICAL INTRAEPITHELIAL NEOPLASIA AND CERVICAL CARCINOMA"

By

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DISSERTATION SUBMITTED TO
SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION AND RESEARCH, KOLAR, KARNATAKA

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF MEDICINE IN

PATHOLOGY

Under the guidance of

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to be submitted to the

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION AND RESEARCH, TAMAKA, KOLAR, KARNATAKA.

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ACKNOWLEDGEMENT

"It always seems impossible until it's done"- Nelson Mandela.

With this saying and a deep sense of gratitude and humility, I express my thanks and indebtedness to my respected teacher and guide, ***Dr. KALYANI R,*** *Professor and HOD of Pathology, Sri Devaraj URS medical college, Kolar who meticulously handpicked this topic for me, graced my study officially and informally by her constant support and expert advice. Her encouragement, sense of punctuality, research-oriented approach, the painstaking effort to weed out errors, meticulous attention to fine details, her patience and affection during the entire course of this study leaves me permanently indebted to her. I dedicate the good part of this work to her.*

Thanks to ***Dr. SHEELA SR,*** *Professor and HOD of OBG, R.L Jalapa hospital Kolar, for consenting to be my co-guide and providing cases. It was her encouragement and critical appraisal that made me able to reach the destination. She has been so kind to me that I never hesitated to trouble her as often as I needed.*

I am thankful to my Professor, Department of Pathology ***Dr. CSBR PRASAD****, for his able guidance, patience and supervision throughout this study.*

I express my deep sense of gratitude and humble thanks to ***Dr. T.N. SURESH, Dr. SUBHASHISH DAS and Dr. MANJULA K,*** *Professors of pathology, for their advice and encouragement throughout the study. I am gratefully indebted for their support.*

I thank ***Dr. SWAROOP RAJ*** *Associate Professors of pathology for his constant guidance, encouragement and going out of his way to help me anytime of the day.*

I thank ***Dr. HEMALATHA,*** *Associate Professors of pathology****, Dr. SHILPA MD, Dr. SUPREETHA MS, Dr. YASHASWINI R and Dr. GEETHA****, Assistant Professors of Pathology, for their constant guidance, encouragement and going out of their way to help me anytime of the day.*

I thank my senior and friend, ***Dr. ANKITA BAGHEL,*** *for patiently putting up with my constant questions and doubts, for always comforting me and providing me sage advice whenever things felt overwhelming.*

I thank my seniors, ***Dr. NISHIT OJHA, Dr. SHUBHRA RAMCHANDANI, Dr. KARTHIK KR, Dr. ARGHA BARUAH, Dr. SWATI PANDEY, Dr. SULAGNA MANNA*** *and* ***Dr. RAJINI T*** *for helping me in understanding the right approach to the subject and for keeping me on my toes with their thought provoking questions.*

I thank my colleagues, ***Dr. PRADEEP MITRA, Dr. CHANDANA CHENNA REDDY, Dr. MANAN BHARATKUMAR SHAH*** *for their constant support in completing this study and for providing the useful tips and clues in completing this vast work. They have taken care of me and taught me like a teacher when needed.*

I thank all my juniors with the special mention to ***Dr. PREETI UTNAL, Dr. VARSHASHREE R, Dr. GAURAV KHICHARIYA, Dr. PRIYANKA ARVIND PRASAD, Dr. ANKIT ANAND YADAV and Dr. SONIA ARORA*** *for helping me with daily department works so I could finish my thesis on time.*

All hard work put in was accompanied with a lot of good times and memories. I am very grateful for my friends who made this herculean task feel like a breeze, ***Dr. KIMI SHETTY and Dr. SHAMIM BENAZIR.***

I will forever be grateful to ***Mr. HUZEFA MOIZ IGATPURIWALA*** *for being there for me whenever I needed him (irrespective of the time of the day), for being a constant source of unconditional encouragement and selfless support, and for providing creative solutions to my problems throughout my study.*

I express my sincere thanks to ***Dr. MAHESH,*** *for his invaluable help in statistical analysis without which my study would have been incomplete.*

I am also thankful to all the ***Technical Staff,*** *especially* ***Mr. VIRENDRA, Mr. SHANKAR, Mrs. SUMATHI, Ms. ASHA, Mr. KRISHNAPPA*** *and* ***Mr. MAHENDRA*** *for their invaluable help without whom, this study would not have been possible, and I also thank my non-technical staff* ***Mr. PARTHA*** *and* ***Mr.***

JAYARAM *for their help and assistance throughout my duration of course.*

I am infinitely obliged and forever indebted to my parents, ***Mr. KHURSHEED ALI MEHDI*** *and* ***Mrs. SHEERIN MEHDI*** *for being my backbone and for always putting my needs above theirs. I am thankful for my sister,* ***Ms. MENA KHURSHEED MEHDI*** *and brother* ***Mr. MOHAMMED KHURSHEED MEHDI****, for their constant support throughout my study and helping me whenever I needed.*

Finally, I owe all my success and growth to ***THE ALMIGHTY*** *and* ***THE AHLUL-BAYT (a.s.).***

Last but not the least, I also owe my sincere gratitude to:

- *The authors, whose work has been quoted and referred in this text,*
- *Patients whose surgical specimens are the real backbone of the study.*

THANKS TO ALL AND ONE!

Dr. HAJRA KHURSHEED MEHDI

LIST OF ABBREVIATIONS

S.No.	Abbreviation	Expansion
1.	HPV	Human Papilloma Virus
2.	HR-HPV	High Risk - Human Papilloma Virus
3.	NILM	Negative for Intraepithelial neoplasia/Malignancy
4.	CIN	Cervical Intraepithelial Neoplasia
5.	CSC	Cancer Stem Cells
6.	HSIL	High Grade Squamous Intraepithelial Lesion
7.	SCJ	Squamo-Columnar Junction
8.	TZ	Transformation Zone
9.	CIS	Carcinoma In-Situ
10.	SCC	Squamous Cell Carcinoma
11.	LSIL	Low Grade Squamous Intraepithelial Lesion
12.	LAST	Lower Anogenital Squamous Terminology
13.	LCR	Long Control Region
14.	SIL	Squamous Intraepithelial Lesion
15.	WD-SCC	Well Differentiated Squamous Cell Carcinoma
16.	MD-SCC	Moderately Differentiated Squamous Cell Carcinoma
17.	PD-SCC	Poorly Differentiated Squamous Cell Carcinoma
18.	DES	Diethylstilbestrol
19.	CCSC	Cervical Cancer Stem Cell
20.	ABCG2	ATP-binding cassette sub-family G member 2
21.	ALDH1	Aldehyde dehydrogenase 1
22.	OPN	Osteopontin

LIST OF ABBREVIATIONS

23.	HA	Hyaluronic Acid
24.	Cdk-4	Cyclin dependent kinase - 4
25.	HIER	Heat Induced Epitope Retrieval
26.	TBS	Tris Buffer Solution
27.	DAB	Di- Amino Benzidine
28.	FIGO	International Federation of Gynaecology and Obstetrics
29.	DPX	Distyrene Plasticizer Xylene
30.	HPF	High Power Field
31.	n	Number of cases
32.	WHO	World Health Organization
33.	TNM	Tumor, Node and Metastasis

ABSTRACT

Background:

Cervical cancer is the second most commonly occurring malignancy among women in the world. It is the most commonly reported gynaecological malignancy in India and is also one of the major causes of cancer related morbidity. In India, the average age for cervical cancer incidence is 50-60 years. The peak age for HSIL incidence is 40-50 years. In South India, the prevalence of cervical cancer accounts for 17.55% of all reported cancer cases among the female population. The incidence of HR-HPV infection peaks around 25 years of age, which coincides with the peak age for sexual activity. More than 90% of HSIL and virtually all cases of cervical cancer are associated with HR-HPV infection. Stem Cells exist in niches in the cervical tissue at this squamo-columnar junction, which probably when infected with HR-HPV or affected by any risk factors, undergo malignant transformation to Cancer Stem Cells (CSC). They are believed to be the starting point of carcinogenesis and play a role in cancer relapse and metastasis. The study of CSCs in context with CIN and Carcinoma Cervix are not many. More over the expression of CSC markers are not well defined with respect to the stages of carcinogenesis in the cervix. The present study aims to see the association of HR-HPV genome integration with cervical epithelial cells (p16) and proliferation of these cells (p16 + Ki-67) with expression of CSCs (CD44), to determine the stage at which the CSCs are expressed.

Aim of the study:

1. To observe and correlate the expression of p16, Ki-67 and CD44 in normal, High grade squamous intraepithelial lesion (HSIL) and carcinoma cervix using immunohistochemistry.

2. To compare the expression of p16, Ki-67 and CD44 with the clinico-pathologic parameters of carcinoma cervix.

Methods:

The study was carried at The Department of Pathology, R.L.Jalappa Hospital and Research Centre attached to Sri Devaraj Urs Medical College, Tamaka, Kolar, during the period of July 2016 to June 2018. The study included 26 cases each of normal, HSIL and carcinoma cervix

cases. Immunohistochemistry was done using antibodies against p16, Ki-67 and CD44. Their expression were documented and analysed. Expression of p16, Ki-67 and CD44 were correlated with normal, HSIL and the clinico-pathological parameters of carcinoma cervix. Statistical correlation was done using Chi-square test or Fischer's exact test and Mann Whitney U test. A p value of less than 0.05 was considered significant.

Results:

The mean age of the cases in the normal, HSIL and carcinoma groups were 42.3±9.3 years, 47.0±13.4 years and 50.4±10.3 years, respectively. 61.5% cases were positive, and 7.7% cases were ambiguous for p16 expression in HSIL cases. 11.5% cases were strongly positive, 53.8% cases were positive, and 34.6% cases were weakly positive for Ki-67 expression in HSIL cases. 42.3% cases were strongly positive, 42.3% cases were positive, and 15.4% cases were weakly positive for CD44 expression in HSIL cases. 92.3% cases were positive, and 7.7% cases were ambiguous for p16 expression in carcinoma cases. 73.1% cases were strongly positive, and 26.9% cases were positive for Ki-67 expression in carcinoma cases. 65.4% cases were strongly positive, 30.8% cases were positive, and 3.8% cases were weakly positive for CD44 expression in carcinoma cases. Statistically significant correlation was seen in p16, Ki-67 and CD44 expression between the three group. Statistically significant correlation was seen in p16 expression and FIGO stage and lymph node involvement in carcinoma cervix cases. No statistically significant correlation was seen between Ki-67 expression and the various clinicopathologic parameters. Statistically significant correlation was seen in CD44 expression and lymph node involvement in carcinoma cervix cases. Statistically significant correlation was seen between p16, Ki-67 and CD44 expression when compared with each other.

Conclusion:

The results of this study showed that expression of p16, Ki-67 and CD44 increases as the lesion progresses from normal to HSIL to carcinoma cervix. There was a significant positive correlation seen in p16 and CD44 expression with the lymph node involvement in carcinoma cervix. In addition, there was a significant positive correlation seen between p16 expression and the FIGO

stage of carcinoma cervix. Whereas, Ki-67 expression showed no statistical correlation with any of the clinico-pathologic parameters. These findings can be used to assess the prognosis of cervical carcinoma and the development of targeted therapy against cervical cancer stem cells.

Keywords : p16, Ki-67, CD44, Cervical cancer, Cancer Stem Cells.

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INTRODUCTION

INTRODUCTION

Cervical cancer is the second most commonly occurring cancer of the female reproductive tract and the most common cause of cancer related death in females.[1] 99.7 % of these cervical cancers are associated with Human Papilloma Virus (HPV), particularly high risk-HPV (HR-HPV). The infection often occurs at the squamo-columnar junction (the transition zone).[2]

HR-HPV infection is the most important risk factor in cervical carcinoma. On a global scale, HPV infection is seen in approximately 11.4% of general population. In Indian population, 4.7% and 1.3% cases of Negative for Intraepithelial Neoplasm/Malignancy (NILM) test positive for HPV 16 and HPV 18 respectively. While in reactive cases the incidence is higher where 22.6% cases test positive for HPV 16 and HPV 18 infections.[3-5] HR-HPV is associated with cervical intraepithelial neoplasia (CIN) and carcinoma. HR-HPV is associated with 36.4% of CIN 1. 74.3% of CIN 2 and CIN 3 is associated with HR-HPV.[6] In India 87.8% to 96.67% of cervical cancers are associated with HPV.[5-10]

Infection by HR-HPV may be in the form of a transient infection or may persist in the host cells in its episomal form or it may integrate with the host genome.[4] It causes risk of carcinoma cervix once the viral DNA integrates with the host genome. This causes the expression of viral mRNA proteins E1 to E8. Viral mRNA proteins E6 and E7 are crucial to inhibit P53 and Rb genes respectively.[4,11,12] P53 and Rb genes are important checkpoints that prevent normal cells from transforming into dysplastic cells. The expression of E7 in cervical epithelial cells is enough to initiate oncogenesis.[11,12]

Stem Cells exist in niches in the cervical tissue at this squamo-columnar junction, which probably when infected with HR-HPV or affected by any risk factors, undergo malignant transformation to Cancer Stem Cells (CSC). These CSCs have the properties of multilineage differentiation, self-renewal, slow cycling capacity, recurrence and tumorigenicity.[13,14] They are believed to be the starting point of carcinogenesis and play a role in cancer relapse and metastasis.[14]

The study of CSCs in context with CIN and Carcinoma Cervix are not many.[15-18] More over the expression of CSC markers are not well defined with respect to the stages of carcinogenesis in the cervix.[19]

The present study aims to see the association of HR-HPV genome integration with cervical epithelial cells (p16) and proliferation of these cells (p16 + Ki-67) with expression of CSCs (CD44), to determine the stage at which the CSCs are expressed.

AIMS AND OBJECTIVES OF THE STUDY

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REVIEW OF LITERATURE

REVIEW OF LITERATURE

ANATOMY:

The uterus is anatomically divided into three distinct parts i.e. corpus, isthmus and cervix. The uterine cervix is the inferior part of the uterus. Thus, connecting the corporis uteri to the vaginal canal. The part of the cervix which protruded into the vagina is called the external os. The endocervix is the isthmus of the cervix and opens internally into the uterus and externally into the vagina via the external os.[20]

HISTOLOGY:

ECTOCERVIX

The ectocervix is lined by mature non-keratinizing stratified squamous epithelium, which is similar to the lining epithelium of the vagina. This epithelial lining is further divided into three zones i.e. Germinal/basal/parabasal cell layer, midzone/intermediate cell layer and superficial zone/superficial cell layer. The continuous regeneration of the epithelium is the function of germinal cell layer.[20-22] The midzone forms the major component of the epithelial lining. The superficial layer is one with the most mature cell.[20]

The germinal cell layer is made up of two cell populations. (1) The basal cell, which is placed perpendicular to the basal lamina. This type of cell acts as stem cells and are major contributor to the epithelial regeneration. (2) The parabasal cell, which is one to two cell thick layer just above the basal layer. This layer frequently shows mitotic figures indicating active regeneration.[20,23-25]

ENDOCERVIX

The endocervix is lined by monolayered mucin secreting columnar epithelium. It lines both the endocervical canal as well as the endocervical glands.[20]

THE TRANSFORMATION ZONE

The junction between the endocervical columnar epithelium and the ectocervical stratified squamous epithelium is called the **Squamocolumnar junction (SCJ)** of the cervix. The SCJ is of two types i.e. the original SCJ and the physiologic SCJ. The original SCJ is present at the time of birth while the physiologic SCJ develops at the time of menarche. The area between these two types of SCJ is called **The Transformation Zone (TZ)**.[20]

The TZ is the site for squamous metaplastic epithelium. Almost all cervical carcinomas and precursor lesions arise from this area.[20]

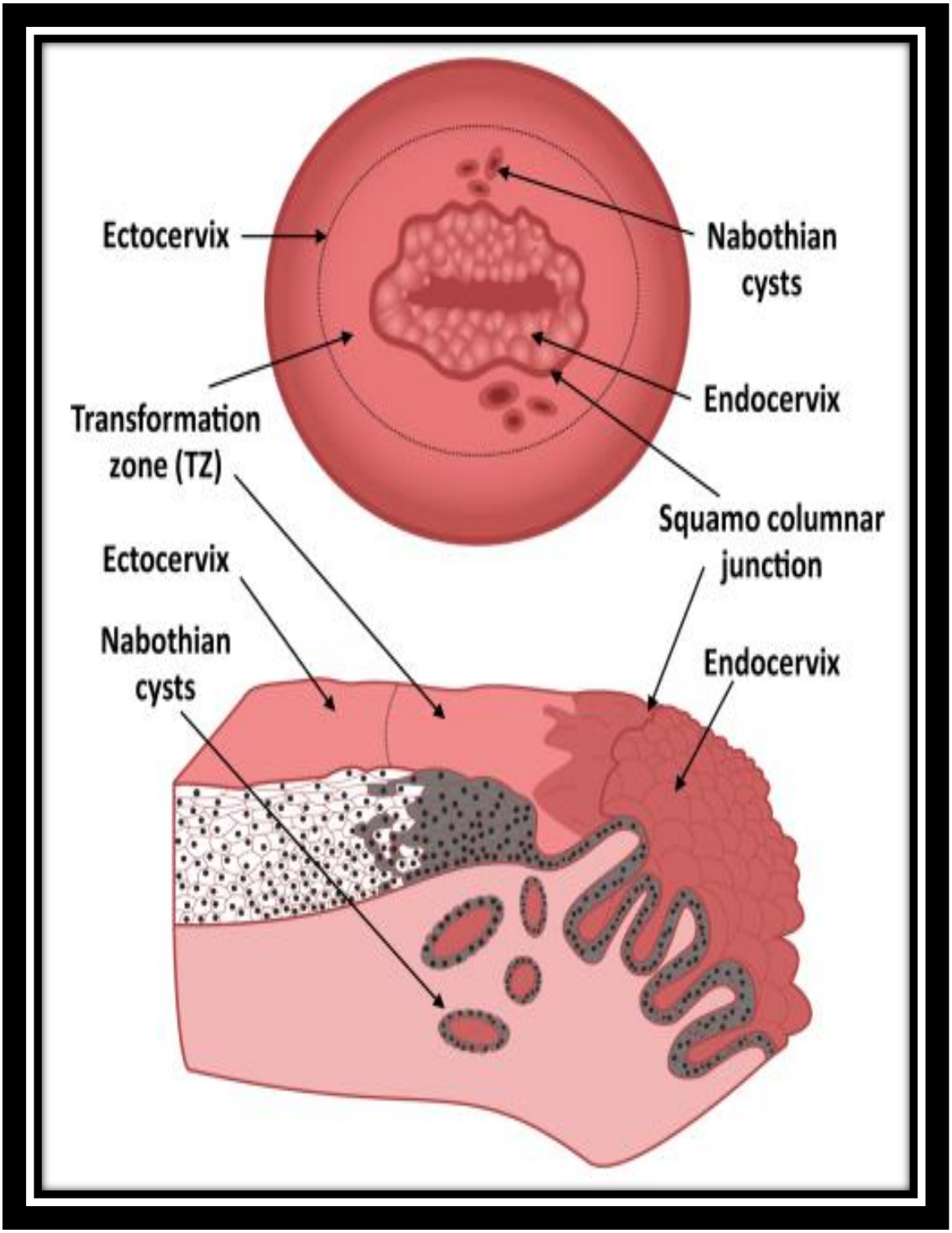


Figure 1: Schematic representation of the Transformation Zone. (Top) Colposcopic view of the cervix showing the TZ with Nabothian. (Bottom) Cross-section view of the cervix showing TZ and SCJ between the endocervix and the ectocervix.[26]

PATHOLOGY OF CERVIX:

INFLAMMATORY DISEASES:

Inflammation of the cervix is called cervicitis. Based on etiology, cervicitis is divided into groups i.e. non-infectious cervicitis and infectious cervicitis.[20]

NON-INFECTIOUS CERVICITIS

It is a non-specific inflammatory response to chemical or mechanical trauma. Most commonly caused by trauma due to pessaries, intrauterine contraceptive devices, diaphragms, foreign bodies and tampons. Iatrogenic causes can be due to surgical intervention and instrumentation. Another cause can be chemical irritation caused secondary to douching.[20,27,28]

Microscopically, it shows stromal edema, congested blood vessels, neutrophilic inflammatory infiltration of the epithelium and stroma. These features are characteristic of acute cervicitis. Chronic cervicitis shows predominantly lymphoplasmacytic infiltration of the epithelium and stroma. Granulation tissue, stromal fibrosis and occasional histiocytes can also be noted. Follicular cervicitis is the term reserved when lymphoid follicles are seen underneath the cervical epithelium.[20,29]

INFECTIOUS CERVICITIS

The most important organisms causing infectious cervicitis are as follows:

A) Bacteria:

- Chlamydia trachomatis
- Neisseria gonorrhoea
- Group B streptococcus

- Gardenella vaginalis
- Mycobacterium tuberculosis

B) Virus:

- Human papilloma virus
- Herpes simplex virus

C) Fungi:

- Candida
- Aspergillus

D) Protozoa and Parasites:

- Trichomonas vaginalis
- Amoeba
- Schistosomes[20]

Infectious cervicitis can be of two types depending on the type of epithelium that is affected i.e. endocervitis and ectocervicitis.[20,29]

PRECANCEROUS CONDITIONS

Until early 1970s, precancerous lesions of squamous epithelium was divided into dysplasia and carcinoma in situ (CIS). In 1973, Richart introduced the idea that all precursor lesions of cervical squamous cell carcinomas (SCC) represented a singular disease process and called it Cervical Intraepithelial Neoplasm (CIN).[30,31]

CIN terminology divides cervical precancerous lesions into three distinct groups:

CIN 1 – previously called mild dysplasia

CIN 2 – previously called moderate dysplasia

CIN 3 – previously called severe dysplasia or CIS [30,31]

In cytology, the Bethesda System uses low grade squamous intraepithelial lesion (LSIL) for CIN 1 and koilocytic atypia, and high grade squamous intraepithelial lesion (HSIL) for CIN 2 and CIN 3. This system is widely used for cytological reporting of pap smears.[32]

In 1994, Wright and Kurman proposed a similar two tiered system for histopathological reporting of cervical precancerous lesions.[31,33] In 2012, the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology formed a consensus and recommended the use of the two-tiered systems (i.e. LSIL and HSIL) according to the Lower Anogenital Squamous Terminology (LAST) for histopathological reporting of squamous precancerous lesions.[34]

LSIL

LSIL is characterised by the combined histological features of nuclear atypia, koilocytosis, multinucleation and epithelial hyperplasia. All these features are pathognomic HPV associated changes and are limited to the lower one third of the squamous cell layer. Koilocytes are characterised by perinuclear cytoplasmic halo which is accompanied by thickened cytoplasmic membrane. Prospective studies show that a approximately 80% LSIL cases regress spontaneously and do not progress to high grade lesion and carcinoma.[35] LSIL is commonly associated with polyploidy.[28,31,36,37]

HSIL

HSIL (CIN 2 and CIN 3) are characterised by presence of atypia in layers of squamous epithelium. Atypia is more when compared to LSIL; more significantly seen in the basal and parabasal cells. Another important feature is the presence of atypical mitotic figures. Additionally, there is marked cellular crowding, anisonucleosis, nuclear pleomorphism and loss of polarity. HSIL is often associated with aneuploidy.[29,36,31] About 8% of women over the age of 30 years with HSIL progress to develop cervical carcinoma.[38]

INVASIVE CERVICAL CANCER

Cervical cancer is the second most common form of cancer and cancer related death in women.[39] Every year in India, 122,844 women are diagnosed with cervical cancer and 67,477 women die from the same. Also, India has the highest age standardised incidence of cervical cancer in South Asia.[5] In Kolar, Karnataka, Carcinoma cervix amounts for 17.55% of all cancer cases in the female population.[3]

ETIOLOGY

1. SEXUAL ACTIVITY

Early onset of sexual activity (specially less than 16 years of age) is one of the most important factor.[29,31] Menopause is associated with reduced risk for carcinoma cervix. Multiple sexual partners are considered a risk factor.[31]

2. SEXUALLY TRANSMITTED DISEASE

HPV infection is the single most important etiological factor. HR-HPV (16, 18, 31 and 45) account for more than 80% of carcinoma cervix.[40] Also associated with cervical cancer are Herpes Simplex Virus and chlamydia trachomatis. However, their mechanisms are not fully understood.[31]

3. EARLY AGE OF PREGNANCY

Women with an early age of pregnancy, between 15 - 19 years of age have a two-fold increased risk for cervical cancer compared with those who get pregnant after the age of 25 years. The increased risk of HPV associated cervical cancer in early age of onset of sexual activity and first pregnancy can be explained by the influence of steroid hormones on HPV infection and also the immune response to the

infection in the pre-adolescent and adolescent age group. The susceptibility of TZ to HPV infection is believed to be associated with the relative ease of denudation of the epithelium in this age group. Thus, facilitating exposure of the basal layer of TZ to HPV even with minimal trauma.[41-43]

4. PARITY

Lower risk of cervical cancer was reported in nulliparous women than multiparous women., and among parous women. A steady trend of increased risk is associated with an increasing number of full-term pregnancies.[44]

5. LOW SOCIO-ECONOMIC STATUS

Low socio-economic status is associated with an increased risk of developing cervical cancer. Cancer screening is believed to be influenced by a consistent low socio-economic status owing to a relative lack of knowledge and timely information regarding the recommended cancer screening guidelines and a scarcity of financial aids to afford the available routine screening.[45]

6. SMOKING

Szarewski in a review report found a positive association between cervical cancer and smoking. One of the possible mechanism is the secretion of smoke and tobacco byproducts such as nicotine and cotinine in the cervical mucous. Thus, affecting the number and distribution of immune cells like Langerhan's cells in the cervical microenvironment.[31,46-48]

7. ORAL CONTRACEPTIVES

Steroid hormones bind to specific HPV DNA sequences present within the transcriptional regulation region i.e LCR (Long Control Region). It either leads to an

increase in the transcription of E6 and E7 genes of HR-HPV.[31] Estrogen undergoes hydroxylation and yields catechol estrogen such as, 2-hydroxyestrogen, 4-hydroxyestrogen and 16-hydroxyestrogen. An increased conversion of estradiol to 16-hydroxyestrone and estriol has been believed to be a risk factor for cervical cancer. Conversely, 2-hydroxyestrogens has antiproliferative effect and is linked with a decreased risk for cervical cancer. 16-Hydroxyestrone binds to the estrogen receptor and thus prolongs the effect of estrogen. This particular action is highly enhanced in HR-HPV immortalized cervical cells. Thus, 16-hydroxylation and HR-HPV enhance the action of one another in promoting cell proliferation. Figure 1 shows the interaction of estrogen metabolism with HR-HPV on cell proliferation.[49]

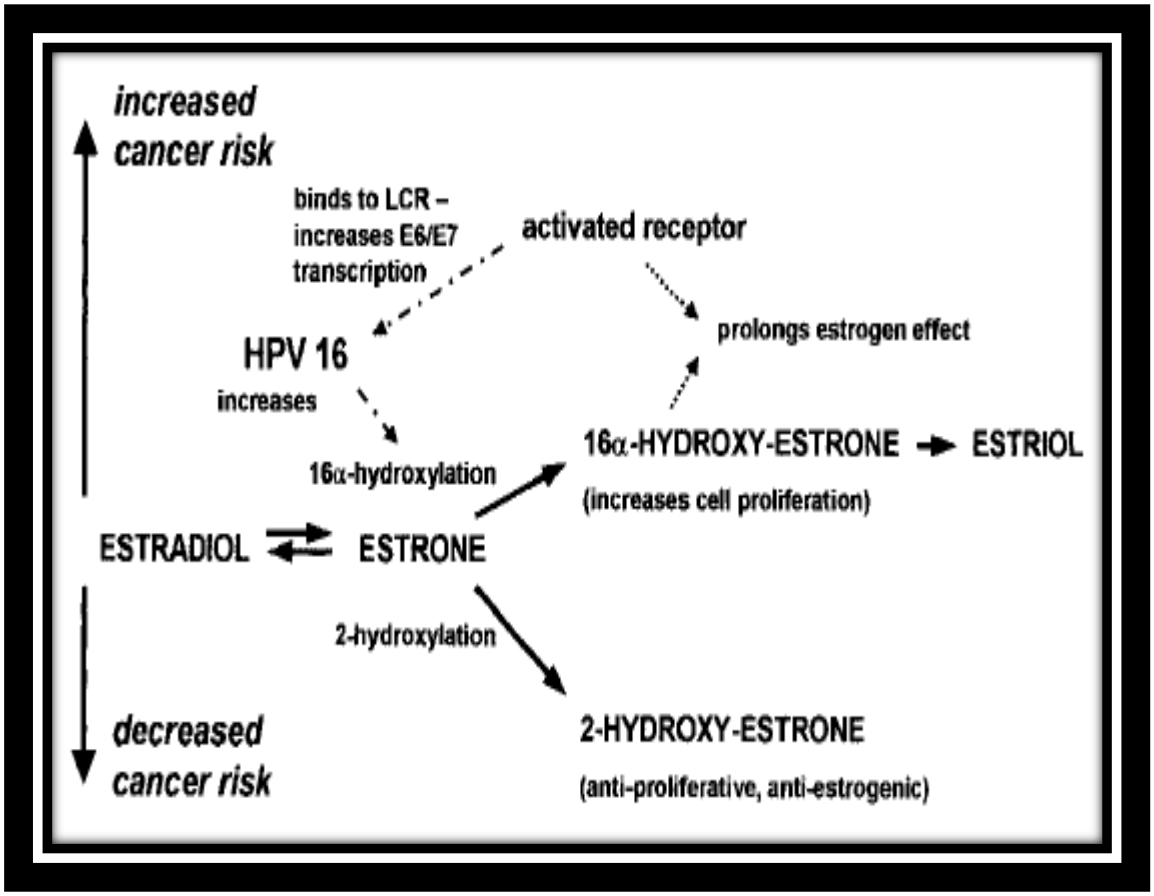


Figure 2: The interaction of estrogen metabolism with HR-HPV on cell proliferation.[49]

8. IMMUNOSUPRESSION

Cellular immune response plays an important deciding factor if HPV infection regresses or progresses to squamous intraepithelial lesion or carcinoma.[31] Human Leucocyte Antigen DRB1*1301 was found to provide a protective action against HPV induced carcinoma cervix.[31,50] Also, incidence of cervical cancer is found to be increased in renal transplant recipients and in HIV infected women.[31] HIV is thought to target the mismatch repair genes thus progressing via the microsatellite instability pathway and leading to HIV associated cervical cancer. Viral protein is also postulated to bind specifically to the cell protein of the DNA or cellular proteins and thereby leading to a defect in the replication process. On the other hand, HIV negative cases progress via the loss of heterozygosity pathway. Also, HIV proteins have an enhancing effect on the HPV proteins, and thus indirectly contributing to disturbance in cell cycle progression.[51]

WHO CLASSIFICATION OF UTERINE CERVICAL TUMORS (2014) [39]

EPITHELIAL TUMOURS

Squamous cell tumours 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 tumours

- 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 nevus

Malignant melanoma

GERM CELL TUMOURS

Yolk sac tumour

LYMPHOID AND MYELOID TUMOURS

Lymphomas

Myeloid neoplasms

SECONDARY TUMOURS

SQUAMOUS CELL CARCINOMA (SCC)

MICROINVASIVE SCC

It is a type of invasive SCC diagnosed only on microscopy, not on macroscopy. Invasion does not exceed the maximum depth of 5mm and doesn't extend horizontally more than 7mm. Thus, microinvasive SCC corresponds with FIGO Stage IA.[39,52,53]

Usually microinvasive SCC is asymptomatic and the patients have a grossly normal cervix or may have a non-specific presentation like chronic cervicitis or erosion. Colposcopically, areas of microinvasive SCC show acetowhitening similar to HSIL, and may contain one or more foci of bizarre surface branching vessels.[54]

Microscopically, microinvasive SCC is seen as tongues of malignant tumor cells invading the basement membrane and entering the cervical stroma. The rest of the cervical epithelium shows features of Squamous Intraepithelial Lesion (SIL). The cells within the microinvasive front show better differentiation compared to SIL. Occasionally, focal areas of keratinisation may be seen in the microinvasive foci. One of the most reliable criteria for microinvasion is the ragged contour of the invading margin.[39,54]

INVASIVE SCC

Cervical cancer is the second most common cause of cancer in women after breast cancer, affecting approximately 0.5million women world-wide.[39] Cervical cancer statistics show an average annual rise of 0.6%. A major proportion of the cases belong to low socio-economic countries. With widespread implementation of pap

smear screening and public health measures, these numbers have dropped in the recent years.[39,54]

CLINICAL FEATURES

Women with stage I tumors are most commonly asymptomatic, especially in tumors with endophytic growth. These patients are usually detected by an abnormal pap smear on routine pap screening.[54]

The presenting symptoms of invasive carcinoma of the cervix mainly depends on the size and stage of the lesion. Most of these tumors present with abnormal vaginal bleeding. The most common and significant presenting features are postcoital bleeding or following douching. Other frequently encountered complaints include intermittent spotting, serosanguinous discharge, and frank haemorrhage. A minority (10-20%) of the patients also complain of blood tinged foul smelling discharge and pain radiating to the sacral region. Constitutional symptoms such as generalised weakness, pallor, malaise, weight loss, pedal edema, rectal pain, dysuria and haematuria are commonly encountered symptoms of locally advanced or metastatic cervical cancer.[39,54]

MACROSCOPY

Cervical cancer on visual inspection appear as an exophytic or endophytic lesion. On palpation, induration of the cervix can be detected. Most of the cervical carcinomas present as exophytic, fungating, polypoidal or papillary growth. Early lesions present as a focal induration, ulceration, or an elevated granular area that bleeds on touch.[39,54]

Endophytic carcinomas on the other hand are either ulcerative or nodular. They usually tend to grow within the endocervical canal and invade the cervical stroma deeply resulting in an enlarged, indurated, barrel-shaped cervix. Endophytic growth, therefore, is not visible and sampling is usually not possible. Such lesions are clinically occult and present at an advanced stage.[39,54]

MICROSCOPY

Invasive SCC has varying growth patterns, cell type and degree of differentiation. Virtually all variants of SCC cervix have HPV etiology. Invasive SCC is characterized infiltration of the stroma by neoplastic cells in the form of irregular and ragged anastomosing tongues or cords. In others, the tumor may invade in the form of individual cells. Tumor cells are usually polygonal or round with abundant eosinophilic cytoplasm and well-defined cell membranes. Intracellular bridges may be variably visible. The nuclei may be uniform or pleomorphic with coarse chromatin and occasional mitotic figures. Cervical SCC are broadly divided into two major groups i.e. Keratinising and Non-keratinising.[39,54]

KERATINISING SCC

Keratinizing SCC are characterized by varying size and configuration of nests and cords of well-differentiated squamous cells. Keratin pearls are composed of keratinised squamous cells arranged in concentric nests and are the defining feature of keratinizing carcinomas. The neoplastic cells have abundant amount of eosinophilic cytoplasm and prominent intracellular bridges. Cytoplasm may show dense individual cell keratinisation. The nuclei are enlarged and hyperchromatic. Prominent nucleoli are not seen. Mitotic figures are occasionally seen.[39,54]

NON-KERATINISING SCC

The tumor cells are predominantly arranged in sheets or nests. The cells demonstrate intercellular bridging and individual cell keratinisation, but lack keratin pearl formation. Nuclear pleomorphism and mitotic figures are more marked. The nuclei are comparatively larger and irregular with coarse granular chromatin and prominent nucleoli. The cell borders are not well defined. Small cell non-keratinising SCC is identified by nests and sheets of small non-keratinising basaloid cells with scant amount of cytoplasm. The nuclei are uniform hyperchromatic with numerous mitotic figures.[39,54]

Traditionally, cervical SCC is graded using modified Broder's system based on the degree and extent of keratinisation, mitosis and cellular atypia into three groups;

- Well differentiated SCC (Grade 1)
- Moderately differentiated SCC (Grade 2)
- Poorly differentiated SCC (Grade 3) [53,55-60]

Cervical SCC can also be classified as;

- Keratinising SCC – Well differentiated SCC (WD-SCC)
- Non-keratinising large cell SCC – Moderately differentiated SCC (MD-SCC)
- Non-keratinising small cell SCC – Poorly differentiated SCC (PD-SCC)[55,61]

BASALOID SCC

This variant is composed of tumor cells arranged in nests. The cells are basal type with high mitotic activity. Comedo necrosis and geographic necrosis is a common finding in this high grade variant of SCC.[39,54]

VERRUCOUS SCC

Verrucous carcinoma is a seldom highly differentiated variant of SCC. Clinically, it resembles condyloma and is a slow growing carcinoma. It is characterised by hyperkeratotic, warty surface and a pushing invasive borders. The cells lack cytological atypia, koilocytes change and mitotic figures. There is dense inflammatory infiltration at the epithelial stromal junction. This variant has high incidence of recurrence but rarely metastasises. The prefered treatment of choice is wide local excision.[39,54]

WARTY (CONDYLOMATOUS) SCC

Warty (condylomatous) carcinoma is a variant of SCC showing marked condylomatous

changes. This variant shows warty surface and appears similar to condyloma on low power. At the deep margin it shows typical features of SCC. Tumor cells have vacuolated cytoplasm and nuclear changes resemble koilocytotic atypia. This variant is less aggressive compared to well-differentiated SCC of the cervix.[39,54]

PAPILLARY SCC

Microscopically, papillary SCC is composed many layers of atypical epithelial cells arranged in papillary pattern. The cells are more basaloid and resemble those of a HSIL. They hyperchromatic, oval nuclei with scant amounts of cytoplasm. Mitotic figures are frequently seen. Also, focal areas of squamous differentiation may be seen with underlying infiltrative invasive border. Papillary SCC have a similar behaviour as that of invasive SCC.[39,54]

SQUAMOTRANSITIONAL SCC

This variant is similar to its counterpart found in the urinary bladder. The cells are arranged in papillary projections. These cells are oval with long axis perpendicular to the basement membrane and flatten out as the cells reach the surface. The prognosis and behaviour are similar to papillary SCC and invasive SCC.[39,54]

LYMPHOEPITHELIOMA LIKE CARCINOMA

This variant is typically well circumscribed and are composed of undifferentiated squamous cells arranged in ill-defined islands. Surrounding stroma shows dense lymphocytic inflammatory infiltration. The cells often show syncytial appearance. This variant has a better prognosis compared to the typical SCC. Epstein Barr Virus is believed to be a causative factor for this variant of SCC. HPV may also play a possible role in the pathogenesis.[39,54]

ADENOCARCINOMA

Adenocarcinoma comprises 10-25% of cervical carcinomas.[37] It is associated with long term use of oral contraceptives. HR-HPV is associated with 94% of adenocarcinoma. Most common being HPV-18. Abnormal uterine bleeding with a mass is the most common presentation seen in 75% of the patients. Some women may present with vaginal discharge.[39,54]

MACROSCOPY

Exophytic fungating or polypoidal growth is seen in more than 50% of the cases. Other cases show nodular or diffuse infiltrative growth. In 15% of the cases, gross lesion may not be visualised.[29,39,54]

MICROSCOPY

ENDOCERVICAL ADENOCARCINOMA, USUAL TYPE

This variant is the most common and constitutes 90% of all cervical adenocarcinoma. The tumor shows well to moderately differentiated cells arranged in complex glandular pattern. The cells are round to oval, lack mucin secretion and show characteristic pseudostratification. The nuclei are elongated, hyperchromatic and show prominent micronucleoli. Floating mitotic figures are seen.[29,39,54]

MUCINOUS CARCINOMA

This variant shows mucinous differentiation and is subdivided into gastric type, intestinal type and signet ring cell type.[29,39,54]

VILLOGLANDULAR CARCINOMA

This variant shows villous-papillary fronds lined by endocervical type columnar cells showing mild to moderate atypia and lacks mucin. Mitotic figures and pseudostratification are often seen.[29,39,54]

ENDOMETRIOID CARCINOMA

Endometrioid carcinoma of the cervix is by definition composed of tumor cells that resemble those of uterine primary adenocarcinomas. The cells of endometrioid carcinomas of the cervix tend to show stratification and have round to oval nuclei aligned perpendicular to the basement membrane of the endocervical gland. The tumor cells lack mucin and have scantier cytoplasm compared to usual type endocervical adenocarcinomas. Small foci of squamous epithelium are frequently encountered. It is crucial to differentiate this variant from cervical extension of a primary uterine endometrial adenocarcinoma. Primary uterine endometrial adenocarcinomas are bulky that show myometrial invasion before cervical extension is noted. However, primary cervical adenocarcinomas show cervical enlargement in the absence of uterine enlargement. It can be differentiated from endometrial carcinoma by using p16.[29,39,54]

CLEAR CELL CARCINOMA

This variant makes up for 4% of cervical carcinomas and is associated with in utero diethylstilbestrol (DES) exposure. It may also occur in women not exposed to DES. The microscopic features are made up of three basic pattern i.e. solid, tubulocystic, and papillary. The tumor cells have abundant clear to granular eosinophilic cytoplasm. Cytoplasmic clearing is due to glycogen accumulation. The nuclei are highly pleomorphic, hyperchromatic and project into the lumen giving the cells a hobnail appearance. The differential diagnosis for clear cell carcinoma of cervix include Arias-Stella reaction and microglandular hyperplasia. Arias-Stella reaction, which is associated with pregnancy, lack mitotic figures and the classic

patterns seen in clear cell carcinoma. Microglandular hyperplasia is commonly seen in the women of reproductive age, whereas clear cell carcinomas commonly occur in older women.[29,39,54]

SEROUS CARCINOMA

Serous carcinoma is a rare variant of cervical adenocarcinoma that is similar to its counterpart seen in the endometrium. Primary cervical serous carcinoma is a diagnosis of exclusion after extension from other gynaecological sites is ruled out. This variant shows cells arranged in complex papillary pattern. These cells show high grade nuclear atypia. Also seen are psammoma body formation.[29,39,54]

MESONEPHRIC CARCINOMA

This variant shows tubular glands lined by cuboidal epithelium lacking mucin and containing hyaline secretion in their lumina. Other patterns seen are retiform, solid, branching, papillary, spindle cell and ductal pattern. The cells show variable atypia and increased mitotic activity.[29,39,54]

ADENOCARCINOMA ADMIXED WITH NEUROENDOCRINE CARCINOMA

This rare variant shows cervical adenocarcinoma with neuroendocrine differentiation.[29,39,54]

OTHER EPITHELIAL TUMORS

ADENOSQUAMOUS CARCINOMA

Adenosquamous carcinoma is defined as malignant epithelial tumor composed of glandular cells and malignant squamous cells. It can occur in young as well as old women. The squamous component shows well differentiated squamous cells showing keratin pearls or individual cell keratinisation. Sufficient glandular differentiation of the adenocarcinoma component should be seen to make the diagnosis of adenosquamous carcinoma.[29,39,54]

GLASSY CELL CARCINOMA

This tumor is a poorly differentiated adenosquamous carcinoma and accounts for 1% of cervical carcinomas. The cells are characteristically uniform, large and polygonal with fine ground glass-type of cytoplasm. The cells have a well-defined cell membrane and prominent nucleoli. The ground glass cytoplasmic appearance is due to abundant intracytoplasmic filaments and dilated rough endoplasmic reticulum. Numerous mitotic figures are seen. Dense lymphoplasmacytic inflammatory infiltration is seen in the stroma. This variant has an extremely aggressive clinical course and is associated with a poor response to radiotherapy.[29,39,54]

ADENOID CYSTIC CARCINOMA

Adenoid cystic carcinomas make up less than 1% of cervical adenocarcinomas. Grossly, these tumors present as hard masses that may be ulcerative or friable. Microscopically, it is similar to the counterpart seen in salivary glands. The tumor is composed of small basaloid cells with scant cytoplasm arranged in nests. The

characteristic sieve-like cribriform appearance is due to hyaline globules, acini formation and cysts. Peripheral palisading and lymphovascular invasion are frequently seen. This variant has an aggressive behaviour as it is often associated with local recurrences and metastatic spread.[29,39,54]

ADENOID BASAL CARCINOMA

Adenoid basal carcinoma is usually asymptomatic and is an incidental finding in the colposcopic biopsy done for a co-existing squamous intraepithelial lesion. Grossly, no mass or growth is usually identified. Microscopically, this tumor is made up of small basaloid cells arranged in lobular nests and cords. The periphery of the nests show nuclear palisading. The central area of the nest may show cystic space filled with necrotic debris or may show squamoid or glandular differentiation. Adenoid basal carcinomas can usually be differentiated from adenoid cystic carcinomas by the lack of hyaline globules and scant mitotic figures.[29,39,54]

UNDIFFERENTIATED CARCINOMA

This carcinoma shows tumor cells arranged in sheets and lacking squamous and glandular differentiation.[29,39,54]

NEUROENDOCRINE TUMORS

LOW GRADE NEUROENDOCRINE TUMOR

This group of tumors exhibit neuroendocrine differentiation and includes carcinoid tumor and atypical carcinoid tumor.[29,39,54]

- **CARCINOID TUMOR**

  This grade 1 low grade neuroendocrine tumor is characterised by organoid growth pattern. The cells show granular "salt and pepper" chromatin and abundant eosinophilic cytoplasm.[29,39,54]

- **ATYPICAL CARCINOID TUMOR**

  This grade 2 neuroendocrine tumor shows greater degree of nuclear atypia and increased mitotic activity. Areas of necrosis can also be seen.[29,39,54]

HIGH GRADE NEUROENDOCRINE TUMOR

This group of neuroendocrine tumor includes small cell neuroendocrine carcinoma and large cell neuroendocrine carcinoma.[29,39,54]

- **SMALL CELL NEUROENDOCRINE CARCINOMA**

  This tumor is composed of monotonous small cells with hyperchromatic nuclei showing characteristic nuclear moulding and scant cytoplasm. Abundant mitotic figures and apoptotic bodies are seen. Extensive areas of necrosis, Lymphovascular invasion and perineural invasion are also frequently noted.[29,39,54]

- **LARGE CELL NEUROENDOCRINE CARCINOMA**

  This tumor is composed of large cells with abundant cytoplasm, round to ovoid nuclei showing prominent nucleoli. These cells are arranged in diffuse, trabeculae, cords and in organoid pattern. Increased mitotic count is usually seen.[29,39,54]

Table 1: TNM AND FIGO STAGING OF CERVICAL CARCINOMA[39,62]

TNM	FIGO	
T - Primary Tumor		
Tx		Primary tumour is not assessable.
T_0		No evidence of primary tumour.
Tis		Pre-invasive carcinoma (Carcinoma in situ).
T_1	**I**	**Tumour limited to the cervix (extension to corpora uteri should be not considered).**
T_{1a}	**IA**	Invasive carcinoma diagnosable only on microscopy. Stromal invasion extending to a maximum depth of 5.0 mm and a horizontally spreading to a maximum of 7.0 mm or less.
T_{1a1}	**IA1**	Depth of invasion is 3.0 mm or less and horizontal spread is 7.0 mm or less.
T_{1a2}	**IA2**	Depth of invasion more than 3.0 mm but not extending more than 5.0 mm with a horizontal spread not exceeding 7.0 mm.
T_{1b}	**IB**	Grossly visible lesion limited to the cervix or microscopic lesion greater than T1a/Stage IA2.
T_{1b1}	**IB1**	Grossly visible lesion not exceeding 4.0 cm in greatest dimension.
T_{1b2}	**IB2**	Grossly visible lesion exceeding 4.0 cm in greatest dimension.
T_2	**II**	**Tumour invades beyond uterus but not extending to the pelvic wall and/or to lower third of vagina**
T_{2a}	**IIA**	Tumour with no parametrial invasion.
T_{2a1}	**IIA1**	Grossly visible lesion not exceeding 4.0 cm in greatest dimension.

sT_{2a2}	**IIA2**	Grossly visible lesion exceeding 4.0 cm in greatest dimension.
T_{2b}	**IIB**	Tumour with parametrial invasion.
T_3	**III**	**Tumour extends upto pelvic wall, extends upto lower one third of vagina, and causes hydronephrosis and/or non-functioning kidney.**
T_{3a}	**IIIA**	Tumour extends upto lower one-third of vagina.
T_{3b}	**IIIB**	Tumour extends upto pelvic wall, causes hydronephrosis and/or non-functioning kidney.
T_4	**IV**	**Tumour invades bladder mucosa or rectal mucosa or extends beyond the confines of true pelvis.**
N – Regional Lymph Node		
N_x		Regional lymph nodes is not assessable.
N_0		No metastasis to regional lymph node.
N_1		Metastasis to the regional lymph node present
M – Distant Metastasis		
M_0		No distant metastasis
M_1		Distant metastasis present.

PROGNOSTIC FACTORS:

The most important factor influencing the prognosis in cervical carcinoma is stage. Histologic grading and typing have very little direct prognostic influence on survival in any stage. The other important pathologic prognostic factors include tumor size in the greatest dimension, depth of invasion, parametrial involvement, lymphovascular invasion and lymph node status.[54]

CERVICAL CANCER STEM CELLS (CCSC)

Cancer stem cells (CSC) are hypothesised to be a small population of tumor cells residing in the niches within the tumor.[13,14] These CSC have tumorigenic properties, multilineage differentiation potential and self-renewal capacity.[13,14,63-65]

They are characterised by their capacity to undergo asymmetrical cell division giving rise to two distinct and different daughter cells. One daughter cell mimics the parent CSC and the other daughter cell has only few features of CSC. Asymmetrical cell division and the property of self-renewal helps CSC to maintain homeostasis within the tumor population.[14,66] As a result, CSC are believed to have tumor initiating properties and are hypothesised to play a very essential role in cancer relapse and distant metastasis.[14,67,68] Thus, CSC are now becoming promising targets for cancer treatment and prevention of recurrence.[14]

CCSC are thought to exist in the niche in the SCJ of the cervix which when infected by HR-HPV undergo malignant transformation. The niche is a conducive microenvironment for CSC to reach optimal balance between activation, renewal and differentiation. Arguments favouring this hypothesis are the following: Firstly, stem

cells have the required mechanisms for self-renewal inherently activated. Secondly, unlike normal mature cells in tissues with a high turnover, stem cells persist for longer periods of time. This means that there is a greater chances for mutations to accumulate in stem cells than in mature cell types. Therefore, probably the target cell for high risk HPV infection is the stem cell of the uterine cervical epithelium. The CCSC markers that are currently studied are ABCG2 (ATP-binding cassette sub-family G member 2), ALDH1 (Aldehyde dehydrogenase 1), CD133, CD49f, OCT4, OPN (Osteopontin), SOX2, CD44, C-KIT, and NANOG.[14] In the current study we use CD44 as a CSC marker.

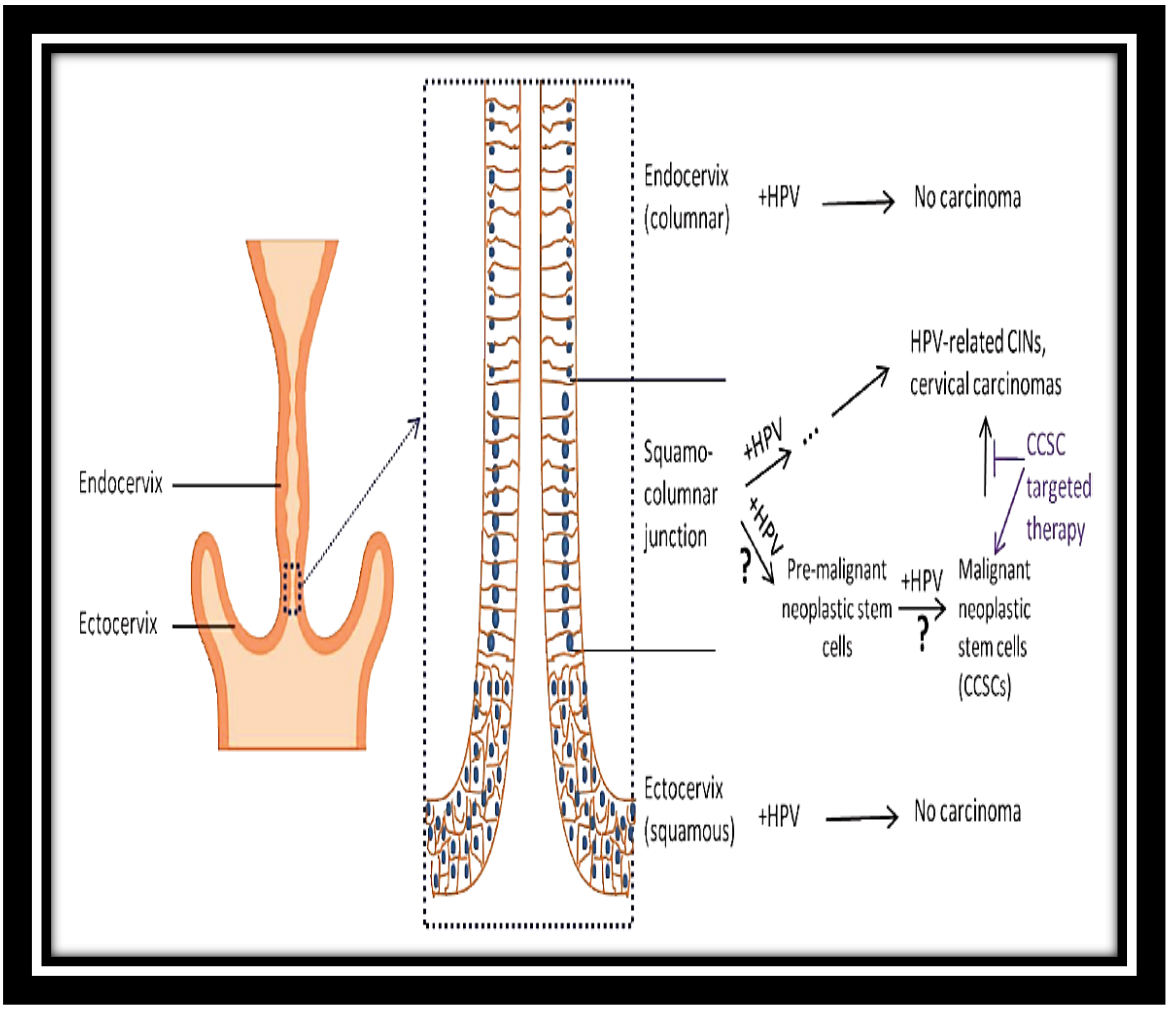


Figure 3: CCSC and cervical carcinogenesis. HPV related carcinogenesis occurs specifically in the cells located SCJ. The HPV infected cells produce

carcinomatous clones and propagate the CCSC. Targeting the CCSC may help to prevent the propagation of HPV related CIN and carcinoma.[14]

CD44

Human cluster of differentiation 44 (CD44) a transmembrane glycoprotein that binds to hyaluronic acid (HA). CD44 has four functional domains that regulate a number of cellular functions like cell-matrix adhesion, proliferation of cells, signal transduction pathway, migration, and apoptosis.[67,68] This implies that a disturbance in the function of CD44 and its expression plays a significant role in the behaviour of a malignant tumor.[10] In recent years, CD44 has been recognised as a biomarker of CSC in acute myeloid leukemia, colorectal carcinoma, gastric carcinoma, hepatocellular carcinoma, non-small cell lung carcinoma, melanoma, multiple myeloma, nodular sclerosing hodgkins lymphoma, esophageal SCC, oral SCC, pancreatic adenocarcinoma, thyroid carcinoma, ovarian carcinoma, urothelial carcinoma and breast carcinoma.[69,71-73] CD44 is associated with poor prognosis in cervical carcinoma.[19,71,73]

The physiological function of CD44 in normal cervical epithelium is not fully understood. Several authors report numerous dynamic changes in the expression of CD44 during carcinogenesis and metastasis.[19,74-78] In normal cervical epithelium, CD44 expression is localised in the basal and parabasal cell layers. In malignant cervical epithelium various authors have reported expression of CD44 in the superficial layer as well which also coincides with distant metastasis and the presence of an aggressive disease process.[19,79-81]

Ayhan et al studied the expression of CD44 in FIGO Stage IB cervical carcinoma and found that there was marked expression of CD44 (50%) in the superficial layer of the malignant cervical epithelium.[19] Kainz et al in a univariate analysis of FIGO stage IB to stage III cervical carcinoma found that overexpression of CD44 was associated with poor prognosis and pelvic nodal metastasis.[19,83-85] In a similar study Speiser et al. reported overexpression of CD44 in 200 early-stage cervical cancers was associated with a poor prognosis. Five-year survival rates of 62% and 84% were seen in patients with or without CD44 overexpression, respectively.[19,86]

p16

p16 a protein that has a cell cycle regulatory function and causes tumor suppression in cells with intact cell cycle. It inhibits the cyclin dependent kinase 4 (cdk-4), therefore inactivating pRb. Thus inhibiting the progression of cell cycle at the G1-S checkpoint. Thereby pausing the cell cycle at the checkpoint before entering into the S1 phase.[87] E7 oncoprotein found in HR-HPV functionally inactivates Rb protein. This causes uninhibited activity of cdk-4 that promotes the S phase in the cell. This leads to over expression of p16.[4,87] Therefore, p16 is a biomarker for HR-HPV infection and a surrogate marker for the detection of E7 induced inactivation of Rb protein. In other words, p16 overexpression is suggestive of immortalisation of epithelial cells into cancer cells.[4,87,88]

Only HR-HPV has the integrative ability with the basal and parabasal stem cell genome. Thus, immunohistochemical staining pattern of p16 in HR-HPV infected epithelium is strong and diffuse (block positivity) staining in nuclei as well as in cytoplasm. Contrastingly, the immunostaining pattern in low risk HPV subtypes is weak and focal staining of nuclei and/or cytoplasm limited to the cells in the

superficial layers. p16 is also expressed in normal tissues such as the normal endometrium in proliferative phase, oesophageal squamous cells, breast ductal cells, gastric glands.[87,89]

Ki-67

Ki-67 protein is a cellular proliferative marker. It is expressed in all active phases of the cell cycle with maximum positivity during mitosis. However, it is absent in the resting phase i.e. G0.[4,88] Ki-67 is highly valuable in determining the tumour grade, degree of proliferation and therefore the prognosis.[89-92] It is the gold standard for assessing the proliferative index of a tumor.[4,93,94] Its expression is the normal cervical epithelial lining is limited to the basal and parabasal layers.[90,95] Ki-67 shows nuclear staining on immunohistochemistry. Increased Ki-67 expression and index is noted in HR-HPV infected cells reflecting the increased cell cycle dynamics resulting from the immortalization of the normal cervical cells into cancer cells.[4,93,94] In CIN, Ki-67 expression is associated with the degree of dysplasia and grade of carcinoma. It is therefore indispensable in terms of prognosis and utility in patient follow up.[4,94,96-98]

MATERIALS AND METHODS

MATERIALS AND METHODS

STUDY DESIGN

Case control study

STUDY PERIOD

July 2016 to June 2018

STUDY PLACE

Department of Pathology, Sri Devaraj Urs Medical College attached to R.L. Jalappa Hospital and Research Centre, Tamaka, Kolar.

SAMPLE SIZE

Sample size was estimated based on the difference in proportion of p16 gene among normal subjects and CIN 2 (this difference in proportion gave highest sample size among all the difference in proportions).

$$\text{Sample size} = \frac{r+1}{r} \frac{(p^*)(1-p^*)\left(Z_\beta + Z_{\frac{\alpha}{2}}\right)^2}{(p_1 - p_2)^2}$$

r is ratio of control to cases where 1 represents equal number of case and control.

p* is the average proportion exposed i.e. (proportion of exposed cases + proportion of control exposed)/2.

Z_β is the standard normal variate for power. For eg, for 80% power Z_β is 0.84, for 90% power Z_β value is 1.28. power is decided by the researcher.

$Z_{\frac{\alpha}{2}}$ is standard normal variate for level of significance.

p1 – p2 is the difference in proportion which is expected based on the results of the previous studies.

p1 = proportion in cases

p2 = proportion in control.

p16 in normal subjects at a proportion of 2% and in CIN II at a proportion of 91% using the above formula maximum sample size of 11 subjects in each group was obtained at 90% power and 1% alpha error.[90] With 20% as non-response rate and considering the design effect of 2, a sample size of 26 subjects was obtained in each group. Hence 26 subjects were included in three groups i.e. Normal - 26, HSIL - 26, and Carcinoma Cervix - 26. Total sample size was 26 x 3 = 78 subjects.

INCLUSION CRITERIA

Hysterectomy and cervical biopsy specimens.

EXCLUSION CRITERIA

1. Cases that have undergone preoperative chemotherapy or radiotherapy.
2. Cases with malignancies other than Squamous cell carcinoma cervix.
3. Cases with LSIL (CIN 1).

METHOD

1. Cervical biopsy and hysterectomy cases received between July 2016 to June 2018 were retrieved from the records of Department of Pathology, Sri Devaraj Urs Medical College with histomorphological features of normal cervix, HSIL and Cervical carcinoma. 26 cases each were included in the study.

2. Details of the patient such as the age, presenting symptoms, colposcopic findings, radiological staging, lymph node involvement, parametrial involvement and size of the tumor were collected. All slides were reviewed and designated to their respective group depending on their histomorphological features.
3. Cervical SCC cases were classified as;

   Keratinising SCC – WD-SCC

   Non-keratinising large cell SCC – MD-SCC

   Non-keratinising small cell SCC – PD-SCC[57,58]
4. Additional sections of 4um thickness were cut from these paraffin blocks and subjected to staining with p16, Ki-67 and CD44 according to the standard protocol markers using appropriate positive and negative controls.

   The details of the immunohistochemical markers used in the study are as follows:

Table 2: Antibodies used for Immunohistochemistry.

Antigen	Clone	Species	Producer	Dilution	Control	Stain
Anti-p16[INK4]	Monoclonal	Mouse	Biogenex	Ready to use	SCC	Nuclear stain
Anti-Ki67	Monoclonal	Mouse	Biogenex	Ready to use	Breast	Nuclear stain
CD44	Monoclonal	Mouse	Biogenex	Ready to use	Breast	Cytoplasmic membrane stain

IMMUNOHISTOCHEMISTRY PROCEDURE

1. 3-4mm thick tissue sections were cut and floated on to organosialine coated slide and left on hot plate at 60ºC overnight.
2. Deparaffinization was done using Xylene I and II for 15 min each
3. Dexylenisation was done using absolute alcohol I and II for 1 min each
4. Dealcoholisation was done using 90% and 70% alcohol for 1 min each
5. Hydration was done using tap water for 10 min.
6. Distilled water rinsing was done for 5 min.
7. Antigen Retrieval technique was done using the Heat Induced Epitope Retrieval (HIER) technique. Sections were microwaved at power 10 for 6 minutes in TRIS EDTA buffer at pH 9.0 for 4 cycles.
8. The sections were then washed with Tris buffer solution (TBS) at pH 7.4 for 3 cycles of 5 minutes each.
9. Peroxidase block was done for 30 minutes in dark to block the endogenous peroxidase enzyme.
10. TBS washing was done for 3 cycles of 5 minutes each.
11. Power block was used on the sections for 10 minutes. The sections were not washed by TBS in this step.
12. Sections were covered with primary antibody for 60 minutes.
13. TBS buffer washing was done for 3 cycles of 5 minutes each.
14. Sectioned were covered with Super sensitive poly horse radish peroxidase (secondary antibody) for 30 min.
15. TBS buffer washing was done for 3 cycles of 5 minutes each.
16. SuperEnhancer was used on the sections for 30 minutes.

17. Colour development was done with working colour development solution Di-Amino Benzidine (DAB) for 15 minutes.
18. Distilled water washing was done for 3 cycles of 5 minutes each.
19. The sections were counter stained with Harris haematoxylin for 1 min.
20. The sections were dehydrated, cleared and mounted with DPX.

IMMUNOHISTOCHEMICAL ANALYSIS

Sections were first examined at low magnification (40x magnification) using Olympus CX 21i microscope to identify areas of highest positivity (hotspot). Areas of hotspots were used for interpreting the immunohistochemical staining.

IMMUNOHISTOCHEMICAL INTERPRETATION

CD44

CD44 shows brown cytoplasmic membrane positivity. Two features of immunohistochemical reactions were assessed separately on a semi-quantitative basis (H score) as follows:

1. The staining extent was expressed as the percentage of positively stained cells in 10 high power fields in the hotspot areas in each case. The means of the percentages were calculated and scored as 0% positive cells or positive cells located in the basal layer (0), 1 - 10% positive cells (1), 11% - 40% positive cells (2), 41% - 75% positive cells (3), and ≥76% positive cells (4).

2. The staining intensity was subjectively scored as mild or weak (1), moderate (2), and strong (3).

3. The final score was expressed as a product of the two scores as

- 0 - 1 point – negative (-)

- 2 - 3 points - weakly positive (+)
- 4 - 7 points - positive (++)
- ≥8 points - strongly positive (+++).[99,100]

p16

p16 shows nuclear positivity and the staining was interpreted based on four parameters such as intensity, extent, continuity and location. Intensity was taken as either strong (dark brown) or weak (yellow). Extent was divided into diffuse (expression in more than 50% of the epithelium) and focal (expression in less than 50% of the epithelium). Continuity was either continuous (staining extends laterally over a significant distance) or discontinuous (alternating clusters stained cells). Lastly, location was divided into positive cells seen in the lower one third, two thirds, or entire thickness of epithelium.[16,34,101]

Based on these parameters, the lesions were then categorized into block-positive, negative, and ambiguous. The lesion was taken as block-positive when it fulfilled all criterias as described in LAST; showing strong and diffuse immunoreactivity extending upwards from the basal layers, involving more than one third of the epithelium and showing a continuous involvement. Negative immunostaining was defined as complete absence of staining or weak, focal, and/or discontinuous staining. Cases that did not meet the criteria of block-positive and negative immunostaining were labelled as ambiguous.[16,34,101]

Ki-67

Ki-67 positivity is seen as a brown nuclear positivity. It is interpreted semi-quantitively as H-Score similar to CD44.

1. The staining extent was expressed as the percentage of positively stained cells in 10 high power fields in the hotspot areas in each case. The means of the percentages were calculated and scored as 0% positive cells (0), 1 - 10% positive cells (1), 11% - 40% positive cells (2), 41% - 75% positive cells (3), and ≥76% positive cells (4).

2. The staining intensity was subjectively scored as mild or weak (1), moderate (2), and strong (3).

3. The final score was expressed as a product of the two scores as

- 0 - 1 point – negative (-)
- 2 - 3 points - weakly positive (+)
- 4 - 7 points - positive (++)
- ≥8 points - strongly positive (+++).[102]

RESULTS

&

STATISTICAL ANALYSIS

STATISTICAL ANALYSIS

Data was entered into Microsoft excel data sheet and was analysed using SPSS 22 version software. Categorical data was projected in the form of Frequencies and proportions. **Chi-square test or Fischer's exact test** (for 2x2 tables only) was used as test of significance for qualitative data. **Yates correction** was applied wherever chi-square rules were not fulfilled (for 2x2 tables only).

Continuous data was represented as 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 variables and qualitative variables respectively.

Graphical representation of data: MS Excel and MS word was used to obtain various types of graphs such as bar diagram, Pie diagram and Scatter plots.

p value (Probability that the result is true) of <0.05 was considered as statistically significant after assuming all the rules of statistical tests.

Statistical software: MS Excel**,** SPSS version 22 **(**IBM SPSS Statistics, Somers NY, USA) was used to analyze data. EPI Info (CDC Atlanta), Open Epi, Med calc and Medley's desktop were used to estimate sample size, odds ratio and reference management in the study.

Paired t test or Wilcoxon Signed rank test is the test of significance for paired data.

ANOVA (Analysis of Variance) or Kruskal Wallis test was the test of significance to identify the mean difference between more than two groups for quantitative and qualitative data respectively.

RESULTS

Seventy-eight specimens which consisted of twenty-six specimens of normal cervix, HSIL and cervical carcinoma each were studied during the period of July 2016 to June 2018 in the Department of Pathology in Sri Devaraj Urs Medical College and R. L. Jalappa Hospital & Research Centre, Tamaka, Kolar.

Immunohistochemistry was done in all 78 cases for p16, Ki-67 and CD44. The following data was recorded and analysed.

1. Age distribution
2. Chief complaints
3. Colposcopic findings
4. FIGO staging
5. Lymph node involvement
6. Size of the tumor
7. Histological grade of the tumor
8. p16 expression
9. Ki-67 expression
10. CD44 expression

Table 3: Age wise distribution of HSIL and carcinoma cervix cases in the study.

AGE GROUP IN YEARS	NUMBER OF HSIL CASES	NUMBER OF CARCINOMA CERVIX CASES
21-30	1 (3.9%)	0 (0.0%)
31-40	5 (19.2%)	3 (11.5%)
41-50	11 (42.3%)	7 (26.9%)
51-60	7 (26.3%)	10 (38.5%)
61-70	1 (3.9%)	6 (23.1%)
>70	1 (3.9%)	0 (0.0%)
TOTAL	**26 (100.0%)**	**26 (100.0%)**

There was an increased incidence of HSIL observed in the age group of 41-50 years (42.3%), followed by 50-60 years (26.8%) and 31-40 years (19.2%).

There was an increased incidence of carcinoma cervix observed in the age group of 51-60 years (38.5%), followed by 41-50 years (26.9%) and 61-70 years (23.1%).

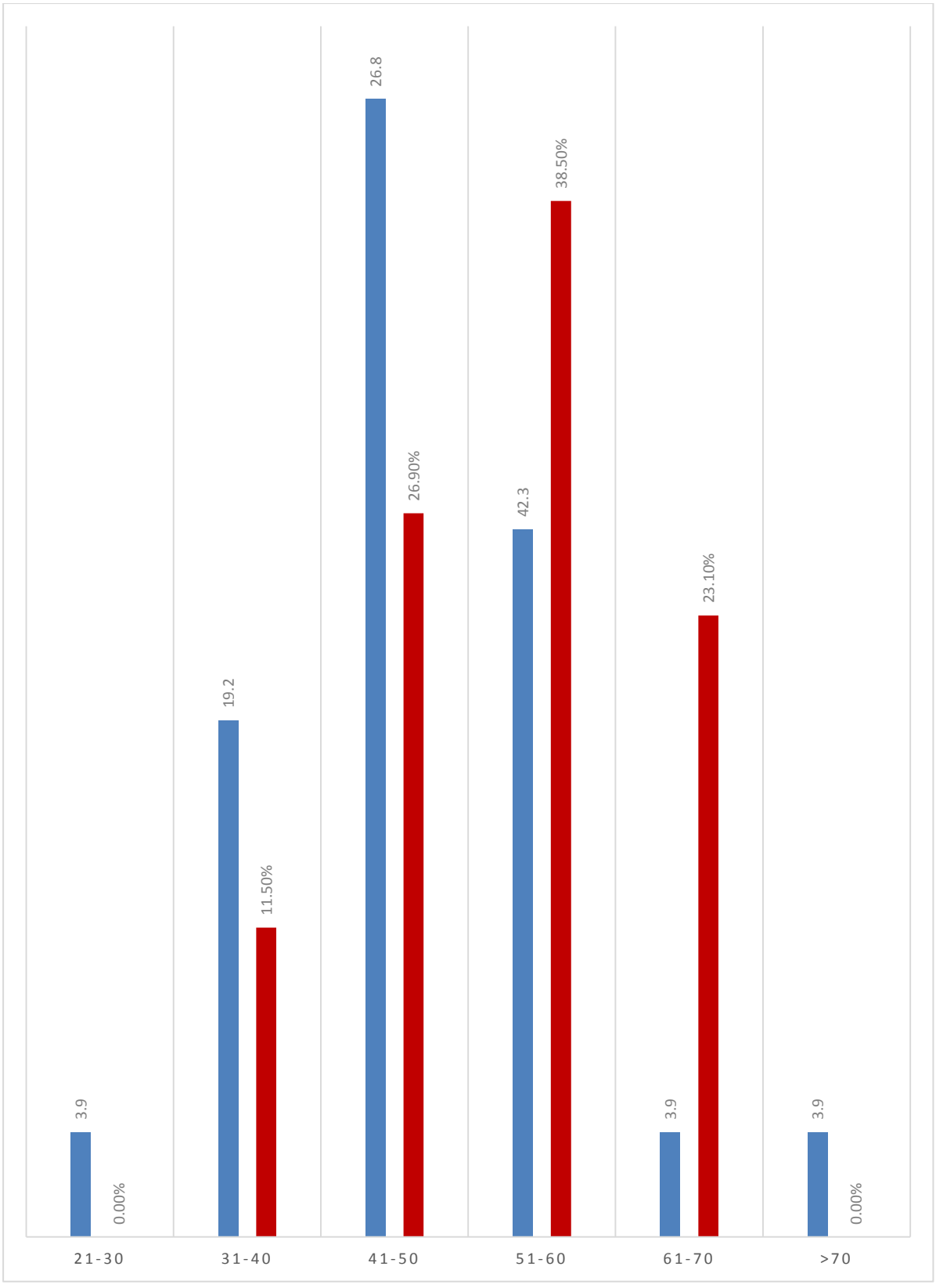


Chart 1: Bar diagram showing Age wise distribution of HSIL and carcinoma cases in the study.

Table 4: Mean age comparison between three groups.

		Age		P value
		Mean	SD	
Group	Normal	42.3	9.3	0.042
	HSIL	47.0	13.4	
	Carcinoma cervix	50.4	10.3	

Mean age of subjects in Carcinoma Cervix group was 50.4 ± 10.3 years, mean age of subjects in HSIL group was 47.0 ± 13.4 years and in normal group was 42.3 ± 9.3 years. The difference in age distribution between normal, HSIL and carcinoma cervix was statistically significant.

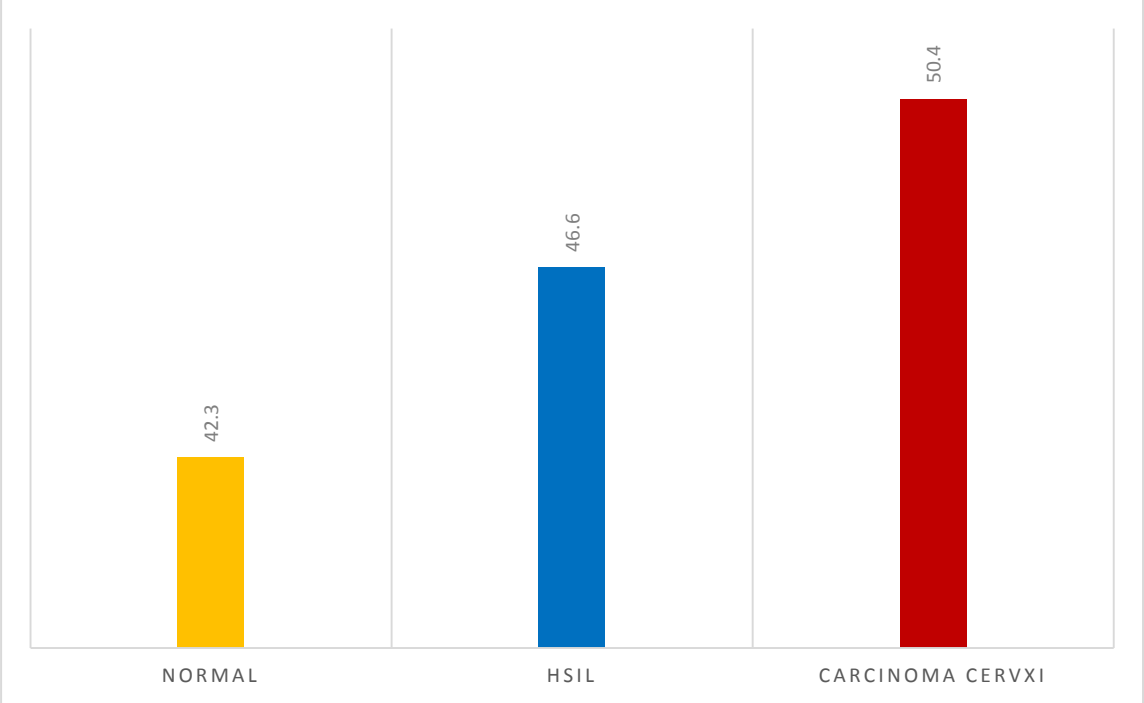
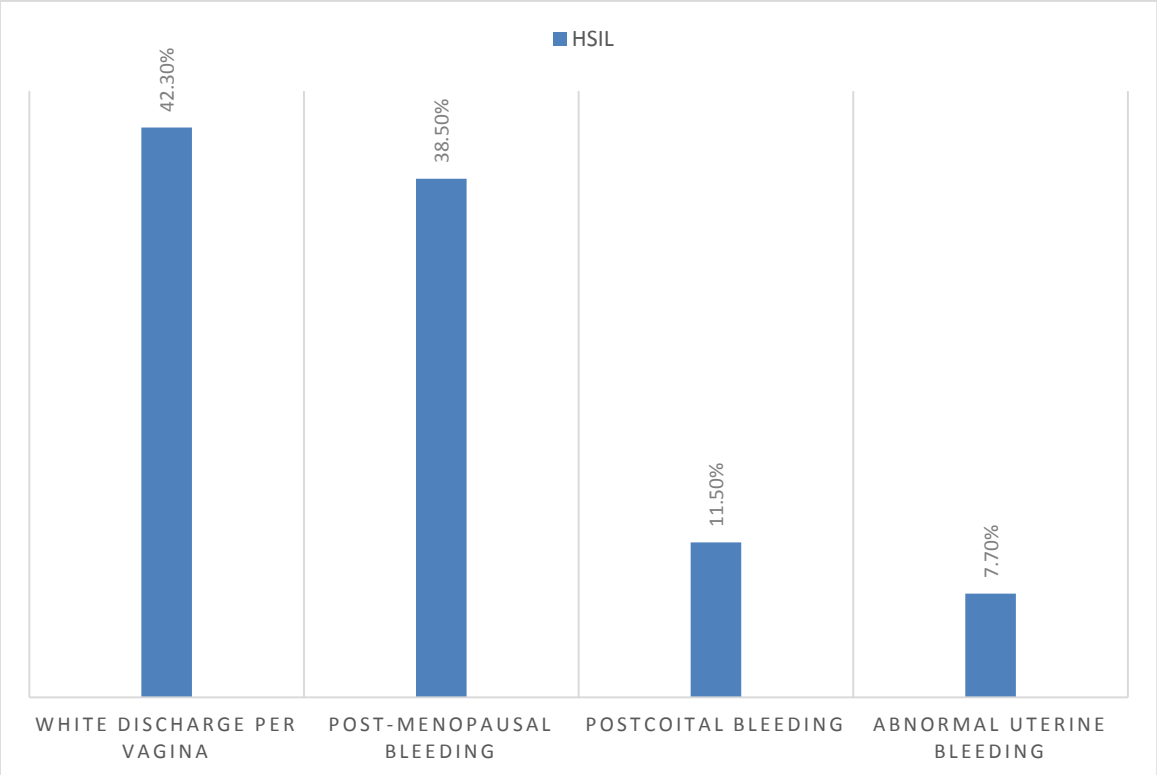


Chart 2: Bar diagram showing Mean age comparison between three groups.

Table 5: Chief complaints encountered in HSIL cases.

HSIL		
	Count (n =26)	**Percentage (%)**
White Discharge Per Vagina	11	42.3%
Post-Menopausal Bleeding	10	38.5%
Postcoital Bleeding	3	11.5%
Abnormal Uterine Bleeding	2	7.7%

In HSIL group, 42.3% had discharge per vagina, 38.5% had post-menopausal bleeding, 11.5% had bleeding per vagina and 7.7% had abnormal uterine bleeding.



In Normal group, 100% had abnormal uterine bleeding.

Chart 3: Bar diagram showing chief complaints encountered in HSIL cases.

Table 6: Chief complaints encountered in carcinoma cervix cases.

Carcinoma cervix		
	Count (n=26)	**Percentage (%)**
-Post-menopausal Spotting	13	50.0%
White Discharge per vagina	8	30.8%
Bleeding Per vagina	4	15.4%
Abnormal Uterine Bleeding	1	3.8%

In Carcinoma Cervix group, 50% had post-menopausal spotting, 30.8% had white discharge per vagina, 15.4% had bleeding per vagina, and 3.8% had abnormal uterine bleeding.

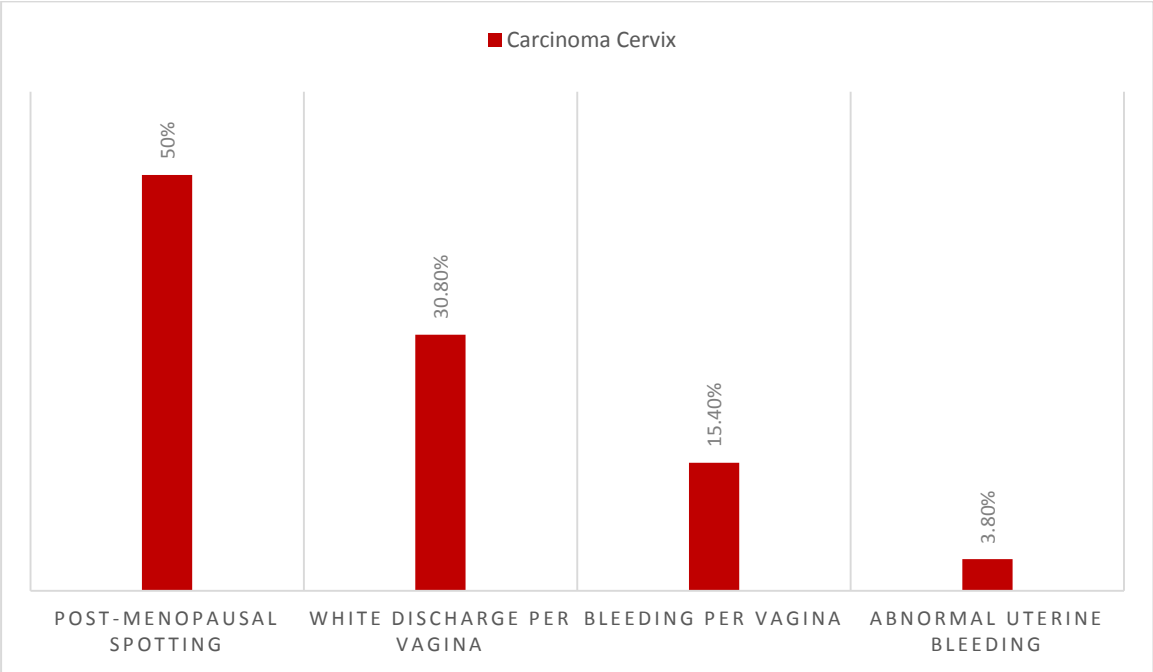


Chart 4: Bar diagram showing chief complaints encountered in Carcinoma cervix.

Table 7: Colposcopy findings encountered in HSIL and carcinoma cervix cases.

COLPOSCOPIC FINDING	NUMBER OF HSIL (%)	NUMBER OF CARCINOMA CERVIX (%)
EROSION	25 (96.2%)	0 (0.0%)
GROWTH	1 (3.8%)	26 (100.0%)
TOTAL	**26 (100.0%)**	**26 (100.0%)**

In HSIL cases, 96.2% had cervical erosion and 3.8% had growth. On colposcopic examination, 100.0% of carcinoma cases had cervical growth. 100.0% of cases in the normal group were unremarkable.

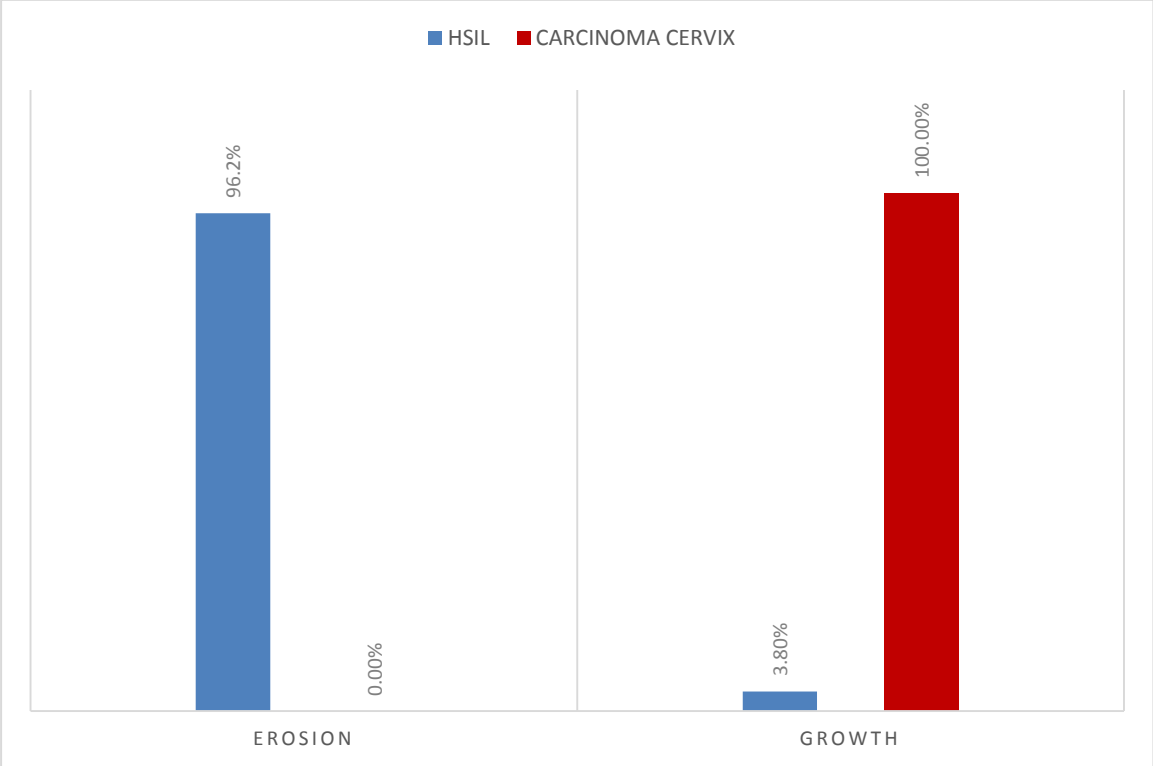


Chart 5: Bar diagram showing colposcopic findings encountered in HSIL and carcinoma cervix cases.

Table 8: Distribution of FIGO Stage of Carcinoma Cervix.

		Carcinoma Cervix	
		Count (n=26)	**Percentage (%)**
FIGO Stage of Carcinoma Cervix	**II A**	8	30.8
	II B	12	46.2
	III A	6	23.0

In Carcinoma cervix cases, the most common stage encountered was Stage IIB which was 46.2%, followed by Stage IIA and Stage IIIA which were 30.8% and 23.0% respectively.

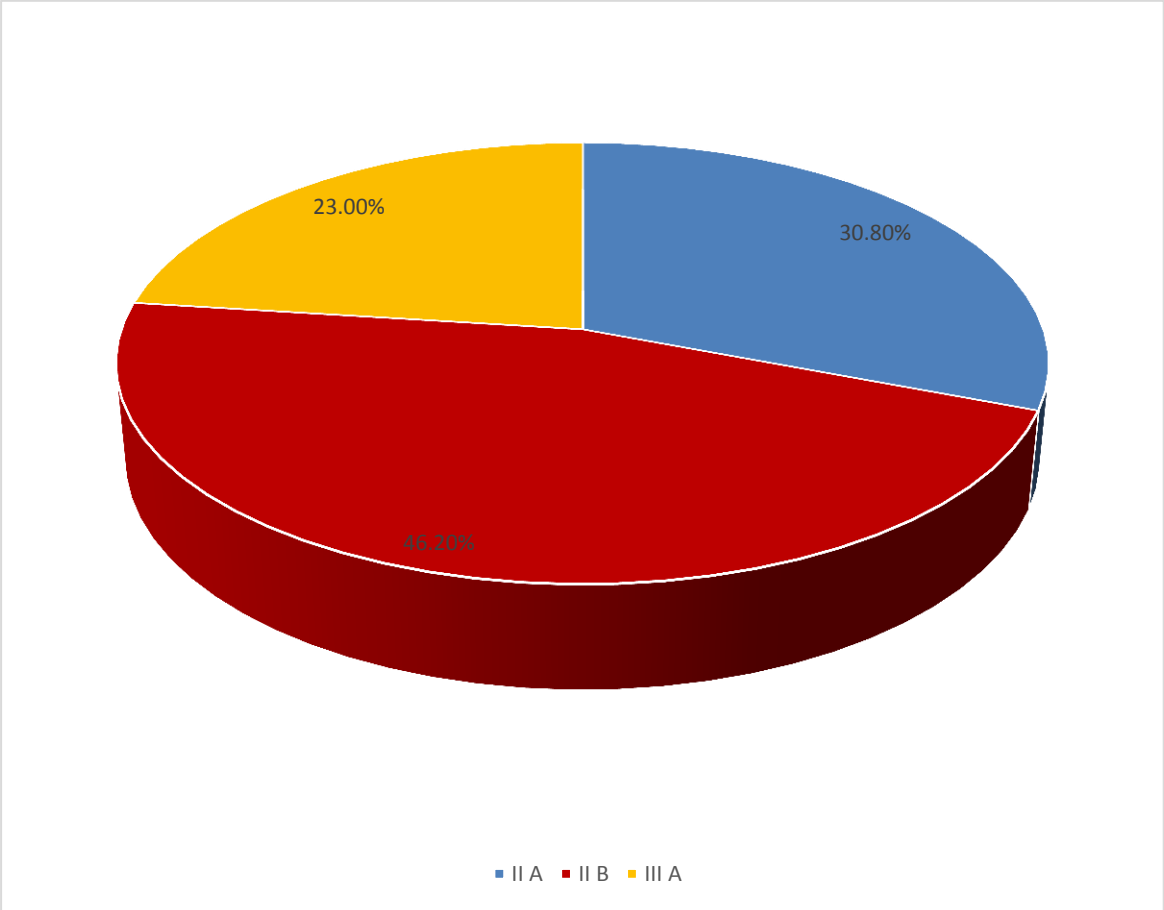


Chart 6: Pie diagram showing distribution FIGO Stage of Carcinoma Cervix.

Table 9: Grade distribution of HSIL cases.

HSIL	Cases (n=26)	Percentage (%)
CIN 2	9	34.6
CIN 3	17	65.4

In HSIL group, 34.6% had CIN 2 and 65.4% had CIN 3.

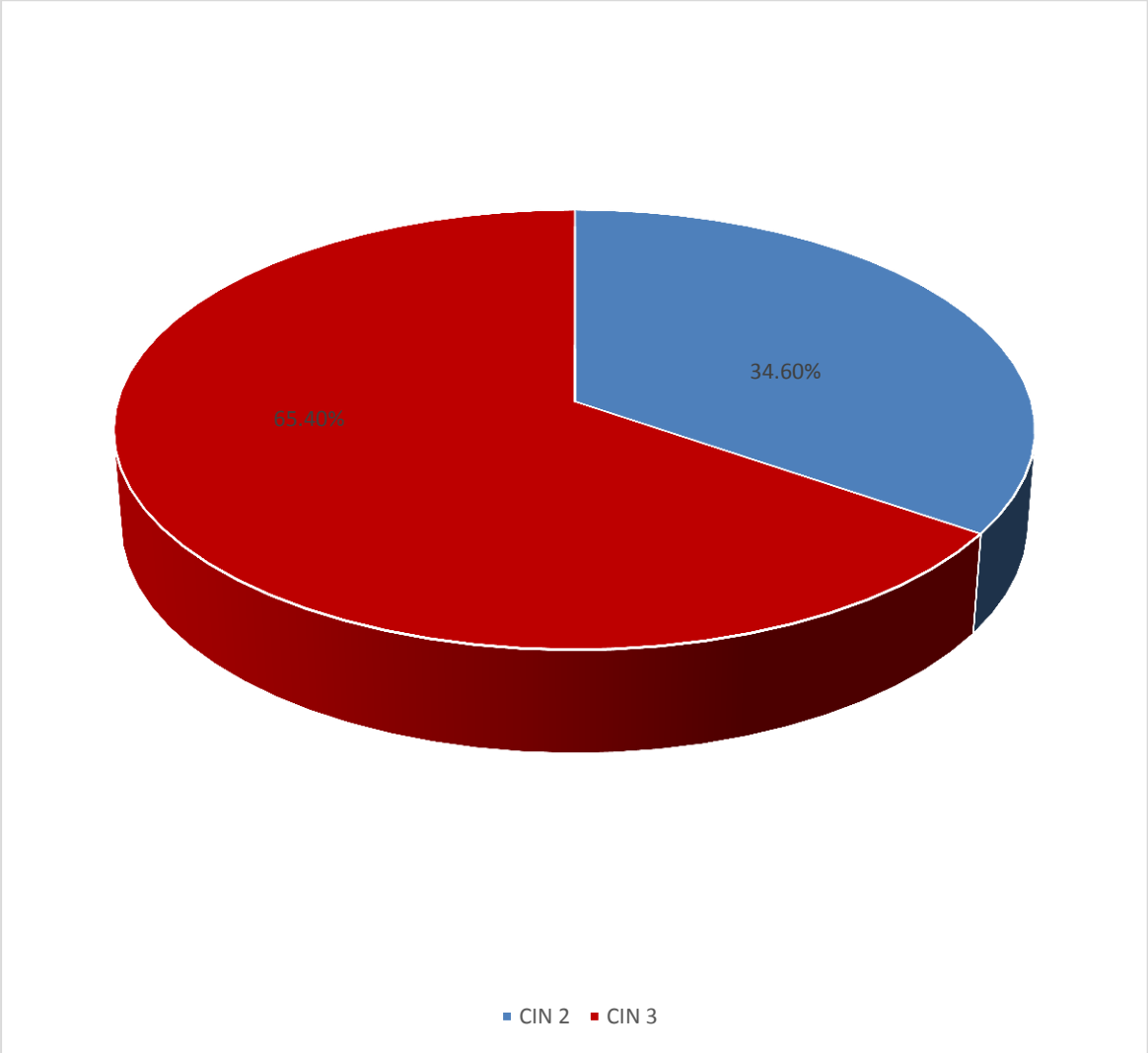


Chart 7: Pie diagram showing grade distribution of HSIL cases.

Table 10: Grade distribution of carcinoma cervix cases.

Grade of SCC	Cases (n=26)	Percentage (%)
Well Differentiated SCC	14	53.8
Moderately Differentiated SCC	8	30.8
Poorly Differentiated SCC	4	15.4

In Ca Cervix group, 53.8% had Well Differentiated SCC, 30.8% had Moderately Differentiated SCC and 15.4% had Poorly Differentiated SCC.

In Normal group, 100% had Chronic Cervicitis.

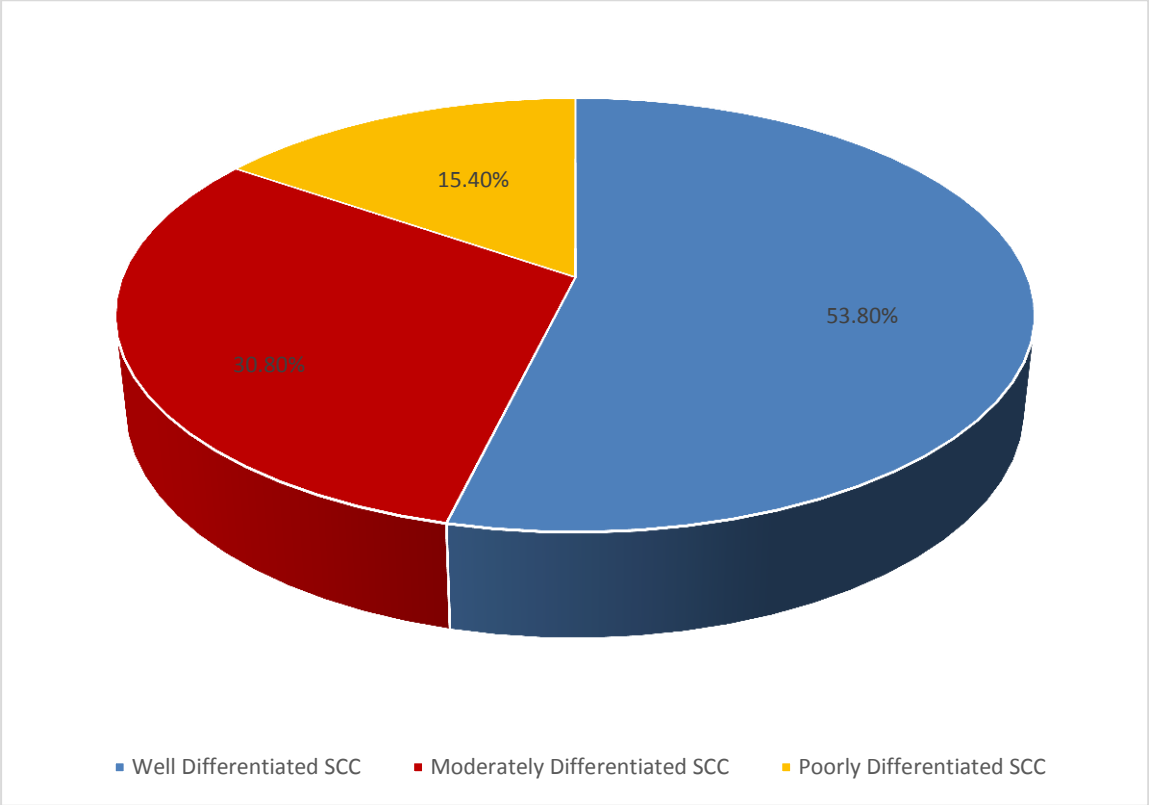


Chart 8: Pie diagram showing grade distribution of carcinoma cervix cases.

Table 11: p16 expression comparison between three groups.

		Group					
		Normal		HSIL		Carcinoma cervix	
		Count (n=26)	Percentage (%)	Count (n=26)	Percentage (%)	Count (n=26)	Percentage (%)
p16	Negative	26	100.0	8	30.8	0	0.0
	Ambiguous	0	0.0	2	7.7	2	7.7
	Positive	0	0.0	16	61.5	24	92.3

$\chi 2 = 55.69$, df = 4, $p < 0.001$

In Carcinoma Cervix group, 92.3% were positive for p16 and 7.7% were ambiguous. In HSIL group, 61.5% were positive for p16, 7.7% were ambiguous for p16 and 30.8% were negative for p16. In Normal group, 100% were negative for p16. There was significant difference in p16 expression between three groups.

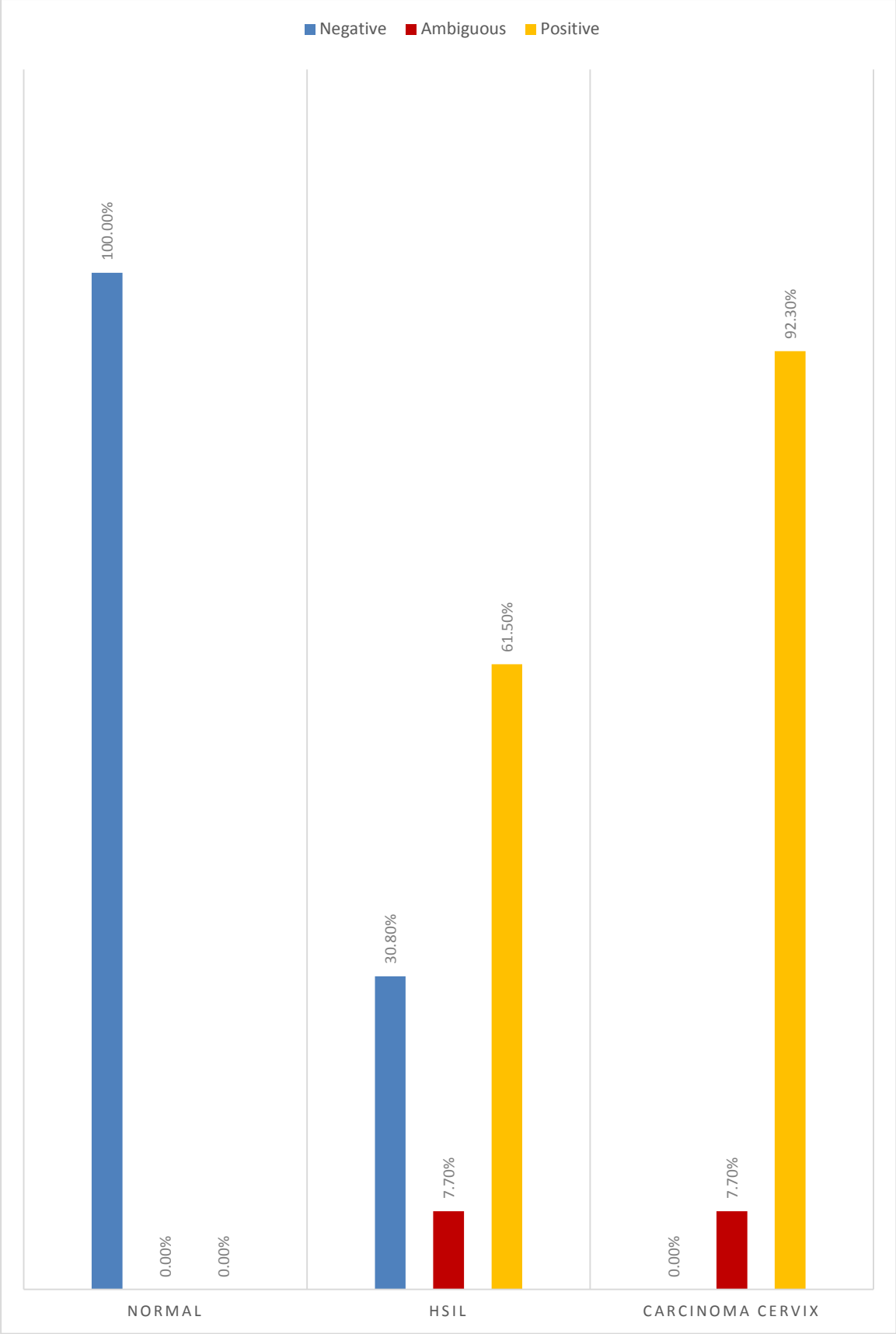


Chart 9: Bar diagram showing p16 expression comparison between three groups.

Table 12: Association between p16 expression and Grade of Carcinoma Cervix.

		Grade of Carcinoma Cervix						P value
		Well Differentiated SCC		Moderately Differentiated SCC		Poorly Differentiated SCC		
		Count (n=14)	Percentage (%)	Count (n=8)	Percentage (%)	Count (n=4)	Percentage (%)	
p16	Ambiguous	2	14.3	0	0.0	0	0.0	0.395
	Positive	12	85.7	8	100.0	4	100.0	

In carcinoma cervix, 85.7% of well differentiated SCC were positive and 14.3% were ambiguous for p16. Whereas, 100.0% of Moderately differentiated SCC and 100.0% of Poorly differentiated SCC were positive for p16. No statistically significant correlation was observed.

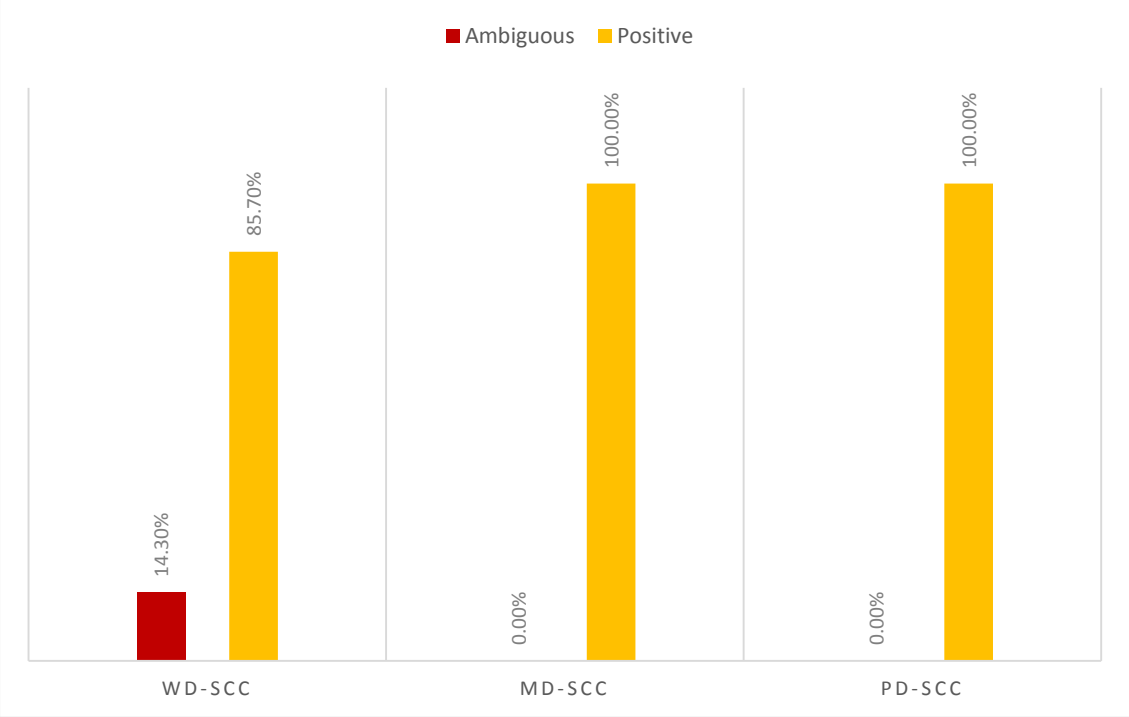


Chart 10: Bar diagram showing association between p16 expression and Grade of Carcinoma Cervix.

Table 13: Association between p16 expression and FIGO stage of Carcinoma Cervix.

		p16						P value
		Negative		Ambiguous		Positive		
		Count (n=0)	Percentage (%)	Count (n=2)	Percentage (%)	Count (n=24)	Percentage (%)	
FIGO Stage	IIA (n=8)	0	0.0%	0	0.0%	8	100.0%	**0.027**
	IIB (n=12)	0	0.0%	0	0.0%	12	100.0%	
	IIIA (n=4)	0	0.0%	2	33.3%	4	66.7%	

In Stage IIA carcinoma, 100.0% were positive for p16. In Stage IIB carcinoma, 100% were positive for p16. In Stage IIIA 66.7% were positive and 33.3% were ambiguous for p16. There was statistically significant association between p16 and FIGO stage of carcinoma cervix.

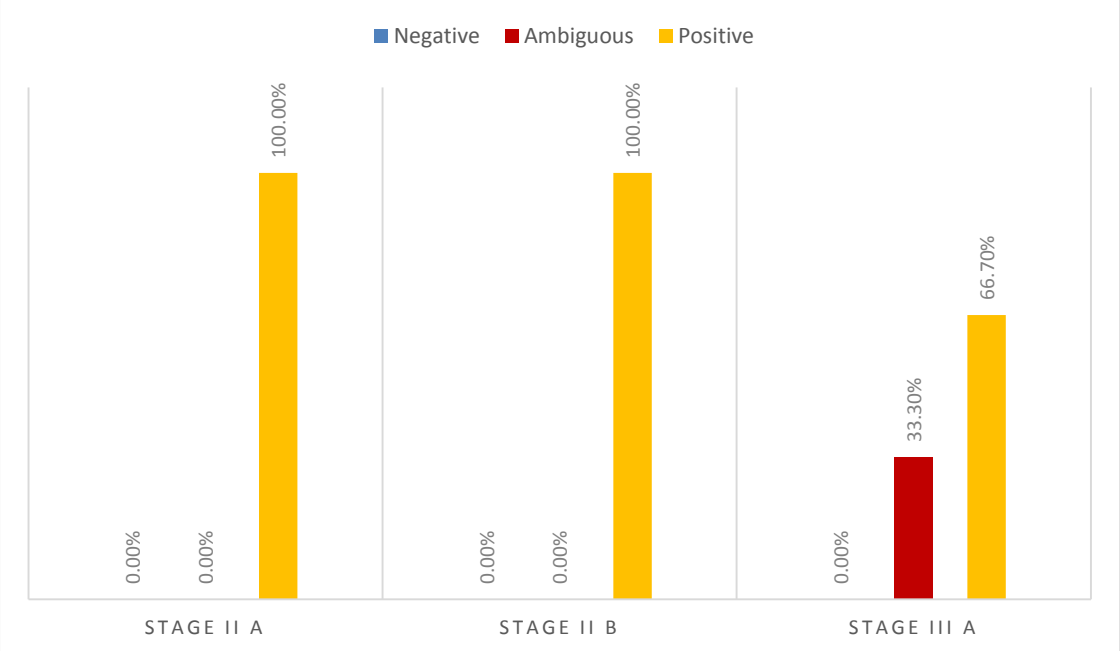


Chart 11: Bar diagram showing association between p16 expression and FIGO stage of Carcinoma Cervix.

Table 14: Association between p16 expression and lymph node status in Carcinoma Cervix.

	Lymph Node Negative		Lymph Node Positive		P value
	Count (n=19)	Percentage (%)	Count (n=7)	Percentage (%)	
Ambiguous (n=2)	2	28.6	0	0.0	**0.015**
Positive (n=24)	5	71.4	19	100.0	

Among the carcinoma cervix cases with lymph node positivity, 100.0% were positive for p16. Among the carcinoma cervix cases with lymph node negativity, 71.4% were positive and 28.6% were ambiguous for p16. There was statistically significant association between p16 and lymph node status of carcinoma cervix.

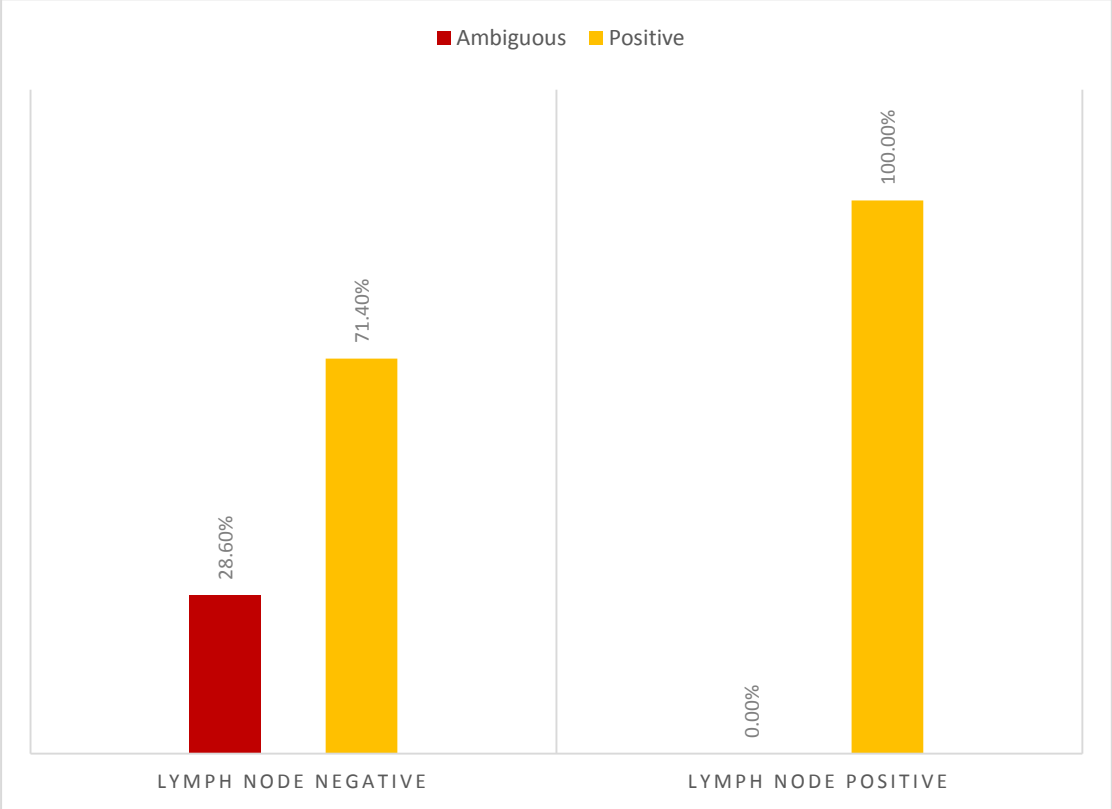


Chart 12: Bar diagram showing association between p16 and lymph node status in Carcinoma Cervix.

Table 15: Association between p16 expression and size of the tumor in Carcinoma Cervix.

	< 3cms		> 3cms		P value
	Count (n=1)	Percentage (%)	Count (n=25)	Percentage (%)	
Ambiguous (n=2)	0	0.0	2	8.0	0.768
Positive (n=24)	1	100.0	23	92.0	

Among the carcinoma cervix cases with tumor size less than 3cms, 100.0% were positive for p16. Among the carcinoma cervix cases with tumor size more than 3cms, 92.0% were positive and 8.0% were ambiguous for p16. No significant association was found between p16 and size of the tumor in carcinoma cervix.

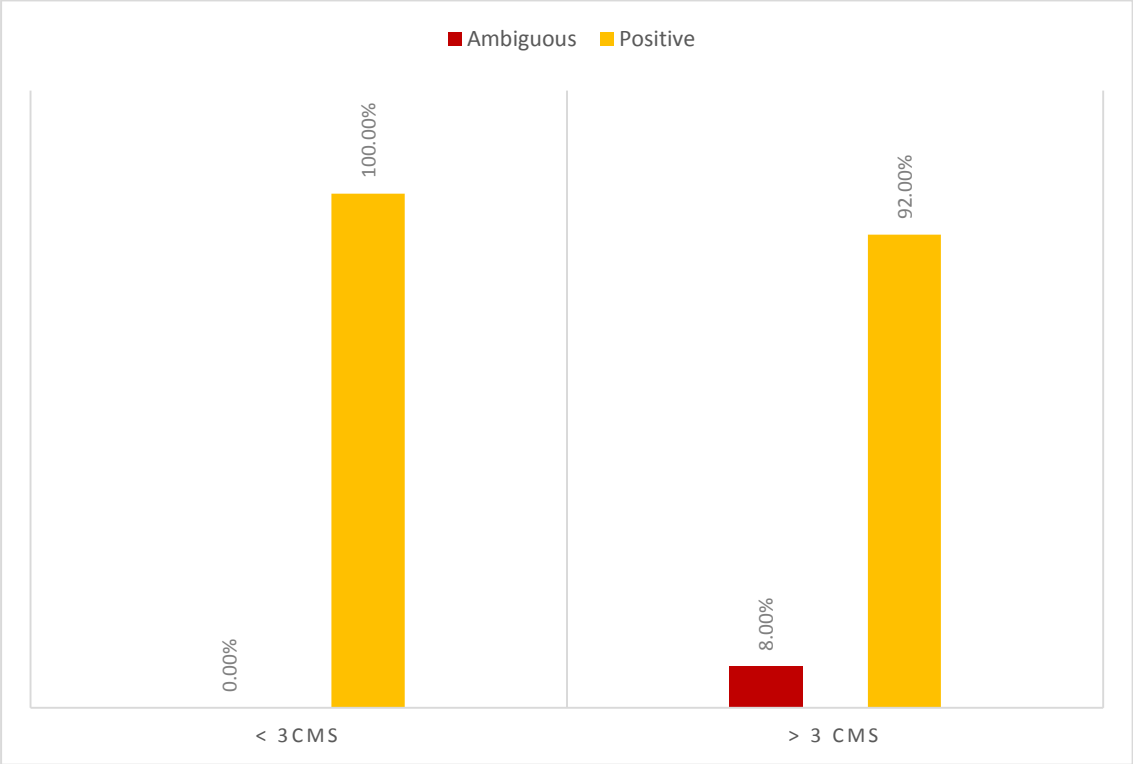


Chart 13: Bar diagram showing association between p16 expression and size of the tumor in Carcinoma Cervix.

Table 16: Ki-67 expression comparison between three groups.

		Group					
		Normal		HSIL		Carcinoma cervix	
		Count (n=26)	Percentage (%)	Count (n=26)	Percentage (%)	Count (n=26)	Percentage (%)
Ki-67	Negative (n=26)	26	100.0	0	0.0	0	0.0
	Weak Positive (n=9)	0	0.0	9	34.6	0	0.0
	Positive (n=21)	0	0.0	14	53.8	7	26.9
	Strong Positive (n=22)	0	0.0	3	11.5	19	73.1

$\chi 2 = 112.45$, df = 6, $p < 0.001$

In Carcinoma Cervix group, 73.1% were strong positive and 26.9% were positive for Ki-67. In HSIL group, 11.5% were strong positive, 53.8% were positive and 34.6% were weak positive for Ki-67. In Normal group, 100.0% were negative for Ki-67. There was significant difference in Ki-67 findings between three groups.

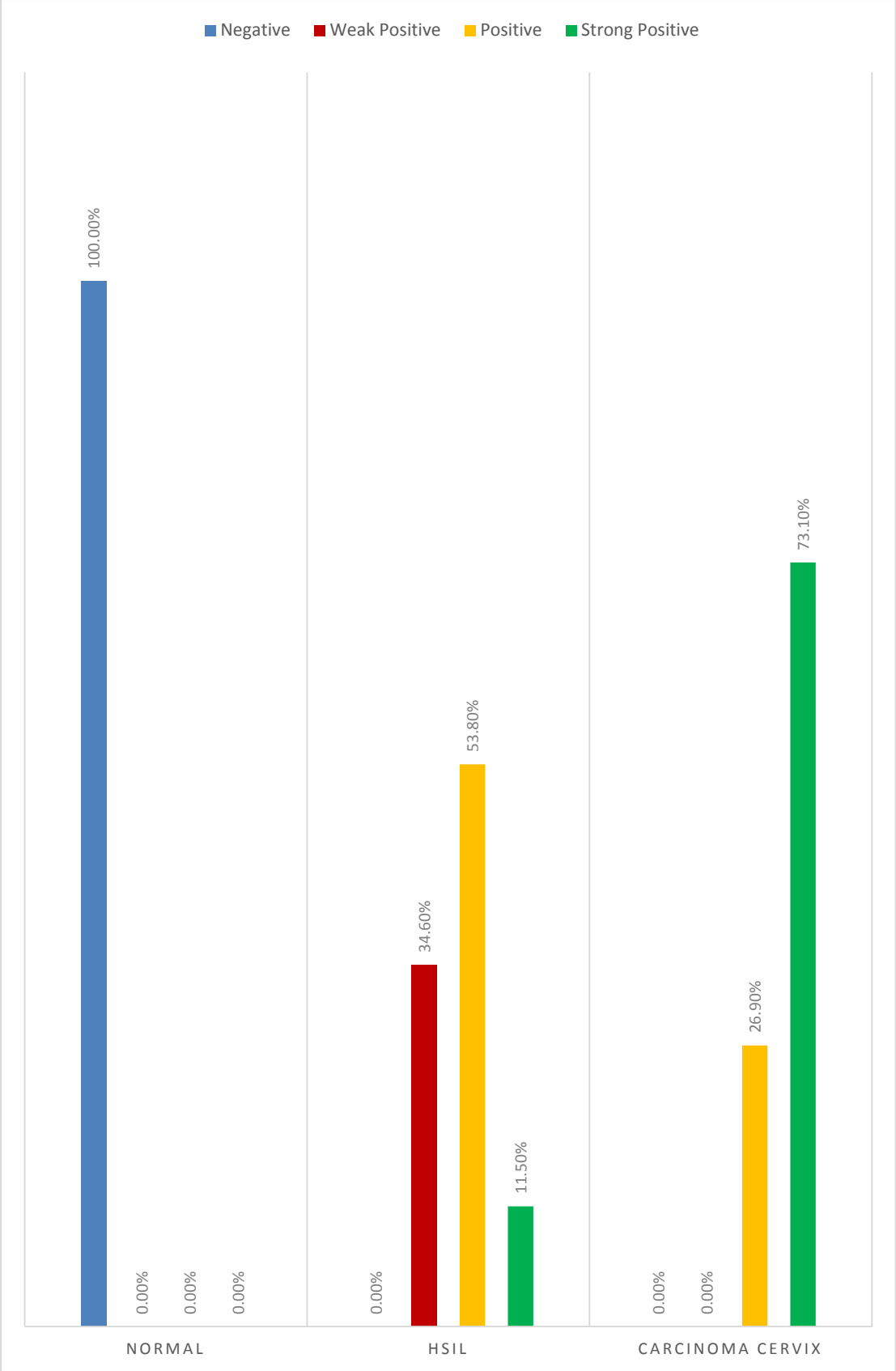


Chart 14: Bar diagram showing Ki-67 expression comparison between three groups.

Table 17: Association between Ki-67 expression and Grade of Carcinoma Cervix.

		Grade of Carcinoma Cervix						P value
		Well Differentiated SCC		Moderately Differentiated SCC		Poorly Differentiated SCC		
		Count (n=14)	Percentage (%)	Count (n=8)	Percentage (%)	Count (n=4)	Percentage (%)	
Ki-67	Positive (n=7)	5	35.7	2	25.0	0	0.0	0.361
	Strong Positive (n=19)	9	64.3	6	75.0	4	100.0	

In carcinoma cervix, among well differentiated SCC, 64.3% of were strongly positive and 35.7% were positive for Ki-67. Among moderately differentiated SCC, 75.0% were strongly positive and 25.0% were positive for Ki-67. Among poorly differentiated SCC, 100.0% were strongly positive for Ki-67. No statistically significant association was observed.

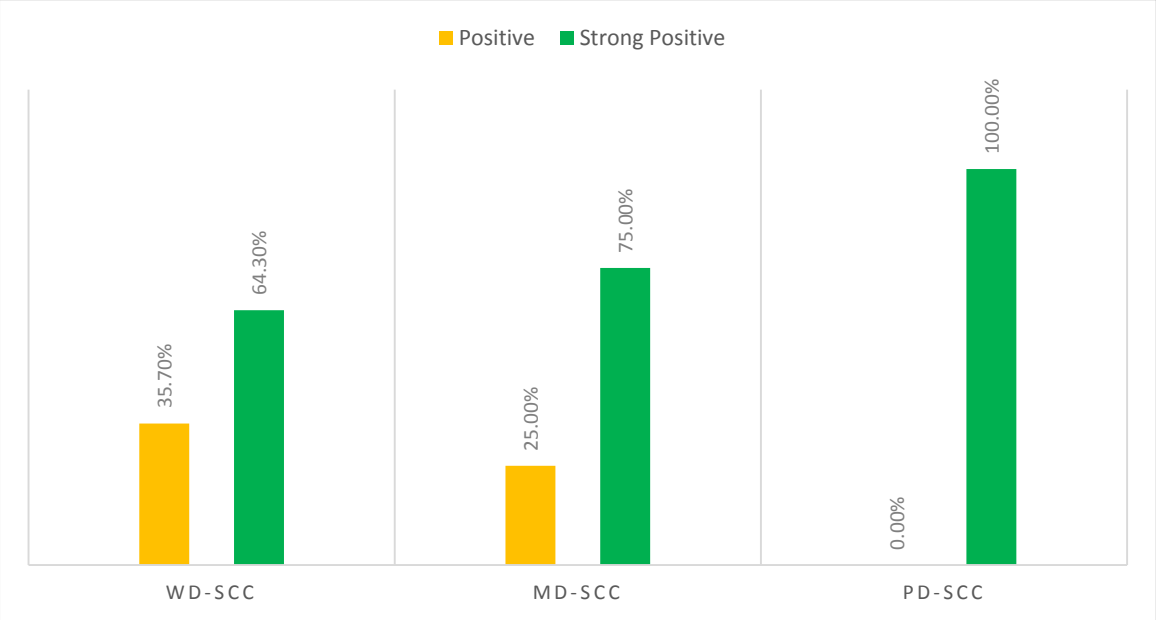


Chart 15: Bar diagram showing association between Ki-67 expression and Grade of Carcinoma Cervix.

Table 18: Association between Ki-67 expression and FIGO stage of Carcinoma Cervix.

		Ki-67				P value
		Positive		Strong Positive		
		Count (n=7)	Percentage (%)	Count (n=19)	Percentage (%)	
FIGO Stage	IIA (n=8)	2	25.0	6	75.0	0.320
	IIB (n=12)	2	16.7	10	83.3	
	IIIA (n=6)	3	50.0	3	50.0	

In Stage IIA carcinoma, 75.0% were strongly positive and 25.0% were positive for Ki-67. In Stage IIB carcinoma, 83.3% were strongly positive and 16.7% were positive for Ki-67. In Stage IIIA 50.0% were strongly positive and 50.0% were positive for Ki-67. No statistically significant association was seen between Ki-67 and FIGO stage of carcinoma cervix.

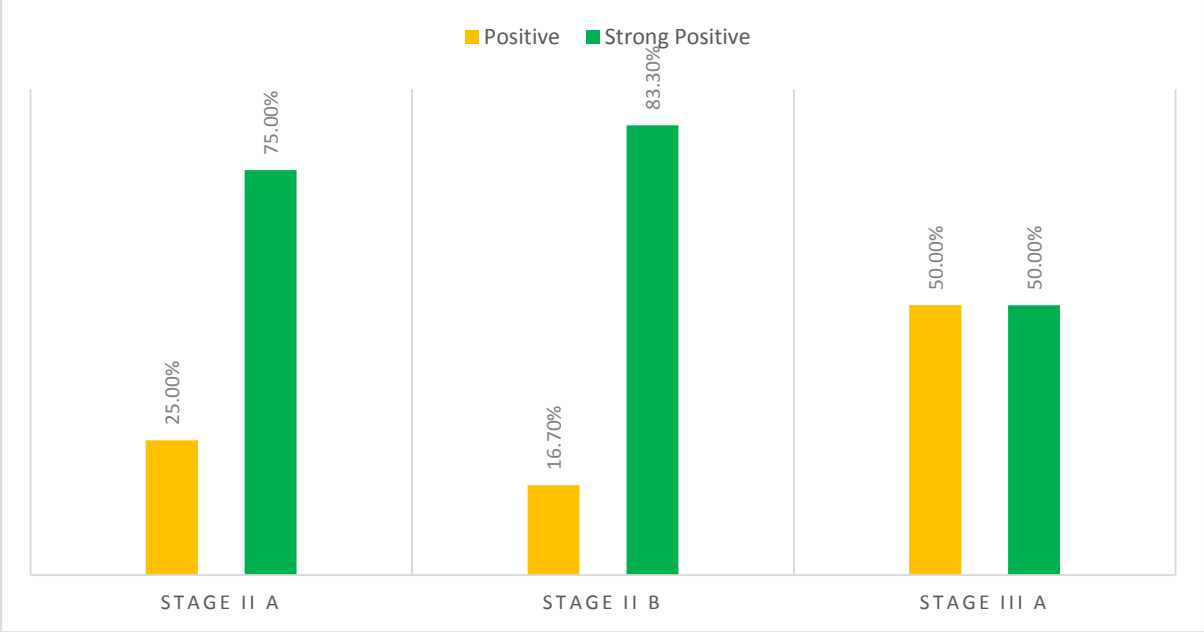


Chart 16: Bar diagram showing association between Ki-67 expression with respect to FIGO stage of Carcinoma Cervix.

Table 19: Association between Ki-67 expression and lymph node status in Carcinoma Cervix.

	Lymph Node Negative		Lymph Node Positive		P value
	Count (n=7)	Percentage (%)	Count (n=19)	Percentage (%)	
Positive (n=7)	1	14.3	6	31.6	0.377
Strong Positive (n=19)	6	85.7	13	68.4	

Among the carcinoma cervix cases with lymph node positivity, 68.4% were strongly positive and 31.6% were positive for Ki-67. Among the carcinoma cervix cases with lymph node negativity, 85.7% were strongly positive and 14.3% were positive for Ki-67. No statistically significant association was observed between Ki-67 and lymph node status of carcinoma cervix.

Chart 17: Bar diagram showing association between Ki-67 expression and lymph node status in Carcinoma Cervix.

Table 20: Association between Ki-67 expression and Size in Carcinoma Cervix group.

	< 3cms		> 3cms		P value
	Count (n=1)	Percentage (%)	Count (n=25)	Percentage (%)	
Positive (n=7)	0	0.0	7	28.0	0.535
Strong Positive (n=19)	1	100.0	18	72.0	

Among the carcinoma cervix cases with tumor size less than 3cms, 100.0% were strongly positive for Ki-67. Among the carcinoma cervix cases with tumor size more than 3cms, 72.0% were strongly positive and 28.0% were positive for Ki-67. No significant association was found between Ki-67 and size of the tumor in carcinoma cervix.

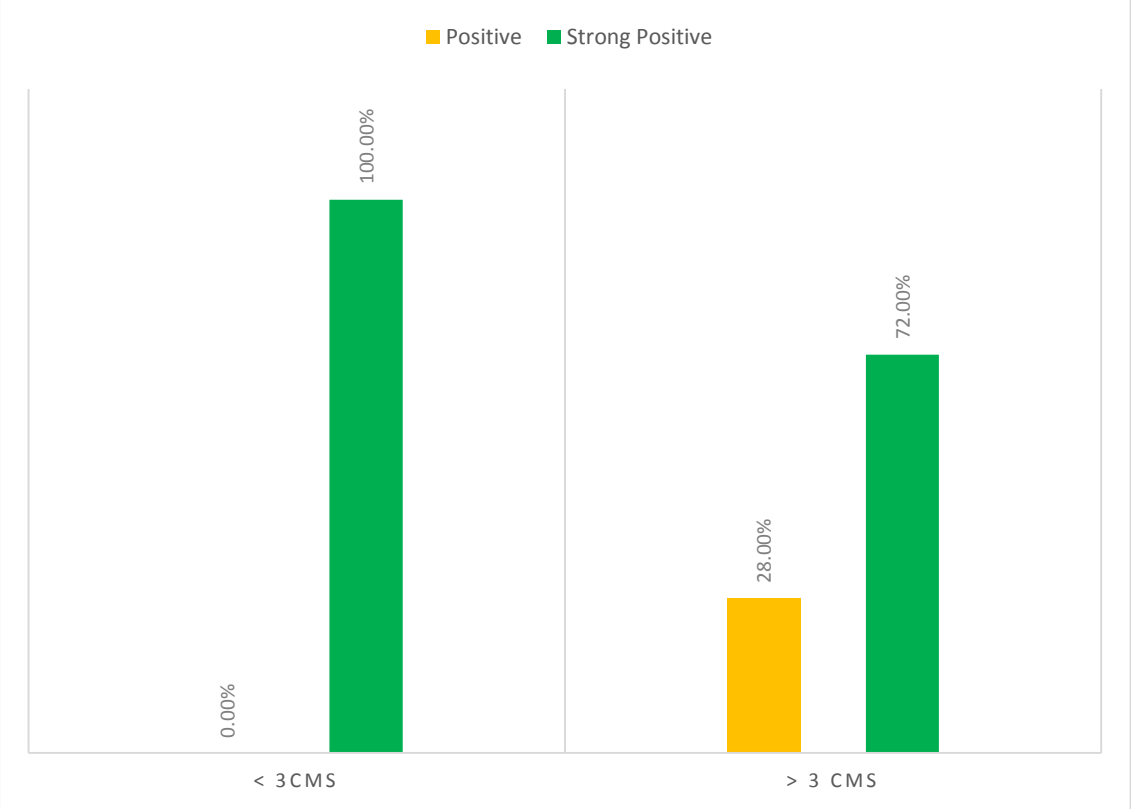


Chart 18: Bar diagram showing association between Ki-67 expression and size of the tumor in Carcinoma Cervix.

Table 21: CD44 expression comparison between three groups.

		Group					
		Normal		HSIL		Carcinoma	
		Count (n=26)	Percentage (%)	Count (n=26)	Percentage (%)	Count (n=26)	Percentage (%)
CD44	Negative (n=26)	26	100.0%	0	0.0%	0	0.0%
	Weak Positive (n=5)	0	0.0%	4	15.4%	1	3.8%
	Positive (19)	0	0.0%	11	42.3%	8	30.8%
	Strong Positive (n=28)	0	0.0%	11	42.3%	17	65.4%

$\chi 2 = 83.33$, df = 6, $p < 0.001$

In Carcinoma Cervix group, 65.4% were strong positive, 30.8% were positive and 3.8% were weak positive for CD44. In HSIL group, 42.3% were strong positive, 42.3% were positive and 15.4% were weak positive for CD44. In Normal group, 100% were negative for CD44. There was significant difference in CD44 findings between three groups.

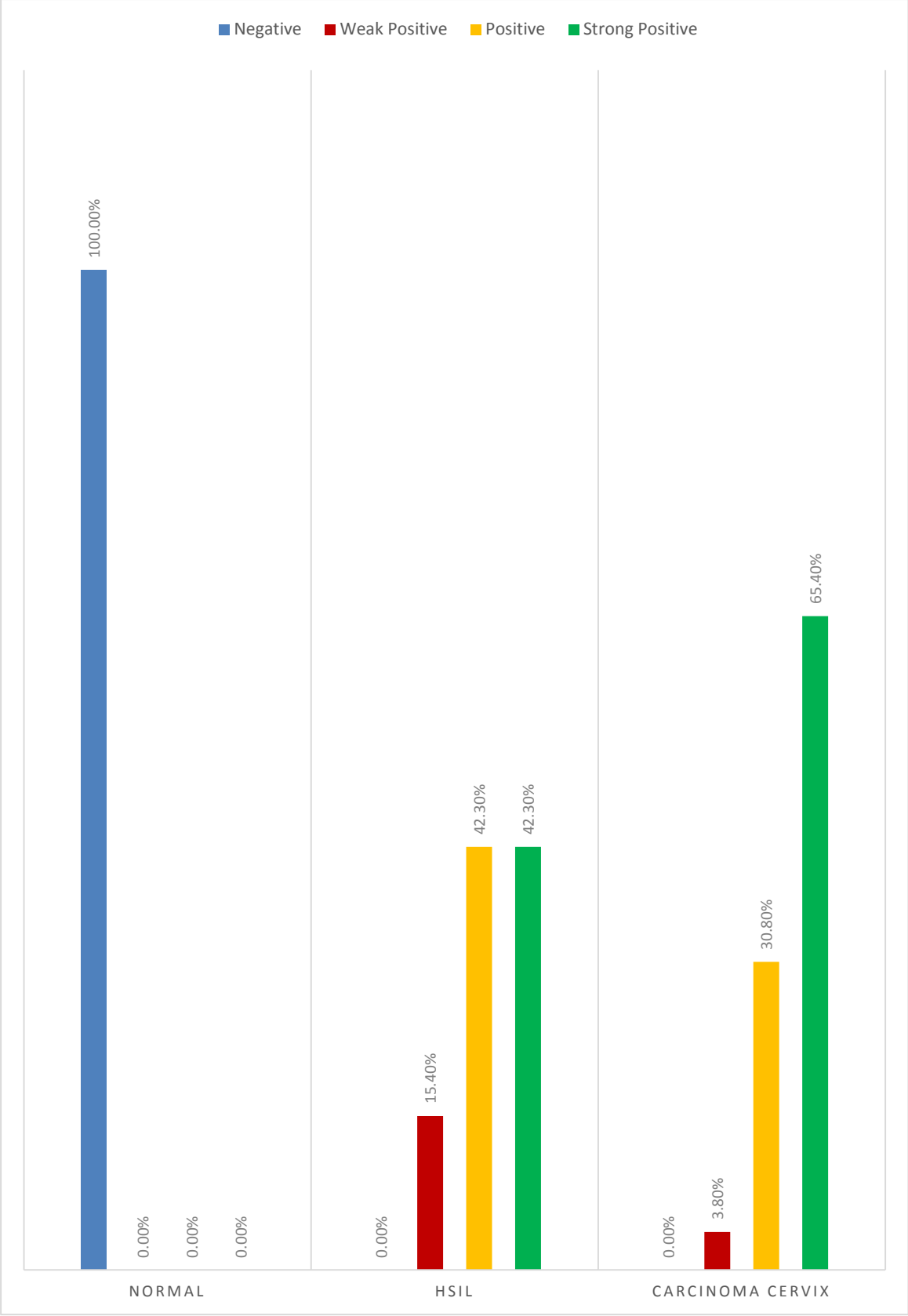


Chart 19: Bar diagram showing CD44 expression comparison between three groups.

Table 22: Association between CD44 expression and Grade of Carcinoma Cervix.

		Grade of Carcinoma Cervix						P value
		Well Differentiated SCC		Moderately Differentiated SCC		Poorly Differentiated SCC		
		Count (n=14)	Percentage (%)	Count (n=8)	Percentage (%)	Count (n=4)	Percentage (%)	
CD44	Weak Positive (n=1)	1	7.1%	0	0.0%	0	0.0%	0.395
	Positive (n=8)	6	42.9%	2	25.0%	0	0.0%	
	Strong Positive (n=17)	7	50.0%	6	75.0%	4	100.0%	

In carcinoma cervix, among well differentiated SCC, 50.0% of were strongly positive, 42.9% were positive and 7.1% were weakly positive for CD44. Among moderately differentiated SCC, 75.0% were strongly positive and 25.0% were positive for CD44. Among poorly differentiated SCC, 100.0% were strongly positive for CD44. These findings were not statistically significant.

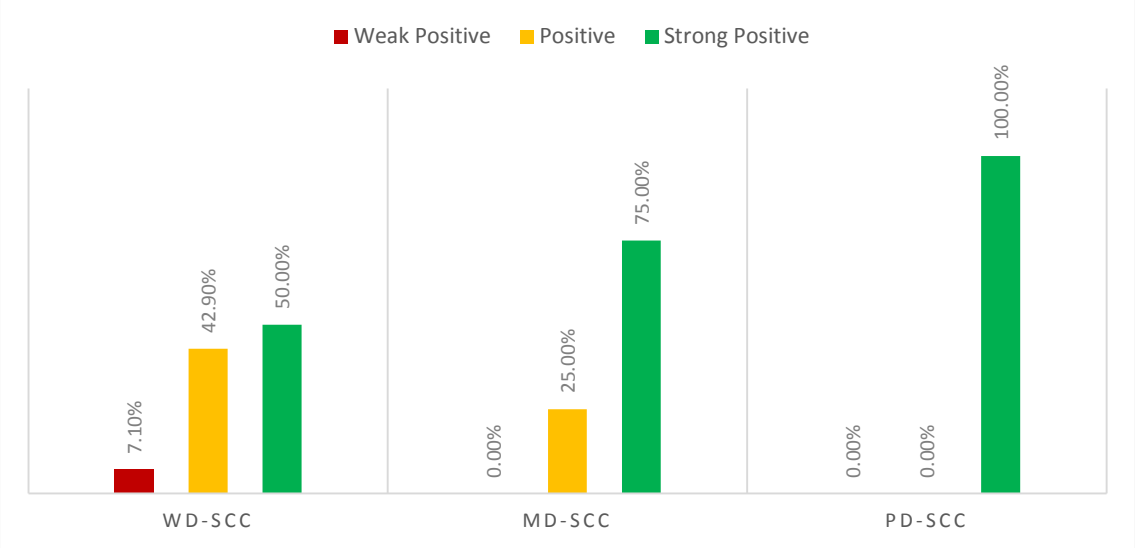


Chart 20: Bar diagram showing association between CD44 expression and Grade of Carcinoma Cervix.

Table 23: Association between CD44 expression and FIGO stage of Carcinoma Cervix.

		CD44						P value
		Weak Positive		Positive		Strong Positive		
		Count (n=1)	Percentage (%)	Count (n=8)	Percentage (%)	Count (n=17)	Percentage (%)	
FIGO Stage	IIA (n=8)	0	0.0	1	12.5	7	87.5	0.232
	IIB (n=12)	0	0.0	5	41.7	7	58.3	
	IIIA (n=6)	1	16.7	2	33.3	3	50.0	

In Stage IIA carcinoma, 87.5% were strongly positive and 12.5% were positive for CD44. In Stage IIB carcinoma, 58.3% were strongly positive and 41.7% were positive for CD44. In Stage IIIA 50.0% were strongly positive, 33.3% were positive and 16.7% were weakly positive for CD44. No statistically significant association was observed between CD44 and FIGO stage of carcinoma cervix.

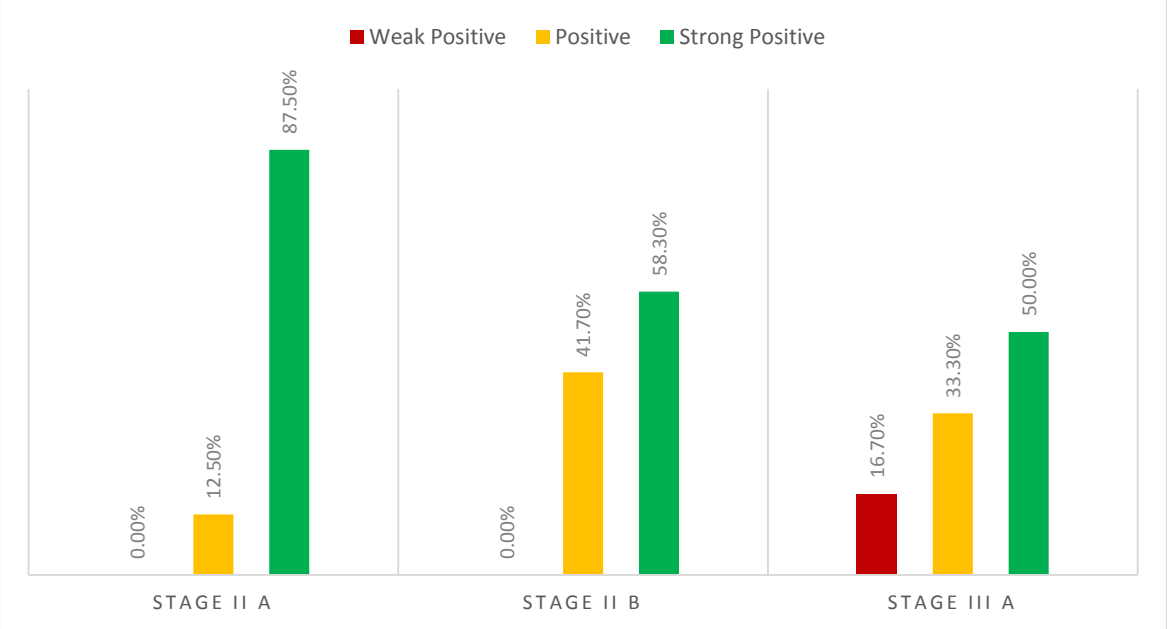


Chart 21: Bar diagram showing association between CD44 expression and FIGO stage of Carcinoma Cervix.

Table 24: Association between CD44 expression and lymph node status in Carcinoma Cervix.

	Lymph Node Positive		Lymph Node Negative		P value
	Count (n=19)	Percentage (%)	Count (n=1)	Percentage (%)	
Weak Positive (n=1)	0	0.0	1	14.2	**0.032**
Positive (n=8)	4	21.1	4	57.2	
Strong Positive (n=17)	15	78.9	2	28.6	

Among the carcinoma cervix cases with lymph node positivity, 78.9% were strongly positive and 21.1% were positive for CD44. Among the carcinoma cervix cases with lymph node negativity, 28.6% were strongly positive, 57.2% were positive and 14.2% was weakly positive for CD44. There was no statistically significant association between CD44 and lymph node status of carcinoma cervix.

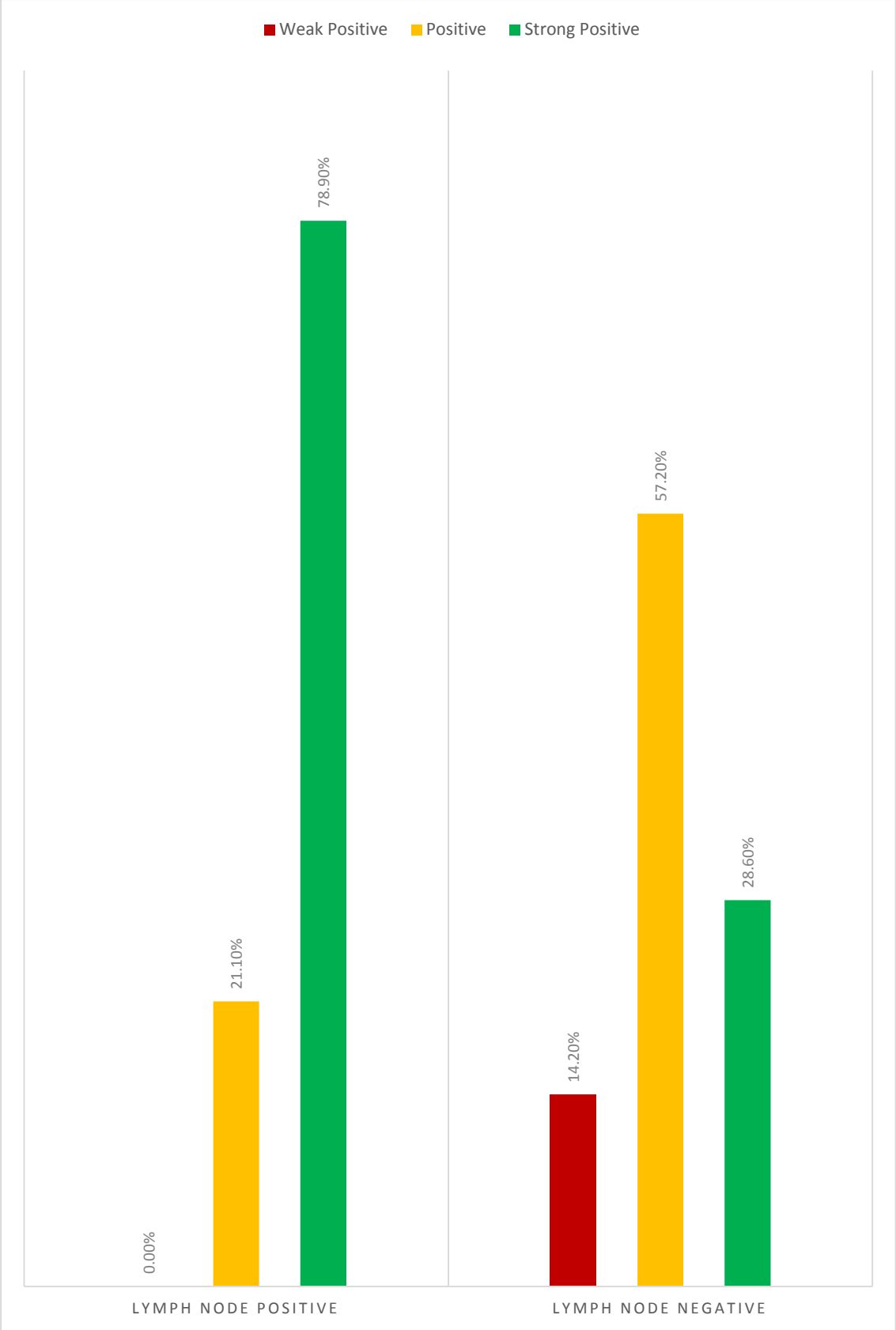


Chart 22: Bar diagram showing association between CD44 expression and lymph node status in Carcinoma Cervix.

Table 25: Association between CD44 expression and Size in Carcinoma Cervix.

	< 3cms		> 3cms		P value
	Count (n=1)	**Percentage (%)**	**Count (n=25)**	**Percentage (%)**	
Weak Positive (n=1)	0	0.0	1	4.0	0.759
Positive (n=8)	0	0.0	8	32.0	
Strong Positive (n=17)	1	100.0	16	64.0	

Among the carcinoma cervix cases with tumor size less than 3cms, 100.0% were strongly positive for CD44. Among the carcinoma cervix cases with tumor size more than 3cms, 64.0% were strongly positive, 32.0% were positive and 4.0% were weakly positive for CD44. There was no significant association between CD44 and size of the tumor in carcinoma cervix.

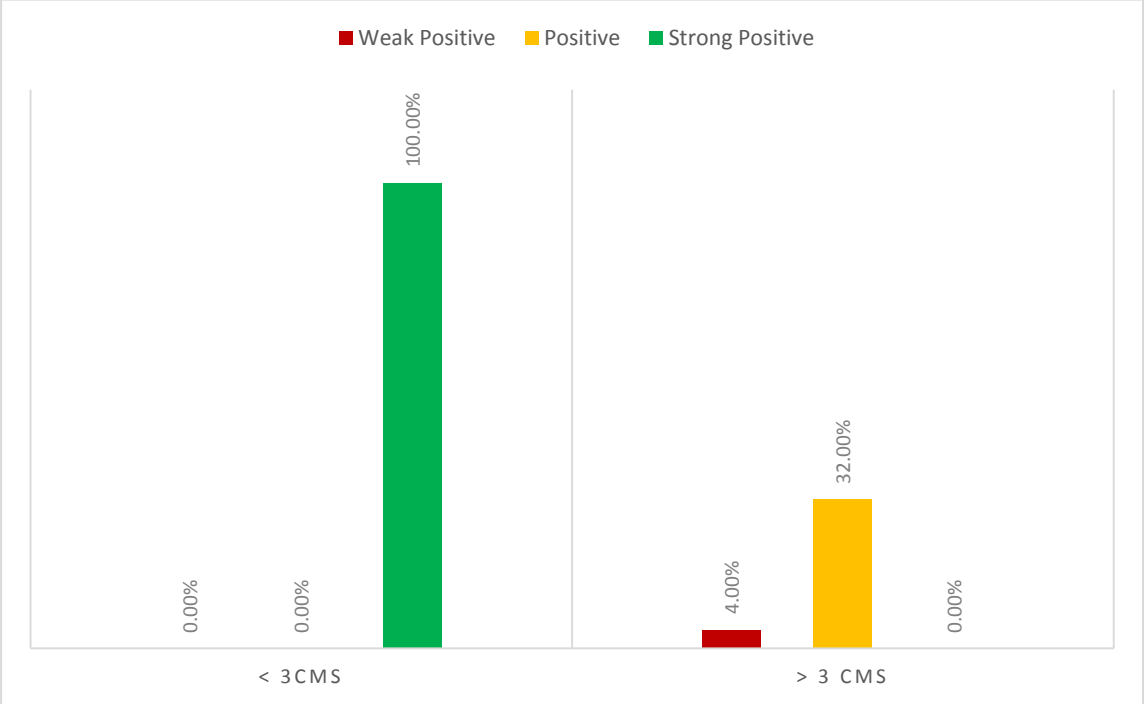


Chart 23: Bar diagram showing association between CD44 expression and size of the tumor in Carcinoma Cervix.

Table 26: Association between p16 and Ki-67 expression.

		p16								
		Normal (n=26)			HSIL (n=26)			Carcinoma (n=26)		
		N (n=26)	A (n=0)	P (n=0)	N (n=8)	A (n=2)	P (n=16)	N (n=0)	A (n=2)	P (n=24)
Ki-67	N (n=26)	26 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	WP (n=10)	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (37.5%)	2 (100.0%)	4 (25.0%)	0 (0.0%)	1 (50.0%)	0 (0.0%)
	P (n=22)	0 (0.0%)	0 (0.0%)	0 (0.0%)	5 (62.5%)	0 (0.0%)	9 (56.3%)	0 (0.0%)	1 (50.0%)	7 (29.2%)
	SP (n=20)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (18.7%)	0 (0.0%)	0 (0.0%)	17 (70.8%)

$\chi 2 = 60.60$, df = 6, p <0.001 **(N – negative, A – ambiguous, P – positive, WP – weak positive, SP – strong positive)**

There was statistically significant association between the expression of p16 and Ki-67.

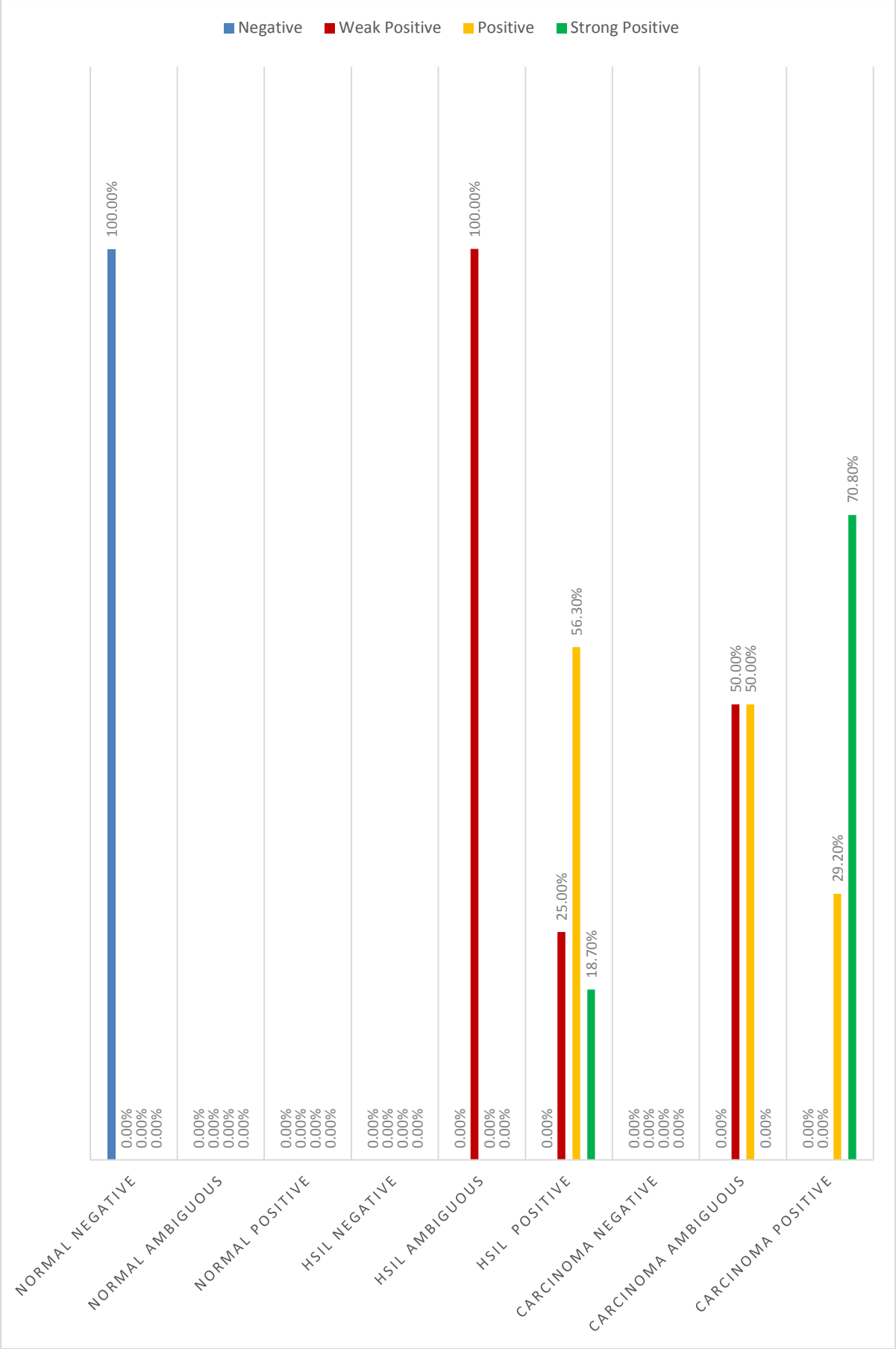


Chart 24: Bar diagram showing association between p16 and Ki-67 expression.

Table 27: Association between p16 and CD44 expression.

		p16								
		Normal (n=26)			HSIL (n=26)			Carcinoma (n=26)		
		N (n=26)	A (n=0)	P (n=0)	N (n=8)	A (n=2)	P (n=16)	N (n=0)	A (n=2)	P (n=24)
CD44	N (n=26)	26 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	WP (n=5)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (12.5%)	0 (0.0%)	3 (18.8%)	0 (0.0%)	1 (50.0%)	0 (0.0%)
	P (n=19)	0 (0.0%)	0 (0.0%)	0 (0.0%)	4 (50.0%)	2 (100.0%)	5 (31.2%)	0 (0.0%)	1 (50.0%)	7 (29.2%)
	SP (n=28)	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (37.5%)	0 (0.0%)	8 (50.0%)	0 (0.0%)	0 (0.0%)	17 (70.8%)

$\chi 2 = 59.72$, df = 6, $p < 0.001$ **(N – negative, A – ambiguous, P – positive, WP – weak positive, SP – strong positive)**

There was statistically significant association between the expression of p16 and CD44.

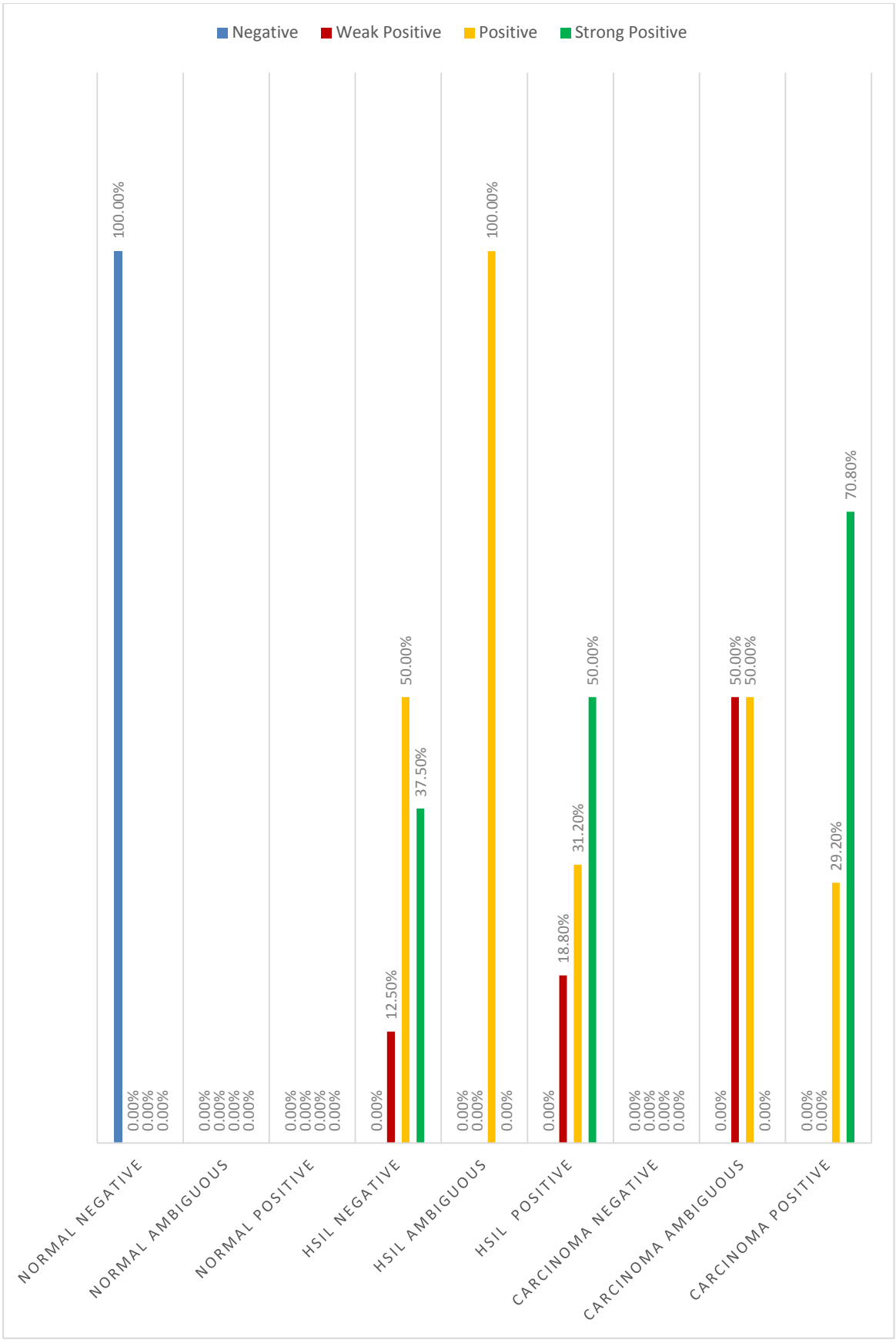


Chart 25: Bar diagram showing association between p16 and CD44 expression.

Table 28: Association between Ki-67 and CD44 expression.

		Ki-67											
		Normal (n=26)				HSIL (n=26)				Carcinoma (n=26)			
		N (n=26)	WP (n=0)	P (n=0)	SP (n=0)	N (n=0)	WP (n=9)	P (n=14)	SP (n=3)	N (n=0)	WP (n=0)	P (n=7)	SP (n=19)
CD44	N (n=26)	26 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	WP (n=5)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	4 (44.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (5.3%)
	P (n=19)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	5 (55.6%)	6 (42.9%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (14.3%)	7 (36.8%)
	SP (n=28)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	8 (57.1%)	3 (100.0%)	0 (0.0%)	0 (0.0%)	6 (85.7%)	11 (57.9)

$\chi 2 = 109.38$, df = 9, p <0.001 **(N – negative, A – ambiguous, P – positive, WP – weak positive, SP – strong positive)**

There was statistically significant association between the expression of Ki-67 and CD44 expression.

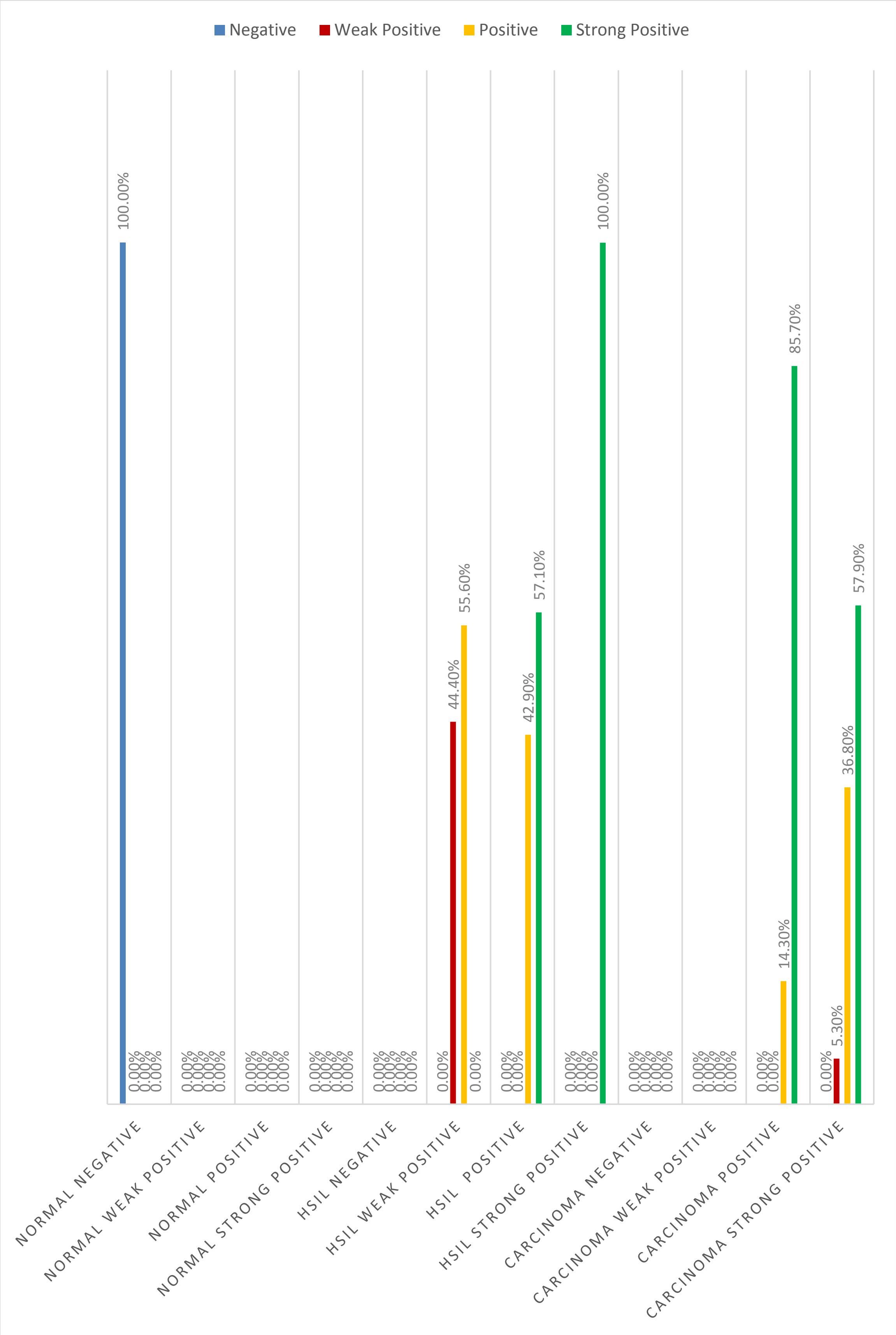


Chart 26: Bar diagram showing association between Ki-67 and CD44 expression.

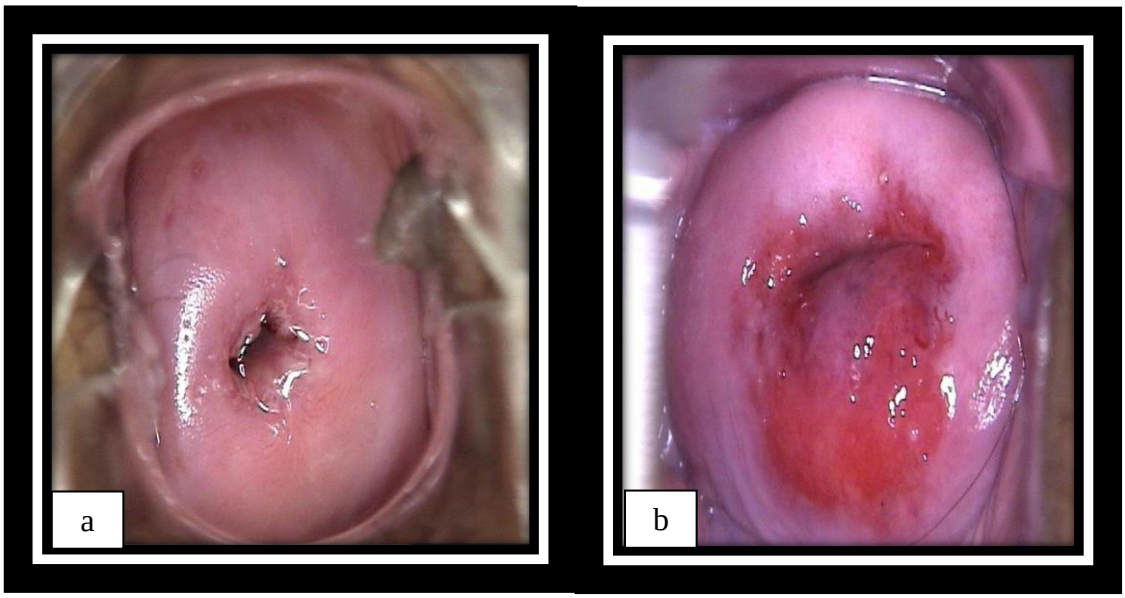


Figure 4: Colposcopic images of (a) normal cervix showing healthy cervix and (b) CIN 2 showing cervical erosion.

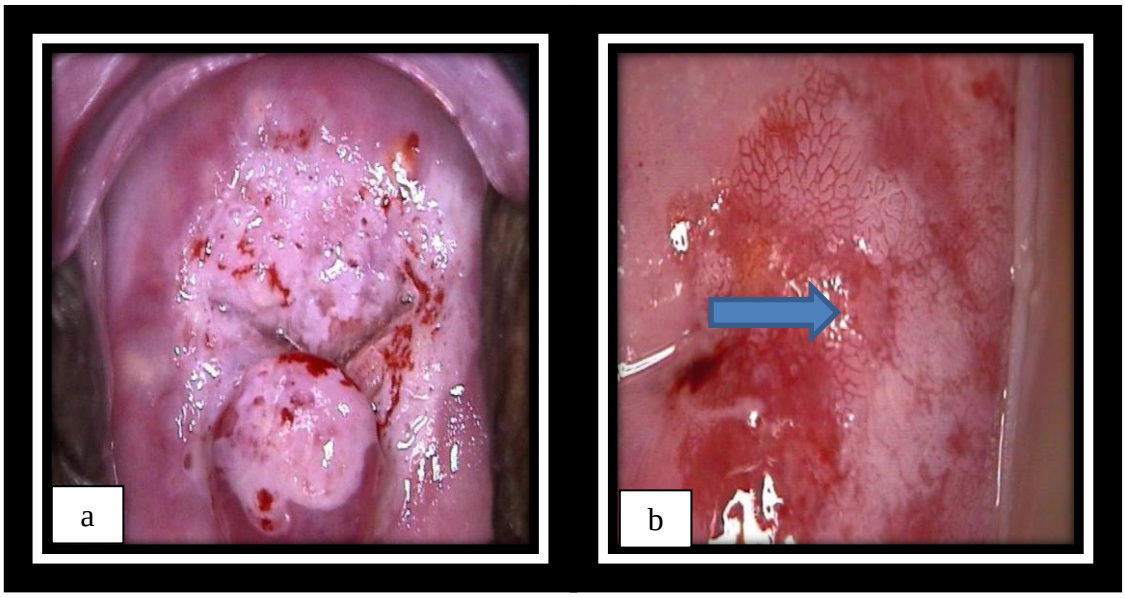


Figure 5: Colposcopic images of (a) CIN 3 showing cervical growth and (b) Carcinoma cervix showing cervical growth and acetowhite positivity (arrow).

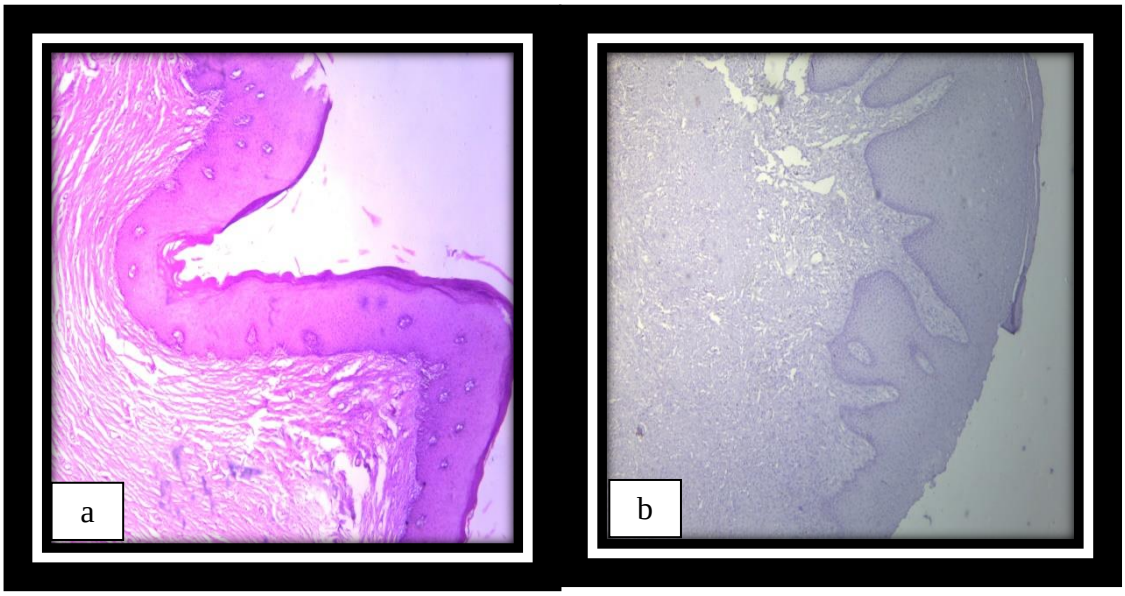


Figure 6: (a) H & E image of normal cervix showing ectocervical lining. (b) Immunohistochemistry p16 expression (negative) in normal cervix.

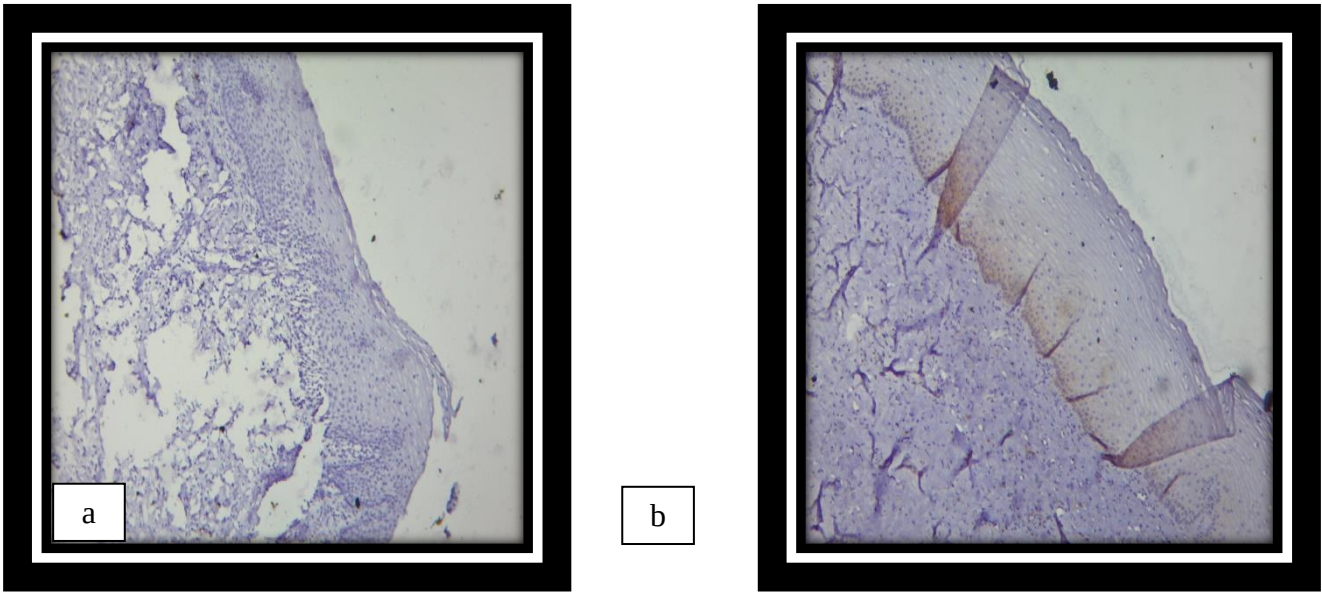


Figure 7: Immunohistochemistry: (a) Ki-67 expression (negative) in normal cervix. (b) CD44 expression (basal / negative) in normal cervix.

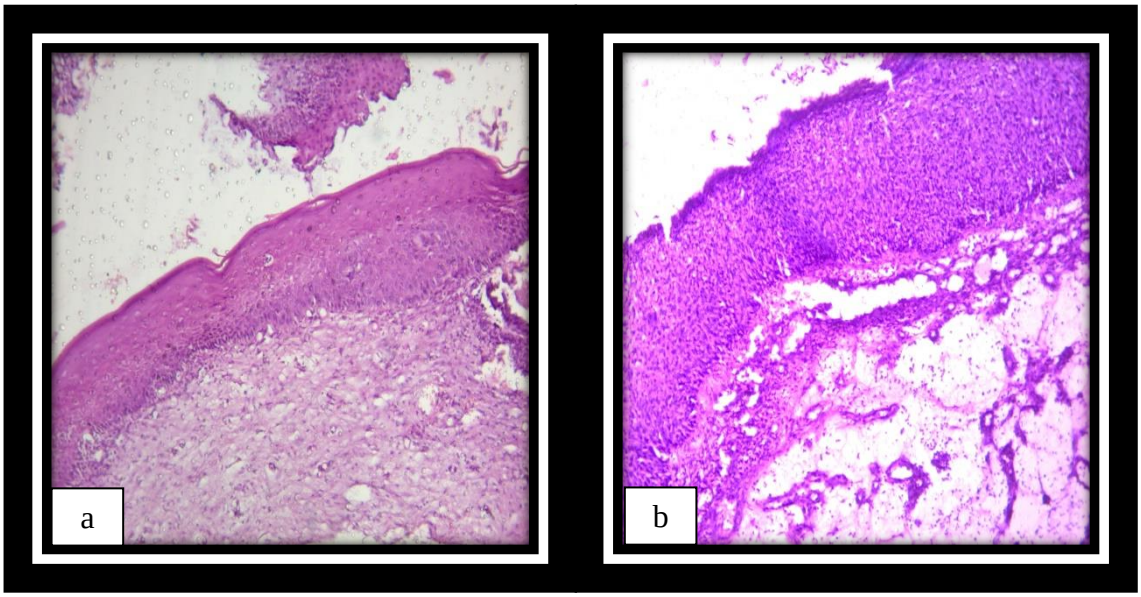


Figure 8: H & E image of HSIL. (a) CIN 2 showing atypia involving the lower two third of the ectocervix. (b) CIN 3 showing atypia involving full thickness of the ectocervix.

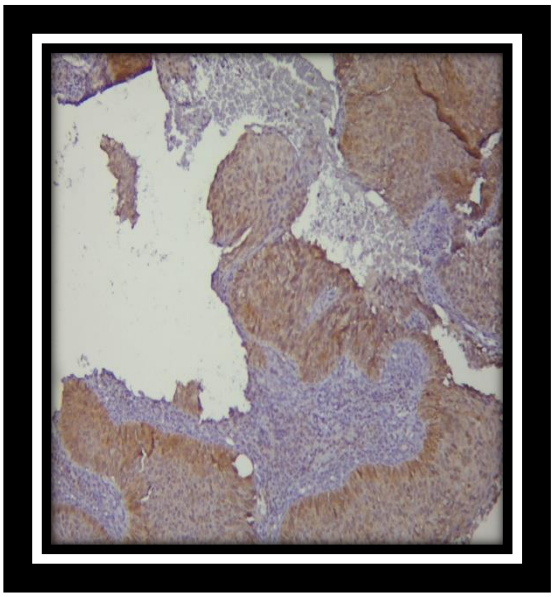


Figure 9: Immunohistochemistry: p16 expression (positive) in HSIL.

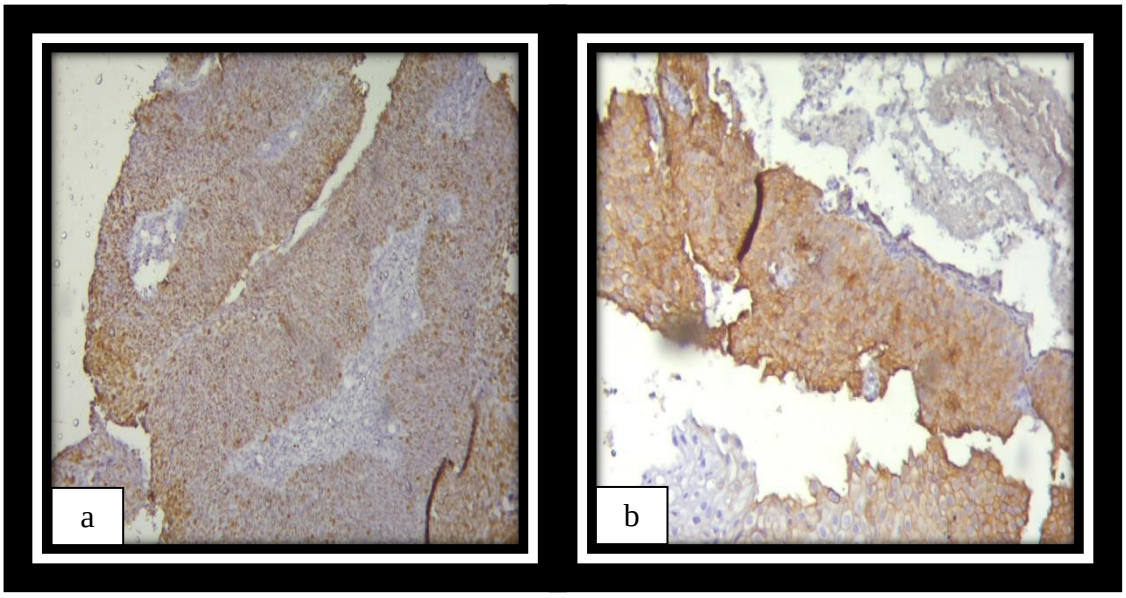


Figure 10: Immunohistochemistry: (a) Ki-67 expression in HSIL. (b) CD44 expression in HSIL. (strong positive)

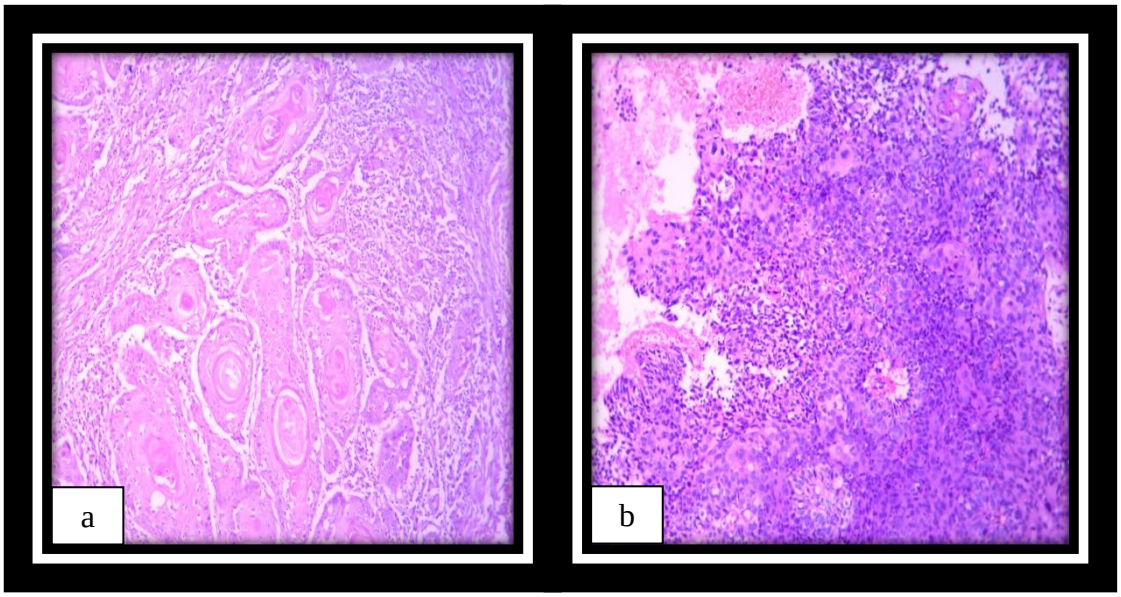


Figure 11: H & E image: (a) Well differentiated squamous cell carcinoma. (b) Moderately differentiated squamous cell carcinoma.

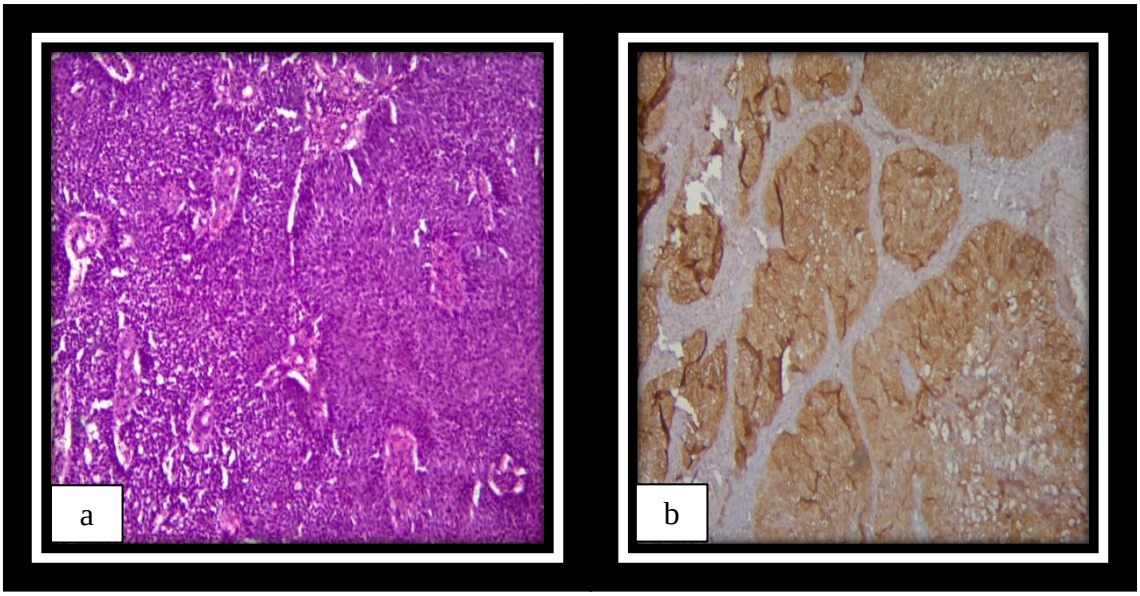


Figure 12: (a) H & E image of Poorly differentiated squamous cell carcinoma. (b) Immunohistochemistry p16 expression (positive) in squamous cell carcinoma.

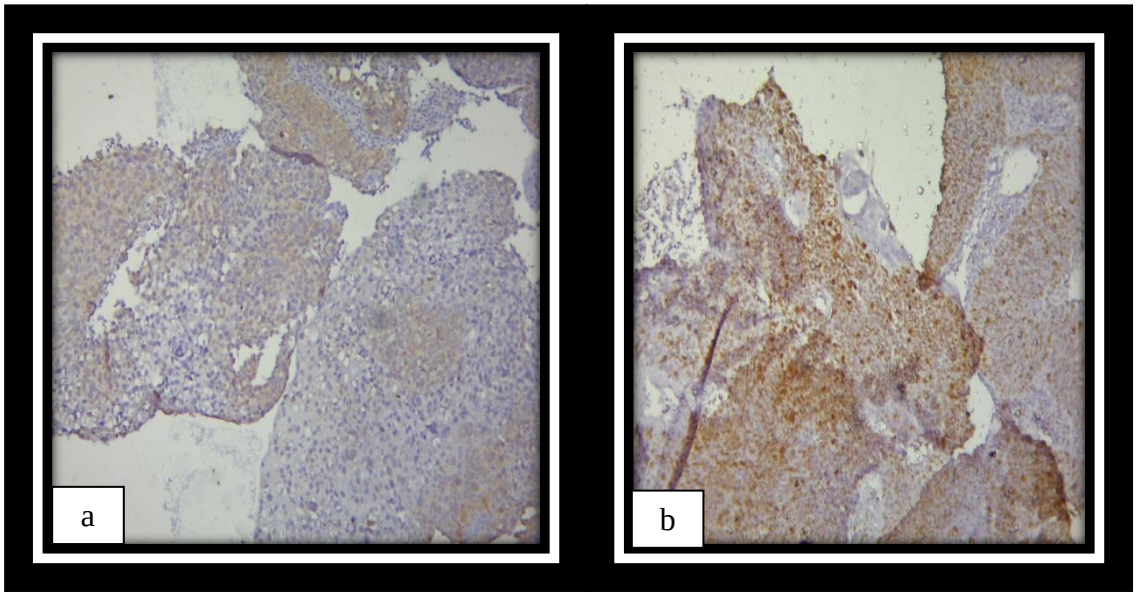


Figure 13: Immunohistochemistry: (a) p16 expression (ambiguous) in squamous cell carcinoma. (b) Ki-67 expression (strong positive) in squamous cell carcinoma.

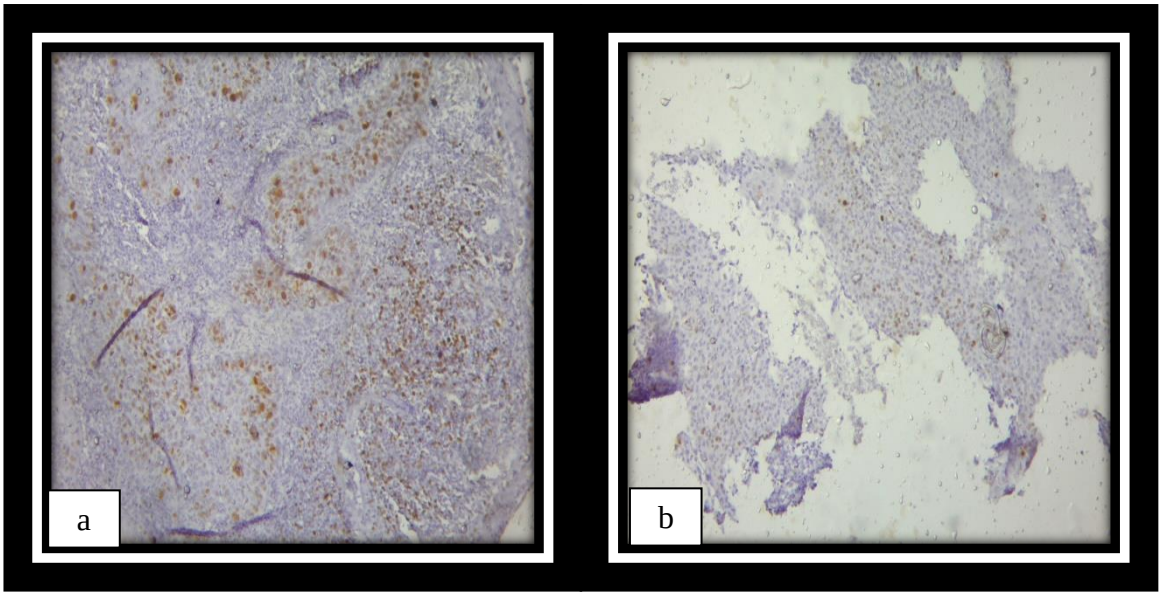


Figure 14: Immunohistochemistry: (a) Ki-67 expression (positive) in squamous cell carcinoma. (b) Ki-67 expression (weak positive) in squamous cell carcinoma.

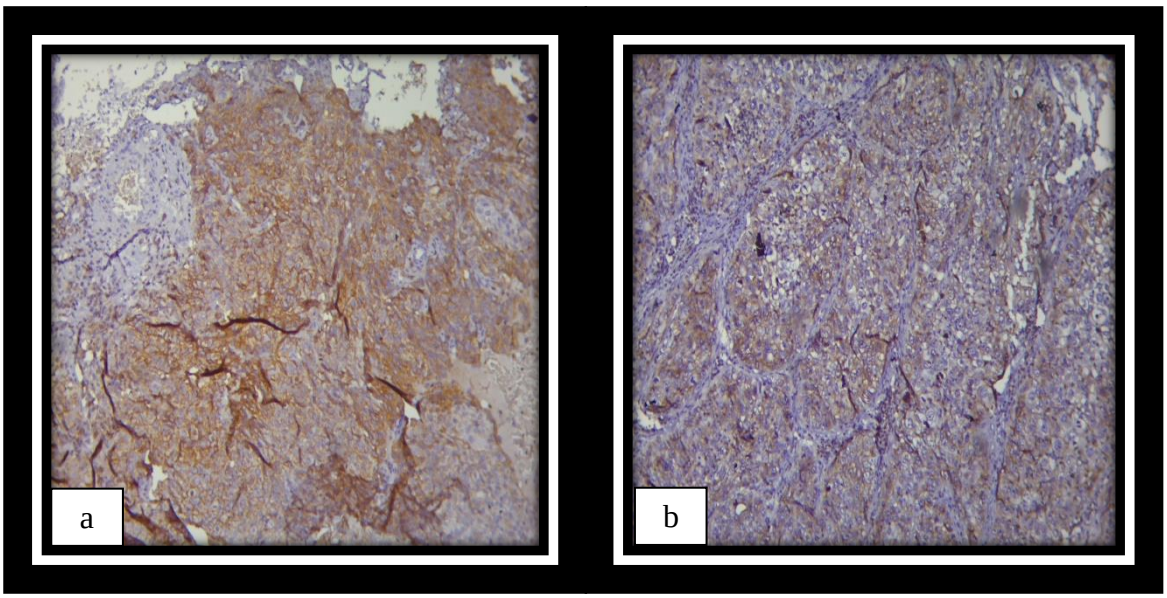


Figure 15: Immunohistochemistry: (a) CD44 expression (strong positive) in squamous cell carcinoma. (b) CD44 expression (positive) in squamous cell carcinoma.

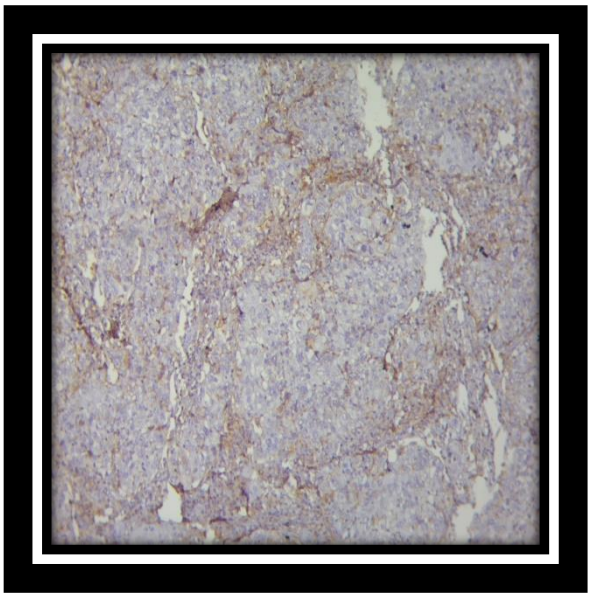


Figure 16: Immunohistochemistry: CD44 expression (weak positive) in squamous cell carcinoma.

DISCUSSION

DISCUSSION

Cervical cancer is the second most commonly occurring malignancy among women in the world. It is the most commonly reported gynaecological malignancy in India and is also one of the major cause of cancer related morbidity.[1] In South India, the prevalence of cervical cancer accounts for 17.55% of all reported cancer cases among the female population. The incidence of HR-HPV infection peaks around 25 years of age, which coincides with the peak age for sexual activity. More than 90% of HSIL and virtually all cases of cervical cancer are associated with HR-HPV infection.[2] In India, the average age for cervical cancer incidence is 50-60 years.[5] The peak age for HSIL incidence is 40-50 years.[103]

AGE DISTRIBUTION:

Table 29: Age distribution of HSIL cases. Comparison with other studies.

	Hebbar et al (2017)	Liu et al (2017)	Present Study (2018)
Number of HSIL cases	**10**	**42**	**26**
Mean age in years	47	39	47.0

In the present study the majority of the HSIL cases belonged to the 41-50 years age group and the mean age reported for HSIL 47.0 ± 13.4. In a study done by Hebbar et al, majority of the HSIL cases belonged to the 40-50 years age group and the mean age was 47 years.[104] In a study done by Liu et al, most of the HSIL cases had a mean age of 39 years.[105]

Table 30: Age distribution of carcinoma cervix cases. Comparison with other studies.

	Weng et al (2012)	Hong et al (2006)	Present Study (2018)
Number of Carcinoma Cervix cases	**62**	**34**	**26**
Mean age in years	52	48.6	50.4

In the present study the majority of the carcinoma cervix cases belonged to the 51-60 years and the mean age was 50.4 ± 10.3. In a study done by Weng et al, the mean age for the carcinoma cervix cases was 52 years.[106] Hong et al reported similar findings where, carcinoma cervix cases had a mean age of 48.6 years.[107] The findings in the present study was in accordance to these studies.

CHIEF COMPLAINTS:

In HSIL group, the most common complaint was discharge per vagina (42.3%), followed by post-menopausal bleeding (38.5%), postcoital bleeding (11.5%) and abnormal uterine bleeding (7.7%). Similar findings were reported in the study done by Gupta et al, where the most common complaint in the HSIL cases was discharge per vagina followed by postcoital bleeding, postmenopausal bleeding and abnormal uterine bleeding.[103]

In carcinoma cervix group, the most common complaint was postmenopausal bleeding (50.0%), followed by discharge per vagina (30.8%), bleeding per vagina (15.4%) and abnormal uterine bleeding (3.8%). Similar findings were reported by Gupta et al, where the most common complaint among the carcinoma group was postmenopausal bleeding (45.5%).[103]

In the present study, abnormal uterine bleeding was the only complaint in the normal group as it included only those cases that has undergone hysterectomy for leiomyoma.

COLPOSCOPIC FINDINGS:

In HSIL group, the most common colposcopic finding was cervical erosion seen in 96.2%. Similar findings were observed in the study done by Gupta et al, where the most colposcopic finding in HSIL cases was cervical erosion followed by cervical hypertrophy.[103]

In carcinoma cervix group, all the cases showed cervical growth (100.0%) on colposcopy. Similar findings were observed by Gupta et al, where the most common colposcopic finding among the carcinoma group was cervical growth, followed by cervical erosion and hypertrophy.[103]

In the present study, all the cases in the normal group showed healthy cervix on colposcopy as these cases underwent hysterectomy for leiomyoma.

p16 EXPRESSION:

Table 31: p16 expression in normal cases. Comparison with other studies.

	Izadi-Mood et al (2012)	Sarma et al (2017)	Present Study (2018)
Negative	39 (100.0%)	15 (100.0%)	26 (100.0%)
Ambiguous	0 (0.0%)	-	0 (0.0%)
Positive	0 (0.0%)	0 (0.0%)	0 (0.0%)
Number of cases	**39**	**15**	**26**

In the present study, all the normal cases were stained negative for p16. Similar observations were made in the study done by Izadi-Mood et al in 2012 and Sarma et al in 2017.[108,109]

Table 32: p16 expression in HSIL cases. Comparison with other studies.

	Izadi-Mood et al (2012)	Sarma et al (2017)	Present Study (2018)
Negative	2 (18.2%)	10 (30.3%)	8 (30.8%)
Ambiguous	0 (0.0%)	-	2 (7.7%)
Positive	9 (81.8%)	23 (69.7%)	16 (61.5%)
Number of cases	**11**	**33**	**26**

In the present study, 61.5% of HSIL cases had shown block positivity for p16 and 30.8% cases were negative for p16 immunostaining. These findings were similar to the findings of Izadi-Mood et al and Sarma et al.[108,109]

However, 7.7% of the HSIL cases in the present study were ambiguously stained for p16. In a study done by Liu et al in 2017 on 220 CIN 2 cases, 23% were ambiguously stained for p16. It was concluded that p16 ambiguous cases were distinct form of HSIL that had an intermediate risk of progression.[105]

Table 33: p16 expression in carcinoma cervix cases. Comparison with other studies.

	Izadi-Mood et al (2012)	**Sarma et al (2017)**	**Present Study (2018)**
Negative	2 (10.0%)	0 (0.0%)	(0.0%)
Ambiguous	3 (15.0%)	-	2 (7.7%)
Positive	15 (75.0%)	26 (100.0%)	24 (92.3%)
Number of cases	**20**	**26**	**26**

In the present study, 92.3% of carcinoma cases had shown block positivity for p16 and 7.7% cases were ambguous for p16 immunostaining. In a study done by Izadi-Mood et al in 2012, 75% of the carcinoma cases were positive, 15% carcinoma cervix cases were ambiguous, and 10% carcinoma cases were negative for p16 immunostaining. In a similar study done by Sarma et al in 2017, all the carcinoma cases showed strong positivity for p16 immunostaining.[108,109] The two ambiguously

positive cases in the present study can be explained by a low sensitivity of immunohistochemistry on paraffin-embedded formalin fixed tissue.[110]

There was significant difference in the p16 expression between the normal, HSIL and carcinoma cervix groups (p value <0.001). The findings in the present study were in conjunction to the findings in the above mentioned studies.

Table 34: p16 expression with respect to the grade of carcinoma cervix. Comparison with other studies.

	Kishore et al (2017)			**Present Study (2018)**		
	WD-SCC	**WD-SCC**	**MD-SCC**	**PD-SCC**	**MD-SCC**	**PD-SCC**
Negative	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Ambiguous	-	-	-	2 (14.3%)	0 (0.0%)	0 (0.0%)
Positive	15 (100.0%)	15 (100.0%)	15 (100.0%)	12 (85.7%)	8 (100.0%)	4 (100.0%)
Number of cases	**15**	**15**	**15**	**14**	**8**	**4**

In the present study, among the well differentiated carcinomas 85.7% were positive and 14.3% were ambiguous for p16 immunostaining. Among the moderately differentiated and poorly differentiated carcinomas, all the cases were positive for p16 immunostaining. There was no statistical difference (p value = 0.395) noted in the p16 expression with respect to the grade of SCC. This was in accordance to the study done by Kishore et al in 2017, where there was no difference in the expression of p16 among the different grades of SCC.[111]

Table 35: p16 expression with respect to the FIGO stage of carcinoma cervix. Comparison with other studies.

	Amaro-Filho et al (2013)		Present Study (2018)	
FIGO Stage	**II**	**III**	**II**	**III**
Negative	1 (3.8%)	2 (8.0%)	0 (0.0%)	0 (0.0%)
Ambiguous	-	-	0 (0.0%)	2 (33.3%)
Positive	25 (96.1%)	23 (92.0%)	26 (100.0%)	4 (66.7%)
Number of cases	**26**	**25**	**20**	**6**

In the present study, there was statistically significant correlation between p16 expression and the stage of carcinoma (p value = 0.027). In a study done by Amaro-Filho et al, low expression of p16 was seen stage I and stage II carcinoma and high expression of p16 was seen in stage III and stage IV carcinoma (p value = 0.023).[112] These findings were similar to the present study with the limitation of stage I and stage IV carcinoma.

In a study done by Weng et al and Son et al, no statistical correlation was seen in the expression of p16 and stage of carcinoma (p value >0.05).[106,113]

Table 36: p16 expression with respect to lymph node involvement in carcinoma cervix. Comparison with other studies.

	Weng et al (2012)		**Son et al (2012)**		**Present Study (2018)**	
	Lymph Node Negative	**Lymph Node Positive**	**Lymph Node Negative**	**Lymph Node Positive**	**Lymph Node Negative**	**Lymph Node Positive**
Negative	11 (28.2%)	13 (56.5%)	6 (20.7%)	3 (50.0%)	0 (0.0%)	0 (0.0%)
Ambiguous	-	-	-	-	2 (28.6%)	0 (0.0%)
Positive	28 (71.8%)	10 (43.5%)	23 (79.3%)	3 (50.0%)	5 (71.4%)	19 (100.0%)
Number of cases	**39**	**23**	**29**	**6**	**7**	**19**

In the present study, significant correlation was seen between p16 expression and lymph node involvement (p value = 0.015). However, the percentage of lymph node negative cases which showed p16 positivity were similar to the findings of Son et al and Weng et al, where no significant correlation was noted in the expression of p16 with respect to lymph node involvement.[106,113]

Table 37: p16 expression with respect to the size of tumor in carcinoma cervix. Comparison with other studies.

	Weng et al (2012)		**Present Study (2018)**	
	< 3cms	**> 3cms**	**< 3cms**	**> 3cms**
Negative	12 (40.0%)	12 (37.5%)	0 (0.0%)	0 (0.0%)
Ambiguous	-	-	0 (0.0%)	2 (8.0%)
Positive	18 (60.0%)	20 (62.5%)	1 (100.0%)	23 (92.0%)
Number of cases	**30**	**32**	**1**	**25**

In the present study, there was no statistical correlation between p16 positivity and size of the tumor (p value = 0.768). Similar findings were seen in the study done by Weng et al.[113]

Ki-67 EXPRESSION:

Table 38: Ki-67 expression in normal cases. Comparison with other studies.

	Hebbar et al (2017)	Amaro-Filho et al (2013)	Present Study (2018)
Negative	3 (100.0%)	28 (65.1%)	26 (100.0%)
Weak Positive	0 (0.0%)	15 (34.9%)	0 (0.0%)
Positive	0 (0.0%)	0 (0.0%)	0 (0.0%)
Strong Positive	0 (0.0%)	0 (0.0%)	0 (0.0%)
Number of cases	**3**	**43**	**26**

In the present study, all the normal cases were stained negative for Ki-67. Similar findings were also seen in the study done by Hebbar et al in 2017.[104] Amaro-Filho et al in a study done in 2013, found that weak Ki-67 positivity was localised in the basal layer of 34.9% of normal cervix. This was explained by the presence of squamous metaplastic cells and regenerative cells which stain positive for Ki-67 immunostaining.[112]

Table 39: Ki-67 expression in HSIL cases. Comparison with other studies.

	Hebbar et al (2017)	Agoff et al (2003)	Present Study (2018)
Negative	1 (5.0%)	0 (0.0%)	0 (0.0%)
Weak Positive	2 (10.0%)	20 (11.3%)	9 (34.6%)
Positive	9 (45.0%)	38 (21.3%)	14 (53.8%)
Strong Positive	8 (40.0%)	120 (67.4%)	3 (11.5%)
Number of cases	**20**	**178**	**26**

In the present study, 11.5% of HSIL cases had shown strong positivity, 53.8% cases were positive and 34.6% were weakly positive for Ki-67 immunostaining. These findings were similar to the findings of Hebbar et al and Agoff et al.[104,114] With the exception of one Ki-67 negative case reported by Hebbar et al which was attributed to the low sensitivity of Ki-67 in CIN 2.[104] A similar rising trend of Ki-67 immunostaining from normal to HSIL was seen in the present study.

Table 40: Ki-67 expression in carcinoma cervix cases. Comparison with other studies.

	Hebbar et al (2017)	Amaro-Filho et al (2013)	Agoff et al (2003)	Present Study (2018)
Negative	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Weak Positive	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Positive	1 (17.0%)	14 (17.1%)	3 (6.7%)	7 (26.9%)
Strong Positive	5 (83.0%)	68 (82.9%)	42 (93.3%)	19 (73.1%)
Number of cases	**6**	**82**	**45**	**26**

In the present study, 73.1% of carcinoma cases had shown strong positivity and 26.9% cases were positive for Ki-67 immunostaining. Similar findings were seen in the findings of Hebbar et al, Amaro-Filho et al and Agoff et al.[104,112,114]

There was significant difference in the Ki-67 expression between the normal, HSIL and carcinoma cervix groups (p value <0.001). All the three studies found a statistically significant rising trend of Ki-67 immunostaining from normal to HSIL to carcinoma as was seen in the present study.

Table 41: Ki-67 expression with respect to the grade of carcinoma cervix. Comparison with other studies.

	Jian Qin-Yu et al (2015)		Present Study (2018)		
	WD-SCC and MD-SCC	**PD-SCC**	**WD-SCC**	**MD-SCC**	**PD-SCC**
Negative	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Weak Positive	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Positive	15 (51.7%)	1 (3.6%)	5 (35.7%)	2 (25.0%)	0 (0.0%)
Strong Positive	14 (48.3%)	27 (96.4%)	9 (64.3%)	6 (75.0%)	4 (100.0%)
Number of cases	**29**	**28**	**14**	**8**	**4**

In the present study, 64.3% of well differentiated carcinoma cases, 75.0% of moderately differentiated carcinoma cases and 100.0% of the poorly differentiated carcinoma cases showed strong positivity for Ki-67 immunostaining. When well differentiated and moderately differentiated carcinoma were grouped together, 68.2% (15/22) showed strong positivity for Ki-67 immunostaining. This finding was in accordance to the findings of Jian Qin-Yu et al. However, no statistically significant association was found between Ki-67 expression and the grade of carcinoma.[115]

Table 42: Ki-67 expression with respect to the FIGO stage of carcinoma cervix. Comparison with other studies.

	Amaro-Filho et al (2013)		Present Study (2018)	
FIGO Stage	**II**	**III**	**II**	**III**
Negative	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Weak Positive	3 (11.6%)	3 (12.0%)	0 (0.0%)	0 (0.0%)
Positive	9 (34.6%)	7 (28.0%)	4 (20.0%)	3 (50.0%)
Strong Positive	13 (50.0%)	15 (60.0%)	16 (80.0%)	3 (50.0%)
Number of cases	**26**	**25**	**20**	**6**

In the present study, 80.0% of stage II carcinoma and 50.0% of stage III carcinoma showed strong positivity for Ki-67 immunostaining. There was no statistical association between the Ki-67 expression and stage of carcinoma. This finding was in accordance with the findings of Ancuta et al (2009).[116] However, Amaro-Filho et al reported a statistically significant association of strong positive Ki-67 immunostaining with the stage of carcinoma.[112]

Table 43: Ki-67 expression with respect to lymph node involvement in carcinoma cervix. Comparison with other studies.

	Jian Qin-Yu et al (2015)		Present Study (2018)	
	Lymph Node Positive	**Lymph Node Negative**	**Lymph Node Positive**	**Lymph Node Negative**
Negative	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Weak Positive	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Positive	1 (4.8%)	13 (36.1%)	6 (31.6%)	1 (14.3%)
Strong Positive	20 (95.2%)	23 (63.9%)	13 (68.4%)	6 (85.7%)
Number of cases	**21**	**36**	**19**	**7**

In the present study, 68.4% of cases with lymph node involvement and 85.7% of cases without lymph node involvement showed strong positivity for Ki-67 immunostaining. This finding was in contrast to the study done by Jian-Qin Yu et al, which reported significant statistical correlation.[115] No statistical association was observed between the Ki-67 expression and lymph node status of carcinoma cases (p value = 0.378).

Table 44: Ki-67 expression with respect to the size of tumor in carcinoma cervix. Comparison with other studies.

	Jian Qin-Yu et al (2015)		**Present Study (2018)**	
	< 3cms	**> 3cms**	**< 3cms**	**> 3cms**
Negative	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Weak Positive	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Positive	13 (28.3%)	3 (27.3%)	0 (0.0%)	7 (28.0%)
Strong Positive	33 (71.7%)	8 (72.7%)	1 (100.0%)	18 (72.0%)
Number of cases	**46**	**11**	**1**	**25**

In the present study, 72.0% of cases with size more than 3cms showed strong positivity for Ki-67 immunostaining. Jian Qin Yu et al reported similar findings of 72.7% strong positivity.[115] However, no statistical correlation was found between the size of the tumor and the Ki-67 expression in the study done by Jian Qin Yu et al and the present study.

CD44 EXPRESSION:

Table 45: CD44 expression in normal cases. Comparison with other studies.

	Rodrigues et al (2004)	**Steidl et al (1998)**	**Present Study (2018)**
Negative	5 (100.0%)	9 (100.0%)	26 (100.0%)
Weak Positive	-	0 (0.0%)	0 (0.0%)
Positive	-	0 (0.0%)	0 (0.0%)
Strong Positive	0 (0.0%)	0 (0.0%)	0 (0.0%)
Number of cases	**5**	**9**	**26**

In the present study, all the normal cases showed no staining or weak positivity located in the basal layer which was interpreted as negative for CD44 immunostaining. Similar findings were also seen in the study done by Rodrigues et al and Steidl et al.[117,118]

Table 46: CD44 expression in HSIL cases. Comparison with other studies.

	Callagy et al (2000)	Rodrigues et al (20040	Present Study (2018)
Negative	1 (4.2%)	3 (5.6%)	0 (0.0%)
Weak Positive	0 (0.0%)	-	4 (15.4%)
Positive	5 (20.8%)	-	11 (42.3%)
Strong Positive	18 (75.0%)	51 (94.4%)	11 (42.3%)
Number of cases	**24**	**54**	**26**

In the present study, 42.3% of HSIL cases had shown strong positivity, 42.3% cases were positive, and 15.4% cases were weakly positive for CD44 immunostaining. These findings were similar to the findings of Callagy et al and Rodrigues et al.[117,119]

Table 47: CD44 expression in carcinoma cervix cases. Comparison with other studies.

	Rodrigues et al (2004)	Steidl et al (1998)	Present Study (2018)
Negative	5 (19.2%)	0 (0.0%)	0 (0.0%)
Weak Positive	-	14 (51.9%)	1 (3.8%)
Positive	-	11 (40.7%)	8 (30.8%)
Strong Positive	21 (80.8%)	2 (7.4%)	17 (65.4%)
Number of cases	**26**	**27**	**26**

In the present study, 65.4% of HSIL cases had shown strong positivity, 30.8% cases were positive, and 3.8% cases were weakly positive for CD44 immunostaining. These findings were similar to the findings of Rodrigues et al and Steidl et al.[117,118]

The cases in HSIL group and carcinoma group that are weak positivity and negative for CD44 immunostaining can be explained by the unstable expression of CD44 gene or failure of the protein to translocate and/or attach to the cell membrane due to the absence of supporting proteins in cases of HSIL and carcinoma.[117]

There was significant difference in the expression of CD44 between the normal, HSIL and carcinoma cervix groups (p value <0.001).

CD44 expression with respect to the grade of carcinoma cervix.

In the present study, no significant correlation as established between the expression of CD44 and the grade of carcinoma. Similar findings were reported by a Costa et al (2001) and Bouda et al (2005).[120,121]

CD44 expression with respect to the FIGO stage of carcinoma cervix.

In the present study, 70.0% of stage II and 50.0% of stage III carcinomas showed strong positivity for CD44 immunostaining. However, there was no statistical correlation between FIGO stage and CD44 expression. Similar findings were reported by Steidl et al (1998).[118]

On the contrary, Dasari et al (2014) compared the serum levels of soluble CD44 with the stage of carcinoma. He found significant increase in the level of soluble CD44 in stages III and IV when compared to stages I and II.[122]

Table 48: CD44 expression with respect to lymph node status is carcinoma cervix. Comparison with other studies.

	Ayhan et al (2001)		Present Study (2018)	
	Lymph Node Positive	**Lymph Node Negative**	**Lymph Node Positive**	**Lymph Node Negative**
Negative	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Weak Positive	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (14.2%)
Positive	11 (47.8%)	19 (48.7%)	4 (21.1%)	4 (57.2%)
Strong Positive	12 (52.2%)	20 (51.3%)	15 (78.9%)	2 (28.6%)
Number of cases	**23**	**39**	**19**	**7**

In the present study, 78.9% of cases with lymph node involvement and 28.6% of cases without lymph node involvement showed strong positivity for CD44 immunostaining. This finding was in contrast to the study done by Ayhan et al, which did not report significant statistical correlation.[19]

The findings of the present study show a statistical association between the CD44 expression and lymph node status of carcinoma (p value = 0.032). This was in accordance to the findings of Dasari et al (2014) who showed significant correlation between the serum levels of soluble CD44 and the lymph node involvement in carcinoma. Serum soluble CD44 levels were higher in patients with lymph node involvement.[122]

Table 49: CD44 expression with respect to size of the tumor in carcinoma cervix. Comparison with other studies.

	Ayhan et al (2001)		Present Study (2018)	
	< 3cms	**> 3cms**	**< 3cms**	**> 3cms**
Negative	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Weak Positive	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (4.0%)
Positive	13 (46.4%)	17 (50.0%)	0 (0.0%)	8 (32.0%)
Strong Positive	15 (53.6%)	17 (50.0%)	1 (100.0%)	16 (64.0%)
Number of cases	**28**	**34**	**1**	**25**

In the present study, 64.0% of cases with size more than 3cms and 100.0% of cases with size less than 3cms showed strong positivity for CD44 immunostaining. There was no statistical association between the size of the tumor and the expression status of CD44. Ayhan et al found significant statistical correlation between the size of the tumor and the CD44 expression.[19] Similarly, Bouda et al also reported a statistical association between CD44 expression and tumor diameter.[121]

COMPARISON BETWEEN CD44 EXPRESSION, p16 EXPRESSION AND KI-67 EXPRESSION:

In the present study significant association was seen between the expression of p16, Ki-67 and CD44 both among themselves and also with the progression of normal cervix to HSIL to carcinoma (p value = <0.001 As far as our knowledge and English literature search goes, this was the first study of it's kind to compare the expression of CD44 with the expression of p16 and Ki-67 along with the prognostic factors of carcinoma cervix.

One of the limitation of this study was the unavailability of lymph node sampling for histopathological evaluation, therefore the radiological lymph node involvement was taken as into consideration. Another limitation was the unavailability of FIGO stage I and IV for evaluation.

The discrepancies observed regarding CD44 expression in various studies can be explained by the altered expression of CD44 gene in HSIL and carcinoma. Further studies on a larger sample size may help in reaching a consensus.

Table 50: An overview of the expression of p16, Ki-67 and CD44 in Normal, HSIL and Carcinoma cervix cases.

		Normal (n=26)	HSIL (n=26)	Carcinoma (n=26)
p16	Negative	26 (100%)	8 (30.8%)	0 (0.0%)
	Ambiguous	0 (0.0%)	2 (7.7%)	2 (7.7%)
	Positive	0 (0.0%)	16 (61.5%)	24 (92.3%)
Ki-67	Negative	26 (100%)	0 (0.0%)	0 (0.0%)
	Weak Positive	0 (0.0%)	9 (34.6%)	0 (0.0%)
	Positive	0 (0.0%)	14 (53.8%)	7 (26.9%)
	Strong Positive	0 (0.0%)	3 (11.5%)	19 (73.1%)
CD44	Negative	26 (100%)	0 (0.0%)	0 (0.0%)
	Weak Positive	0 (0.0%)	4 (15.4%)	1 (3.8%)
	Positive	0 (0.0%)	11 (42.3%)	8 (30.8%)
	Strong Positive	0 (0.0%)	11 (42.3%)	17 (65.4%)

CONCLUSION

CONCLUSION

The results of this study showed that expression of p16, Ki-67 and CD44 increases as the lesion progresses from normal to HSIL to carcinoma cervix. There was a significant positive correlation seen in p16 and CD44 expression with the lymph node involvement in carcinoma cervix. In addition, there was a significant positive correlation seen between p16 expression and the FIGO stage of carcinoma cervix. Whereas, Ki-67 expression showed no statistical correlation with any of the clinico-pathologic parameters. These findings can be used to assess the prognosis of cervical carcinoma and the development of targeted therapy against cervical cancer stem cells.

FURTHER SCOPE OF THE STUDY:

CD44 expression is varied in cervical premalignant and malignant lesions and is relatively a new and less frequently explored finding. More studies have to be done to evaluate the usefulness of CD44 with respect to p16 and Ki-67 as a prognostic marker in cervical cancers. Identification of their expression in larger studies on a more extensive scale could possibly have an important role in the development of targeted therapies in cervical cancers thereby, reducing the morbidity and mortality associated with recurrence and resistance to chemotherapy.

SUMMARY

SUMMARY

A case control study, to correlate the immunohistochemical expression of p16, Ki-67 and CD44 in the normal, HSIL and carcinoma cervix cases, done between the study period of July 2016 and June 2018. The following are the salient features noted:

1. The mean age of the cases in the normal, HSIL and carcinoma groups were 42.3±9.3 years, 47.0±13.4 years and 50.4±10.3 years, respectively.
2. Most common chief complaint in the HSIL and carcinoma groups were white discharge per vagina and post-menopausal bleeding, respectively.
3. Most common colposcopic finding in the normal, HSIL and carcinoma groups were healthy, erosion and growth, respectively.
4. Most common FIGO stage of carcinoma cervix cases was Stage IIB.
5. Most common grade of squamous cell carcinoma cases was well differentiated squamous cell carcinoma.
6. HSIL cases: 61.5% cases were positive, and 7.7% cases were ambiguous for p16 expression. 11.5% cases were strongly positive, 53.8% cases were positive, and 34.6% cases were weakly positive for Ki-67 expression. 42.3% cases were strongly positive, 42.3% cases were positive, and 15.4% cases were weakly positive for CD44 expression.
7. Carcinoma cases: 92.3% cases were positive, and 7.7% cases were ambiguous for p16 expression. 73.1% cases were strongly positive, and 26.9% cases were positive for Ki-67 expression. 65.4% cases were strongly positive, 30.8% cases were positive, and 3.8% cases were weakly positive for CD44 expression.
8. Statistically significant correlation was seen in p16, Ki-67 and CD44 expression between the normal, HSIL and carcinoma cases group.

9. Statistically significant correlation was seen in p16 expression and FIGO stage and lymph node involvement in carcinoma cervix cases.
10. No statistically significant correlation was seen between Ki-67 expression and the various clinicopathologic parameters.
11. Statistically significant correlation was seen in CD44 expression and lymph node involvement in carcinoma cervix cases.
12. Statistically significant correlation was seen between p16, Ki-67 and CD44 expression among themselves.

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ANNEXURE

ANNEXURE-I

INFORMED CONSENT FORM

TITLE- ASSOCIATION OF p16, Ki67 AND CD44 MARKERS IN CERVICAL INTRAEPITHELIAL NEOPLASIA AND CERVICAL CARCINOMA.

I understand that I am free to withdraw from the study at anytime. I have read or it has been read to me and I understand the purpose of the study, the risk and benefits associated. I have had the opportunity to ask questions regarding various aspects of the study and my questions were answered to my satisfaction. I the undersigned agree to participate in this study and authorize for further testing on the surgical specimen and disclosure of my personal information for dissertation.

Subject name and signature/ Thumb impression DATE:

Parents / Guardians name / Thumb impression DATE:

Signature of the person taking consent DATE:

ANNEXURE-II

PROFORMA

TITLE- ASSOCIATION OF p16, Ki67 AND CD44 MARKERS IN CERVICAL INTRAEPITHELIAL NEOPLASIA AND CERVICAL CARCINOMA.

NAME: AGE:

HOSPITAL NO: BIOPSY NO: CASE NO:

NATURE OF SPECIMEN:

MARITAL STATUS:

PARITY:

CHIEF COMPLAINTS:

HISTORY OF PRESENT ILLNESS:

PAST HISTORY:

FAMILY HISTORY:

MENSTRUAL HISTORY:

COLPOSCOPIC FINDINGS:

RADIOLOGICAL FINDINGS:

FIGO STAGE:

SIZE:

LYMPH NODE INVOLVEMENT:

GROSS EXAMINATION:

HISTOPATHOLOGICAL DIAGNOSIS:

GRADE OF SQUAMOUS CELL CARCINOMA:

IMMUNOHISTOCHEMICAL FINDING:

p16 EXPRESSION:

Ki-67 EXPRESSION:

CD44 EXPRESSION:

FINAL IMPRESSION:

ANNEXURE III

KEYS TO MASTER CHART

B	BIOPSY NUMBER
AGE	AGE IN YEARS
TAH	TOTAL ABDOMINAL HYSTERECTOMY
WDPV	WHITE DISCHARGE PER VAGINA
AUB	ABNORMAL UTERINE BLEEDING
PMB	POST MENOPAUSAL BLEEDING
PCB	POST COITAL BLEEDING
PMS	POST MENOPAUSAL BLEEDING
BPV	BLEEDING PER VAGINA
E	EROSION
G	GROWTH
UR	UNREMARKABLE
HPR	HISTOPATHOLOGY REPORT
HPR-G	HISTOPATHOLOGY REPORT AND GRADE
CIN	CERVICAL INTRAEPITHELIAL NEOPLASIA
N	NEGATIVE
P	POSITIVE
WP	WEAK POSITIVE
SP	STRONG POSITIVE
A	AMBIGUOUS
L	LESS THAN 3 CMS
M	MORE THAN 3 CMS
LNI	LYMPH NODE INVOLVEMENT
NC	NORMAL CERVIX

CN	B	AGE	NATURE OF SPECIMEN	CC	COLPOSCOPIC FINDINGS	FIGO stage	LNI	SIZE	GROSS	HPR-G	P16	Ki67	CD44	
1	B/11/17	32	Biopsy	PMS	G	IIA	P	M	g/w to g/b stb 0.5x0.5cms	MD-SCC	P	SP	SP	
2	B/87/17	32	Biopsy	BPV	G	IIIA	N	M	g/w to g/b stb 0.5x0.5cms	WD-SCC	A	SP	WP	
3	B/110/17	40	Biopsy	BPV	G	IIIA	N	M	g/w to g/b stb 1x1cms	WD-SCC	P	SP	P	
4	B/139/17	65	Biopsy	PMS	G	IIIA	N	M	g/w to g/b stb 2x2cms	WD-SCC	P	SP	SP	
5	B/170/17	50	Biopsy	WDPV	G	IIB	P	M	g/w to g/b stb 1x1cms	WD-SCC	P	SP	P	
6	B/205/17	45	Biopsy	WDPV	G	IIIA	P	M	g/w to g/b stb 2x2cms	WD-SCC	P	P	SP	
7	B/209/17	50	Biopsy	WDPV	G	IIB	P	M	g/w to g/b stb 2x2cms	PD-SCC	P	SP	SP	
8	B/919/17	62	Biopsy	PMS	G	IIA	P	M	g/w to g/b stb 0.5x0.5cms	PD-SCC	P	SP	SP	
9	B/1137/17	60	Biopsy	WDPV	G	IIB	P	M	g/w to g/b stb 1x1cms	MD-SCC	P	P	SP	
10	B/1155/17	65	Biopsy	WDPV	G	IIA	P	M	g/w to g/b stb 1x1cms	PD-SCC	P	SP	SP	
11	B/1156/17	50	Biopsy	PMS	G	IIB	N	L	g/w to g/b stb 2x2cms	MD-SCC	P	SP	SP	
12	B/1157/17	30	Biopsy	AUB	G	IIA	P	M	g/w to g/b stb 1x1cms	WD-SCC	P	SP	P	
13	B/1184/17	45	Biopsy	WDPV	G	IIA	P	M	g/w to g/b stb 2x2cms	PD-SCC	P	SP	SP	
14	B/60/17	65	Biopsy	PMS	G	IIB	P	M	g/w to g/b stb 2x2cms	MD-SCC	P	SP	P	
15	B/204/17	45	Biopsy	BPV	G	IIA	P	M	g/w to g/b stb 1x1cms	WD-SCC	P	P	SP	
16	B/2953/16	60	Biopsy	PMS	G	IIA	P	M	g/w to g/b stb 1x1cms	MD-SCC	P	P	SP	
17	B/1519/17	55	Biopsy	BPV	G	IIIA	N	M	g/w to g/b stb 0.5x0.5cms	WD-SCC	A	P	P	
18	B/2088/17	51	Biopsy	PMS	G	IIB	P	M	g/w stb 0.5x0.5cms	MD-SCC	P	SP	SP	
19	B/2248/16	56	Biopsy	PMS	G	IIB	N	M	g/w stb 0.5x0.5cms	WD-SCC	P	SP	P	
20	B/2269/16	55	Biopsy	PMS	G	IIB	P	M	g/w to g/b stb 2x2cms	WD-SCC	P	SP	SP	
21	B/1377/17	47	Biopsy	WDPV	G	IIB	P	M	g/w to g/b stb 2x2cms	WD-SCC	P	SP	SP	
22	B/1609/17	55	Biopsy	PMS	G	IIA	P	M	g/w to g/b stb 0.5x0.5cms	MD-SCC	P	SP	SP	
23	B/1714/17	40	Biopsy	PMS	G	IIB	N	M	g/w to g/b stb 0.5x0.5cms	MD-SCC	P	SP	P	
24	B/1276/18	61	Biopsy	PMS	G	IIIA	P	M	g/w to g/b stb 0.5x0.5cms	WD-SCC	P	P	SP	
25	B/1793/17	42	Biopsy	WDPV	G	IIB	P	M	g/w to g/b stb 0.5x0.5cms	WD-SCC	P	P	SP	
26	B/1770/17	52	Biopsy	PMS	G	IIB	P	M	g/w to g/b stb 0.5x0.5cms	WD-SCC	P	SP	P	

CN	B	AGE	NATURE OF SPECIMEN RECEIVED	CC	COLPOSCOPIC FINDINGS	GROSS	HPR	p16	Ki-67	CD44
1	B/70/17	42	B	WDPV	E	g/w to g/b stb 0.5x0.5cms	CIN 2	N	WP	P
2	B/140/17	47	B	AUB	E	g/w to g/b stb 1x1cms	CIN 3	N	P	P
3	B/462/17	53	B	PMB	G	g/w to g/b stb 2x2cms	CIN 3	P	SP	SP
4	B/725/17	33	B	PCB	E	g/w to g/b stb 2x2cms	CIN 2	N	P	SP
5	B/1126/17	50	B	PMB	E	g/w to g/b stb 1x1cms	CIN 3	P	SP	SP
6	B/1366/17	48	B	PCB	E	g/w to g/b stb 0.5x0.5cms	CIN 3	P	WP	P
7	B/1367/17	45	B	PMB	E	g/w to g/b stb 0.5x0.5cms	CIN 2	A	WP	P
8	B/2028/16	28	TAH	WDPV	E	UR	CIN 2	N	P	P
9	B/2896/16	50	B	WDPV	E	g/w to g/b stb 2x2cms	CIN 3	P	P	P
10	B/1504/17	72	TAH	PMB	E	UR	CIN 3	N	WP	WP
11	B/1436/17	48	B	PMB	E	g/w to g/b stb 2x2cms	CIN 2	P	P	SP
12	B/1519/17	50	B	PMB	E	g/w to g/b stb 1x1cms	CIN 3	P	P	P
13	B/212/18	46	B	WDPV	E	g/w to g/b stb 0.5x0.5cms	CIN 3	N	P	SP
14	B/2277/17	53	B	PMB	E	g/w to g/b stb 0.5x0.5cms	CIN 3	P	WP	WP
15	B/1796/17	31	B	WDPV	E	g/w to g/b stb 1x1cms	CIN 3	N	WP	P
16	B/2390/17	51	B	WDPV	E	g/w to g/b stb 0.5x0.5cms	CIN 3	N	P	SP
17	B/269/17	51	B	AUB	E	g/w to g/b stb 0.5x0.5cms	CIN 3	P	SP	SP
18	B/2853/17	52	B	PMB	E	g/w to g/b stb 1x1cms	CIN 3	P	WP	WP
19	B/2786/17	50	B	WDPV	E	g/w to g/b stb 1x1cms	CIN 3	P	P	SP
20	B/2377/17	53	TAH	PMB	E	UR	CIN 2	A	WP	P
21	B/112/18	61	TAH	PMB	E	UR	CIN 3	P	P	P
22	B/712/18	40	TAH	WDPV	E	UR	CIN 2	P	P	P
23	B/1077/18	52	B	WDPV	E	g/w to g/b stb 1x1cms	CIN 3	P	P	SP
24	B/1107/18	40	B	PCB	E	g/w to g/b stb 1x1cms	CIN 3	P	WP	WP
25	B/968/18	35	B	WDPV	E	g/w to g/b stb 1x1cms	CIN 2	P	P	SP
26	B/2989/16	42	B	WDPV	E	g/w to g/b stb 1x1cms	CIN 2	P	P	SP

CN	B	AGE	CC	COLPOSCOPIC FINDINGS	GROSS	HPR	P16	Ki67	CD44
1	B/1140/17	40	AUB	UR	UR	NC	N	N	N
2	B/1193/17	40	AUB	UR	UR	NC	N	N	N
3	B/1506/17	42	AUB	UR	UR	NC	N	N	N
4	B/1225/17	43	AUB	UR	UR	NC	N	N	N
5	B/1250/17	27	AUB	UR	UR	NC	N	N	N
6	B/1253/17	50	AUB	UR	UR	NC	N	N	N
7	B/1267/17	47	AUB	UR	UR	NC	N	N	N
8	B/1268/17	60	AUB	UR	UR	NC	N	N	N
9	B/1296/17	45	AUB	UR	UR	NC	N	N	N
10	B/1280/17	65	AUB	UR	UR	NC	N	N	N
11	B/1286/17	45	AUB	UR	UR	NC	N	N	N
12	B/1290/17	49	AUB	UR	UR	NC	N	N	N
13	B/1297/17	50	AUB	UR	UR	NC	N	N	N
14	B/1302/17	47	AUB	UR	UR	NC	N	N	N
15	B/1316/17	50	AUB	UR	UR	NC	N	N	N
16	B/1328/17	65	AUB	UR	UR	NC	N	N	N
17	B/1332/17	50	AUB	UR	UR	NC	N	N	N
18	B/1354/17	49	AUB	UR	UR	NC	N	N	N
19	B/1356/17	50	AUB	UR	UR	NC	N	N	N
20	B/1395/17	45	AUB	UR	UR	NC	N	N	N
21	B/1400/17	68	AUB	UR	UR	NC	N	N	N
22	B/1937/16	60	AUB	UR	UR	NC	N	N	N
23	B/1918/16	50	AUB	UR	UR	NC	N	N	N
24	B/2089/16	45	AUB	UR	UR	NC	N	N	N
25	B/1339/17	65	AUB	UR	UR	NC	N	N	N
26	B/1340/17	46	AUB	UR	UR	NC	N	N	N