p16 AS A CIRCULATING BIOMARKER IN INVASIVE SQUAMOUS CELL CARCINOMA OF CERVIX

By

Dr. KALYANI R

(Registration No: 16PhD0401)

THESIS SUBMITTED TO

Sri Devaraj Urs Academy of Higher Education and Research

A Deemed to be University

Tamaka, Kolar, Karnataka.

For fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Under

FACULTY OF MEDICINE

DEPARTMENT OF PATHOLOGY

SRI DEVARAJ URS MEDICAL COLLEGE TAMAKA,

KOLAR, KARNATAKA-563101

INDIA.

SRI DEVARAJ URS MEDICAL COLLEGE,

TAMAKA, KOLAR-563101.

Department of Pathology

DECLARATION BY THE CANDIDATE

I hereby declare that the thesis titled **"p16 AS A CIRCULATING BIOMARKER IN INVASIVE SQUAMOUS CELL CARCINOMA OF CERVIX"** is a bonafide and genuine research work which I have carried out, under the supervision and guidance of Dr Raghuveer C.V, Professor, Department of Pathology, Sri Devaraj Urs Medical College, Sri Devaraj Urs Academy of higher Education and Research, Tamaka, Kolar

Date: Signature of the Candidate

Place: Dr. Kalyani. R.

SRI DEVARAJ URS MEDICAL COLLEGE,

TAMAKA, KOLAR-563101.

Department of Pathology

CERTIFICATE BY THE SUPERVISOR

This is to certify that the thesis entitled **"p16 AS A CIRCULATING BIOMARKER IN INVASIVE SQUAMOUS CELL CARCINOMA OF CERVIX"** is the bonafide record of research work done by Dr Kalyani R, Department of Pathology, Sri Devaraj Urs Medical College, Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnataka, under my guidance and supervision, in fulfilment of the requirement for the degree of **Doctor of Philosophy**.

Date:

Place:

Signature of the Supervisor

Dr. Raghuveer. C. V. MD, DCP,

Professor, Department of Pathology,

SDUMC

Pro Chancellor, SDUAHER,

Kolar, Karnataka.

Former Vice Chancellor, SDUAHER,

Kolar, Karnataka.

Former Registrar & Pro Vice Chancellor,

Yenepoya Deemed to be University.

Former Dean Kasturba Medical College,

Former Dean & Medical Director,

SIMSR, Mangalore.

SRI DEVARAJ URS MEDICAL COLLEGE,

TAMAKA, KOLAR-563101.

Department of Pathology

CERTIFICATE BY THE CO-SUPERVISOR

This is to certify that the thesis entitled **"p16 AS A CIRCULATING BIOMARKER IN INVASIVE SQUAMOUS CELL CARCINOMA OF CERVIX"** is the bonafide research work carried out by Dr Kalyani R, Department of Pathology, Sri Devaraj Urs Medical College, Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnataka, in fulfilment of the requirement for the degree of **Doctor of Philosophy.**

Date:

Place: Kolar

Signature of the Co-Supervisor

Dr. S .R. Sheela. MS

Professor, Department of OBG,

Medical Superintendent,

R.L.Jalappa Hospital & Research Center, Kolar

	SRI DEVARAJ URS MEDICAL COLLEGE, **TAMAKA, KOLAR-563101.** **Department of Pathology**	

ENDORSEMENT BY THE HOD/PRINCIPAL/DEAN

This is to certify that the thesis entitled **"p16 AS A CIRCULATING BIOMARKER IN INVASIVE SQUAMOUS CELL CARCINOMA OF CERVIX"** is the bonafide research work done by Dr Kalyani R, Professor & Head, Department of Pathology, under the guidance of Dr. Raghuveer C.V, Professor, Department of Pathology, Sri Devaraj Urs Medical College, Pro-chancellor, Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnataka, in fulfilment of the requirement for the degree of Doctor of Philosophy.

Signature of the Principal/Dean,
Dr. Sreeramulu.P.N. MS
Professor,
Department of Surgery,
R.L.Jalappa Hospital & Research Center,
Principal & Dean,
SDUMC, SDUAHER, Tamaka, Kolar.

COPY RIGHT

I hereby declare that the Sri Devaraj Urs Academy of Higher Education and Research Center, Tamaka, Kolar ,Karnataka, shall have rights to preserve, use and disseminate the thesis entitled on **"p16 AS A CIRCULATING BIOMARKER IN INVASIVE SQUAMOUS CELL CARCINOMA OF CERVIX."** in print or electronic format for academic and research purposes.

Date: Signature of the Candidate

Place: **Dr. Kalyani. R.**

SDUMC, SDUAHER

ACKNOWLEDGMENT

I thank my respected Supervisor Professor Dr C V Raghuveer: I take this opportunity to express my heart felt gratitude and appreciation for his valuable guidance and supervision supporting throughout the whole process. The result of my hard work with his blessings it came to a shape.

I thank my Co-Supervisor Dr.Sheela S.R, who constantly supported me wherever I required. I am thankful to her.

I extend my heartfelt gratitude for the support given by Faculties and Postgraduates of Department of Pathology.

I thank Dr. Sharath B, Department of Cell Biology and Molecular Genetics, for immense help and support.

I thank Dr. Krishnaveni. C, Department of Anatomy, for all her support.

I thank all the patients of cervical cancer and their families to support this project by giving their samples without which I could not have completed my project.

I thank all administrators and management for giving me this opportunity to proceed with my project successfully. I am very grateful for one and all.

I am thankful to everyone who helped me indirectly and directly in this work to complete Ph.D. successfully.

Date: Signature of the Candidate

Place: Kolar **Dr. Kalyani R**

LIST OF ABBREVIATIONS

Sl. No.	ABBREVIATED	EXPANSION
1.	HR-HPV	High Risk Human Papilloma Virus
2.	DNA	Deoxyribonucleic Acid
3.	CfDNA	Cell free DNA
4.	CtDNA	Circulating tumour DNA
5.	RNA	Ribonucleic Acid
6.	INK family	Inhibitor Kinase family
7.	CDK	Cyclin Dependent Kinase
8.	CIN	Cervical Intraepithelial Neoplasia
9.	LAST	Lower Anogenital Squamous Terminology
10.	LOH	Loss Of Heterozygosity
11.	SCC	Squamous Cell Carcinoma
12.	WDSCC	Well Differentiated SCC
13.	MDSCC	Moderately Differentiated SCC
14.	PDSCC	Poorly Differentiated SCC
15.	NKLCSCC	Non-Keratinizing Large Cell Differentiated SCC
16.	NKSCSCC	Non-Keratinizing Small Cell Differentiated SCC
17.	IHC	Immunohistochemistry
18.	ELISA	Enzyme Linked Immunosorbent Assay
19.	RT-PCR	Real Time Polymerase Chain Reaction
20.	EDTA	Ethylenediaminetetraacetic acid
21.	DAB	Diaminobenzidine

22.	HRP	Horse Raddish Peroxidase
23.	TBS	Tris Buffer Solution
24	DPX	Dibutylphthalate Polystyrene Xylene
25.	HSIL	High Grade Squamous Intraepithelial Lesion
26.	CEA	Carcinoembryonic Antigen
27.	PPV	Positive Predictive Value
28.	NPV	Negative Predictive Value
29.	POCT	Point Of Care Test
30.	AUC	Area Under ROC Curve
31.	SPSS	Statistical Package for the Social Sciences
32.	ROC	Receiver Operating Characteristic Curve
33.	CTC	Circulating tumour cells
34.	Rb	Retinoblastoma
35.	SCJ	Squamocolumnar junction
36.	TZ	Transition zone
37.	IUCD	Intrauterine contraceptive device
38.	CIS	Carcinoma in situ
39.	OCP	Oral contraceptive cells
40.	DES	Diethylstilbestrol
41.	WHO	World Health Organization
42.	NOS	Not otherwise specified
43.	SIL	Squamous intraepithelial lesion
44.	ASCUS	Atypical squamous cell of undetermined significance

45.	DFS	Disease free survival
46.	FDA	Food & Drug administration
47.	CLIA	Clinical laboratory improvement amendments
48.	EGFR	Epidermal growth factor receptor
49.	NSCLC	Non-small cell lung carcinoma
50.	ddPCR	Droplet digital polymerase chain reaction
51.	SDUAHER	Sri Devaraj Urs Academy of Higher Education and Research
52.	ANOVA	Analysis of variance
53.	SD	Standard deviation
54.	FIGO	Federation of International Gynecologists and Obstetricians
55.	OD	Optical density
56.	CI	Confidence interval
57.	ELB	Erythrocyte lysis buffer
58.	TE	Tris EDTA
59.	Ct	Cycle threshold
60.	H&E	Hematoxylin and Eosin

LIST OF CONTENTS

LIST OF THE TABLES

LIST OF THE FIGURES

1. INTRODUCTION

Cervical cancer is the commonest gynecological malignancy worldwide. It has the second highest mortality and morbidity rates among all tumors of female genital tract.[1] Worldwide cervical cancer is the fourth most common cause of cancer and fourth most common cause of deaths in women. Worldwide 570,000 cases of cervical cancer were estimated to have occurred with over 300,000 deaths. New cases of cervical cancer detected in India were 96,922 in year 2018 and deaths due to cervical cancer in India reported were 60,078. In Karnataka alone 5,000 new cases are identified each year. In Bangalore, the capital of Karnataka state, cervical cancer is the second most frequent cancer among women with 850 cases being diagnosed every year.[2] The frequency reported by Kidwai Memorial Institute of Oncology, Bangalore between 2004-05 was 15.9%.[3] As per the National Cancer Registry Programme newsletter, the frequency of cervical cancer between 2001-2011 ranged from 6.2 to 22.6 in India and in Bangalore it was 21.1.[4] In a study done at Kolar, 17% cases were diagnosed as cervical carcinoma among all the cancers in females.[5] Human Papilloma virus (HPV) is a DNA (Deoxyribonucleic acid) Virus. HPV-16 and HPV-18 are the high-risk (HR-HPV) genotypes for cervical carcinoma. Integration of viral genome into the host genome with acquisition of other genetic abnormalities results in malignant transformation.[6]

Routine Pap smear test is a screening test to detect the cervical cancer at precancerous stage. However it is limited by its false negative rates and low sensitivity. HPV DNA test also used as a screening test but has limited specificity.[6] HPV infection of cervix causes integration of viral genome with host genome and release of E7 protein which causes degradation of Retinoblastoma protein and increased expression of p16 gene as feedback mechanism resulting in immortalization of host cells. Thus p16 biomarker is proved as a surrogate marker of cervical cancer in in-situ / non-invasive cancer as well as in advanced

cancer and is expressed in the cervical biopsy.[7,8]

Liquid biopsy is a relatively non-invasive procedure where plasma is used for early detection and diagnosis of cancer. The samples which can be used in liquid biopsy are, blood, plasma, serum, urine, body fluids, saliva, etc. The components of liquid biopsy are; circulating tumour cells (CTC), cell free DNA (cfDNA)/circulating tumour DNA (ctDNA), mRNA (Ribonucleic acid), exosomes and proteins. Liquid biopsy has advantage over tissue biopsy especially in tumours which are difficult to assess through biopsy, where repeated follow-up is required, to trace the genetic alteration in tumour, early detection of resistance for chemotherapy and plan the targeted chemotherapy. In addition, it helps to assess the residual disease and its recurrence.[9]

2. NEED FOR THE STUDY

Cervical cancer is one of the commonest cancers in women worldwide especially in developing countries. It is the commonest cause of cancer deaths in females. The prevalence of cervical cancer in this region is 17% of total cancers in females.[5]

HR-HPV is a proved etiological factor of cervical cancer. Infection of HR-HPV causes integration of viral genome to host genome resulting in release of E7 protein by viral genome which act on host cells and causes degradation / functional inactivation of Retinoblastoma (Rb) protein at the G1/S phase of the cell cycle, resulting in early immortalization of epithelial cells, rapid proliferation of cells, dysplastic changes and finally malignant transformation. Degradation / functional inactivation of Rb protein causes increased expression of p16 through p16–Rb pathway as a feedback mechanism.[10,11]

p16 is an effective biomarker of cervical cancer. It improves the reproducibility of evaluation, detects the precancerous lesions, used as primary screening test and adapted to the computerized image analysis techniques.[8] p16 positive immunohistochemistry (IHC) expression are reported in high grade cervical intraepithelial lesion, squamous cell carcinoma, adenocarcinoma-in-situ and adenocarcinoma. p16 is a surrogate marker of HPV infection and p16 detection may serve as an adjunct test in the diagnosis of in-situ lesions and invasive cervical cancer.[8]

Estimation of p16 protein by Enzyme Linked Immunosorbent Assay (ELISA) on lysed samples of cervical cells are reported. The p16 protein levels had positive association with high grade squamous intraepithelial lesion (HSIL) and levels were low in low grade squamous intraepithelial lesion (LSIL) cases and in normal cervix samples.[12,13] However

there are no study reports regarding estimation of p16 protein in blood samples in cervical cancer.

Cytogenetic studies are done in cervical cancer. Mutation of PIK3CA, KRAS, ZFHX3, KMT2C, KMT2D, NSD1 and RNF213 are reported. Alteration in p16 gene is also reported. One study has reported p16 gene methylation in tissue and plasma samples.[7]

Liquid biopsy is a relatively non-invasive procedure where, plasma / serum, body fluids, saliva and urine are used to detect CTC, cfDNA, ctDNA, mRNA, exosomes and protein. It is useful in early detection of cancer, diagnosis of cancer, assess residual disease and recurrence. However, only a few studies are published with respect to cervical cancer & liquid biopsy.[7] As far as our knowledge goes, there are no studies regarding p16 gene expression in squamous cell carcinoma (SCC) of cervix in paired samples of tissue and plasma to assess role of liquid biopsy.

Hence we have taken the study where tissue and plasma samples (paired samples) in each case were evaluated for p16 protein and p16 gene amplification. The results of p16 protein levels and p16 gene expression of tissue and plasma samples were analyzed and correlated to assess the possible role of the concept of liquid biopsy in SCC of cervix.

3. AIM AND OBJECTIVES OF THE STUDY:

Aim:

To assess the role of p16 biomarker as liquid biopsy in invasive SCC of cervix.

Objectives:

1. To evaluate p16 marker expression in tissue sections by IHC in SCC of cervix.
2. To quantitatively estimate p16 protein in plasma by ELISA in SCC of cervix.
3. To evaluate p16 gene amplification in tissue DNA by RT-PCR (Real Time-Polymerase Chain Reaction) in SCC of cervix.
4. To evaluate p16 gene amplification in the plasma DNA by RT-PCR in SCC of cervix.
5. To analyze the association of findings in tissue with that in plasma.

Research Question:

Can p16 protein in plasma and / or p16 DNA in plasma be used as a circulating biomarker as liquid biopsy in invasive SCC of cervix.

4. REVIEW OF THE LITERATURE

4.1 ANATOMY:

Uterus is divided into three parts anatomically: Corpus, isthmus and cervix. The protruding part of cervix into vagina is called as external os. Endocervix opens internally into uterus and externally opens into vagina.[14]

4.2 HISTOLOGY:

Endocervix:

Endocervical lining consists of monolayered mucin secreting columnar epithelium. Endocervical glands and endocervical canal is also lined by monolayered columnar epithelium.[14]

Ectocervix:

Ectocervix is lined by mature non-keratinizing stratified squamous epithelium. Epithelial layer is divided into three layers: Germinal/parabasal cell layer, intermediate cell layer and superficial cell layer. Germinal cell layer helps in continuous epithelial regeneration. Major part of epithelial lining is formed by midzone. Most mature cell is observed in superficial layer.[14]

TRANSFORMATION ZONE:

Squamo-columnar junction (SCJ) of cervix is defined as the junction between ectocervical stratifiedsquamous epithelium and endocervical columnar epithelium. There are two types of SCJ; original SCJ which is present at birth and SCJ which develops during menarche/physiological. The area between original SCJ and physiological SCJ is called as

the Transformation zone (TZ). All Cervical cancers and precursor lesions of cervix arise from TZ (Figure 1).[14]

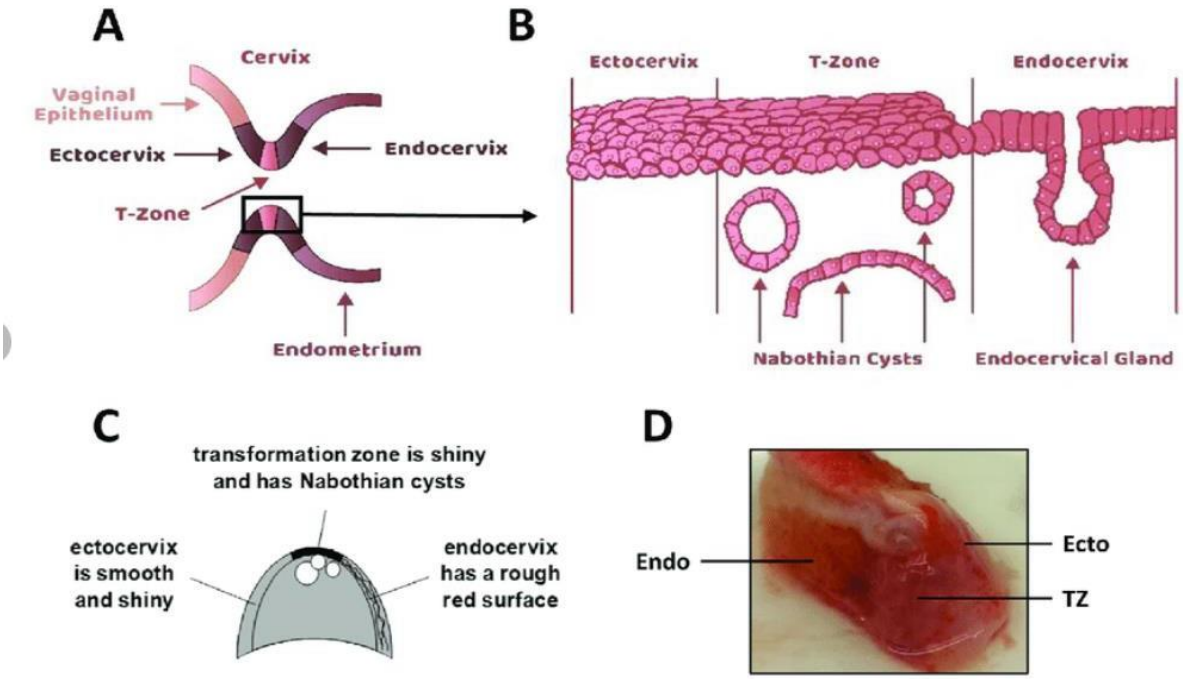


Figure 1: Structure and Histology of Transformation zone (TZ) of cervix.[15]

A. Schematic diagram demonstrating the TZ between ectocervix and endocervix.

B. Histology of the cervical TZ depicting stratified squamous epithelium and Nabothiancysts.

C. Diagram representing the surface features of ectocervix, endocervix and TZ that aid intissue dissection.

The ectocervix surface is smooth, white and shiny, without mucous.

The endocervix surface is red in color, rough surface and covered with mucous.The TZ consists of Nabothian cysts.

D.Photograph of a cervical specimen demonstrating different region.

4.3 CERVIX PATHOLOGY:

INFLAMMATORY DISEASES:

CERVICITIS: is an inflammation of the cervical tissue. Based on the etiology, cervicitis is further subdivided into two subgroups: i.e non-infectious cervicitis and infectious cervicitis.[16]

NON-INFECTIOUS CERVICITIS: It is defined as non-specific inflammatory condition attributed to chemical and mechanical trauma. Common etiological factors are: Pessaries, Diaphragms, Tampons and intrauterine contraceptive devices (IUCD). Iatrogenic cause such as instrumentation can also lead to non-infectious cervicitis. Other causes are chemical irritation which occurs secondarily to douching.[16]

Histomorphology of Non-Infectious

Acute Cervicitis: shows epithelium infiltrated by neutrophils. Stroma also shows neutrophilic infiltration, stromal edema, dilated and congested blood vessels. These features are suggestive of acute cervicitis.[16]

Chronic Cervicitis:

Microscopically shows epithelial infiltration by lymphocytes and plasma cells. Stromal also shows lymphoplasmacytic infiltration. Occasionally fibrosis of stroma, histiocytes and granulation tissue can be observed.[16]

INFECTIOUS CERVICITIS:

Etiological factors:

A) Bacteria: Mycobacterium tuberculosis, Chlamydia trachomatis,Niesseria gonorrhea.

B) **Virus:** Herpes simplex virus, Human papilloma virus

C) Fungus: Candida, Aspergillus

D) Parasites: Amoeba, Schistostomes.[14]

PRECANCEROUS CONDITIONS OF CERVIX:

All precursor lesion of SCC of cervix is represented as a single disease process termed as Cervical intraepithelial neoplasia (CIN).

CIN is divided into three subgroups:

CIN1: Mild dysplasia CIN2: Moderate dysplasia

CIN 3: Carcinoma in situ (CIS) or Severe dysplasia

In cytology, Betheseda system defines LSIL for CIN1 and HSIL for CIN 2 and CIN 3. This system is widely utilized for reporting of cytological pap smears.[17]

College of American Pathologists along with American society for colposcopy and Pathologysuggested utilization of two tier system LSIL and HSIL in accordance with LAST (lower anogenital squamous terminology) 2012 which is used for histopathological reporting of precancerous lesions of SCC of cervix.[18]

LSIL: features consist of koilocytosis, epithelial hyperplasia and nuclear atypia. These features are associated with HPV infection and it is restricted to lower one third of the squamous cell layer. Koilocytes are cells with perinuclear halo and thickened cytoplasmic membrane. 80 % of LSIL cases are self-limiting and do not progress to High grade lesions and carcinoma.[19]

HSIL: CIN 2 and CIN3 features comprise of atypia in squamous epithelial layers affecting intermediate and superficial layers. Atypia is more significantly observed in basal and

parabasal cells along with presence of mitotic figures. Additional features include anisonucleosis, nuclear pleomorphism and loss of Polarity with cellular crowding. Around 8% of HSIL cases develop into cervical carcinoma particularly in women with age more than 30 years.[19]

CERVICAL CANCER:

Cervical cancer is the common cancer of female genital tract. Peak age range for cervical cancer reported is 55 -59 years with average age of presentation of 52 years. The risk factors for carcinoma cervix are HPV infection, sexually transmitted infections, coitus before 18 years of age, multiparity, poor personal hygiene, poor socioeconomic status, smoking, immunosuppressed individuals and women on oral contraceptive pills (OCP). Other risk factors are; Progesterone therapy taken for a long period of time predisposes to adenocarcinoma of cervix and in utero exposure to Diethylstilbestrol (DES).[20]

WHO CLASSIFICATION OF UTERINE CERVICAL TUMOURS: (2020)[21]

I) Squamous epithelial tumors:

A) Mimics of squamous precursor lesions Squamous metaplasia Atrophy of the uterine cervix

B) Squamous cell tumors and precursors Condyloma acuminatum

Squamous intraepithelial lesions of the uterine cervix

SCC, HPV associated, of the uterine cervix

SCC, HPV independent, of the uterine cervix

SCC, Not otherwise specified (NOS) of the uterine cervix

II) Glandular tumors and precursors

A) Benign glandular lesions Endocervical polyp Mullerian papilloma of the uterine cervix Nabothian cyst Tunnel clusters Microglandular hyperplasia Lobular endocervical glandular hyperplasia Diffuse laminar endocervical hyperplasia Mesonephric remnants and hyperplasia Arias

Stella reaction of the uterine cervix Endocervicosis of the uterine cervix Tuboendometrioid metaplasia

B) Adenocarcinomas

Adenocarcinoma in situ, HPV associated, of the uterine cervix Adenocarcinoma, HPV associated, of the uterine cervix Adenocarcinoma in situ, HPV independent, of the uterine cervix Adenocarcinoma, HPV independent, gastric type, of the uterine cervix Adenocarcinoma, HPV independent, clear cell type, of the uterine cervix

Adenocarcinoma, HPV independent, mesonephric type, of the uterine cervix Other adenocarcinomas of the uterine cervix

C) Other epithelial tumors Carcinosarcoma of the uterine cervix

Adenosquamous and mucoepidermoid carcinomas of the uterine cervix Adenoid basal carcinoma of the uterine cervix Carcinoma of the uterine cervix, unclassifiable D) Mixed epithelial and mesenchymal tumors Adenomyoma of the uterine cervix Adenosarcoma of the uterine cervix

E) Germ cell tumors:

Germ cell tumors of the uterine cervix.

SQUAMOUS CELL CARCINOMA (SCC):

MICROINVASIVE SCC:

Diagnosed only by microscopy and not by gross features. Maximum depth of invasion is 5 mm and horizontally does not extend beyond 7 mm. Microinvasive SCC corresponds to Stage IA of FIGO Staging.[22]

Patients are usually asymptomatic. On gross examination cervix is normal or may manifest with erosion or chronic cervicitis. On colposcopy acetowhite areas are noted similar to HSIL

and haphazard branching vessels may be observed. On microscopy, the basement membrane is breached by infiltration of malignant tumor cells into cervical stroma. Rest of the cervical epithelium shows features of squamous intraepithelial lesion (SIL). Ragged shape/contour of the invading margin is considered the most specific criteria for microinvasion.[23]

INVASIVE SCC:

CLINICAL FINDINGS:

Clinical manifestation of patients of invasive carcinoma cervix depends on the stage of lesion and size of the lesion. Most common presentation is abnormal vaginal bleeding. Other most common significant presentation is post-coital bleeding. Other clinical findings include: frank hemorrhage, serosanguinous discharge and intermittent spotting. Locally advanced cervical cancer patients present with pallor, fatigue, pedal edema, weight loss, dysuria and hematuria.[24]

MACROSCOPY OF INVASIVE SCC:

Cervical cancers frequently present as exophytic, polypoidal or fungating growth. Focal ulceration, focal induration, raised granular areas that bleeds on touch are noted in early lesions of cervical cancers. On palpation induration can be noted.[24]

Endophytic cervical carcinomas are ulcerative or nodular. Endophytic carcinoma grows within the endocervical canal resulting in large sized barrel shaped cervix. Endophytic cervical carcinomas are noted in advanced stages of the disease because they have clinically occult manifestation and, in these cases, sampling is not feasible because of the late clinical presentation.[24]

MICROSCOPY OF INVASIVE SCC:

Invasive SCC has variable patterns of growth, different cell type and degree of

differentiation. All variants of SCC of cervix have HPV infection as a common etiological factor.[24]

Microscopically neoplastic cells infiltrate the stroma in form of irregular ragged cords. Tumour cells are polygonal in shape with eosinophilic cytoplasm with a well-defined cell membrane. Intercellular bridges may also be noted. Nuclear pleomorphism is observed with coarse chromatin and mitotic figures can be seen occasionally.[24]

The histomorphology of SCC is classified as keratinized and non-keratinized types. Keratinized type was further classified as well differentiated SCC (WDSCC), moderately differentiated SCC (MDSCC) and poorly differentiated SCC (PDSCC). Non-Keratinized type was further classified as non-keratinised Large Cell (NKLCSCC) and non-keratinised Small Cell (NKSCSCC) types.[21]

OTHER VARIANTS OF SCC:

1) **BASALOID SCC:** Shows tumor cells arranged in nest. Basal cell type with increased mitotic figures. Geographic necrosis is a frequent finding.[24]
2) **VERRUCOUS SCC**: Clinically similar to condyloma. Slow growing cancer. Common characteristic findings are hyperkeratotic, warty surface with pushing borders. Cells do not have atypia and koilocytotic change. Mitotic figures are not seen. Inflammatory cell infiltrates are noted at the junction of epithelium and stroma. Wide local excision is the most preferred treatment. Increased incidence of recurrence is noted but rarely these tumors metastasize.[24]
3) **WARTY SCC:** Also known as condylomatous carcinoma. Features are warty surface resembles to condyloma on low power. Tumor cell shows nuclear changes that is similar to koilocytotic atypia and cell have vacuolated cytoplasm. It is a less aggressive

variant.[24]

4) **PAPILLARY SCC:** On Microscopy, atypical cells are arranged in papillary pattern. Cells are basaloid in nature similar to that of HSIL. Cells have hyperchromatic ovoid nuclei with scant cytoplasm. Mitotic figures are frequently encountered. Focal area of squamous differentiation may be noted.[24]

ADENOCARCINOMA: Constitutes of about 10-25% of cervical carcinoma. It is seen in association with OCP. HR-HPV is observed in 94% of cervical adenocarcinoma cases. Most common HR-HPV is HPV 18. Most common manifestation is abnormal uterine bleeding which is observed in around 75% of patients. Few patients present with vaginal discharge.[24]

MACROSCOPY: In 50 % of the patients, exophytic polypoidal or fungating growth is seen. Other 50% cases show nodular or diffuse infiltrative growth. Grossly, lesion may not be seen in 15% of cases.[24]

MICROSCOPY:

1. **ENDCOERVICAL ADENOCARCINOMA USUAL TYPE:**

   This is the most common variant observed in 90% of Cases. Tumor cells show moderate to well differentiated cells with complex glandular pattern arrangement. Cells are round to ovoid in shape without mucin formation and shows characteristic pseudostratification. Nucleus is elongated and hyperchromatic with prominent nucleoli. Mitotic figures are seen.[24]

2. **VILLOGLANDULAR CARCINOMA:** It is characterized by villous and papillary folds. Lining epithelium consists of endocervical columnar cells demonstrating mild moderate atypia. Mitotic figures are frequently seen.[24]

3. **ENDOMETROID CARCINOMA:** Consists of tumor cells similar to those of primary adenocarcinoma of uterus. Cells show stratification consisting of round to ovoid shaped nuclei. These tumor cell do not have mucin and comprises of scant amount of cytoplasm as compared to endocervical carcinoma of usual type. It can be differentiated from endometrial carcinoma by using p16 marker.[24]

4. **CLEAR CELL CARCINOMA:**

   It comprises of 4% cases of cervical carcinoma. It has association with in-utero DES exposure. It can also occur in women not exposed to DES.

MICROSCOPY: There are three patterns: Solid, papillary and tubulocystic. Tumor cells have abundant clear to granular eosinophilic cytoplasm. Clearing of cytoplasm is due to glycogen accumulation. Nucleus shows high pleomorphism, hyperchromatic nucleus projecting into the lumen giving a Hobnail appearance.[24]

5. **SEROUS CARCINOMA:**

   It is a rare variant of adenocarcinoma. It is a diagnosis of exclusion. Cells are arranged in papillary pattern depicting nuclear atypia. Psammoma bodies can also be noted.[24]

6. **ADENOCARCINOMA ADMIXED WITH NEUROEN DOCRINE CARCINOMA:**

It is a rare variant and shows cervical adenocarcinoma demonstrating neuroendocrine differentiation.[24]

OTHER EPITHELIAL TUMOURS:

1. **ADENOSQUAMOUS CARCINOMA:**

   Malignant epithelial tumor comprising of glandular cells and squamous cells. Occurs in young as well as old women. Squamous cell shows well differentiated squamous cells along- with keratin pearls with individual cell keratinization. Suffice glandular component differentiation of adenocarcinoma component should be noted for

establishing diagnosis of adenosquamous carcinoma.[24]

2. GLASSY CELL CARCINOMA:

It is poorly differentiated adenosquamous carcinoma accounting for 1% cases of cervical carcinoma. Cells are uniform, large and polygonal with ground glass type of chromatin. Cells have a well-defined cell membrane and prominent nucleoli. Dense lymphoplasmacytic infiltrate cells are seen in the stroma.[24]

3. UNDIFFERNTIATED CARCINOMA:

In this variant, tumor cells are arranged in sheets. It lacks squamous differentiation and glandular formation.[24]

4.4 p16 BIOMARKER:

p16 gene is a tumour suppressor gene, located on chromosome 9 band p21.3. It belongs to inhibitor kinase family (INK4 family) of cyclin dependent kinase (CDK) inhibitor proteins.[25,26] In healthy person it functions as a tumour suppressor gene acting on the cell cycle in association with CDK4/6 and Rb protein at the rate limiting step G1/S phase of the cell cycle (Figure 2).[10,11]

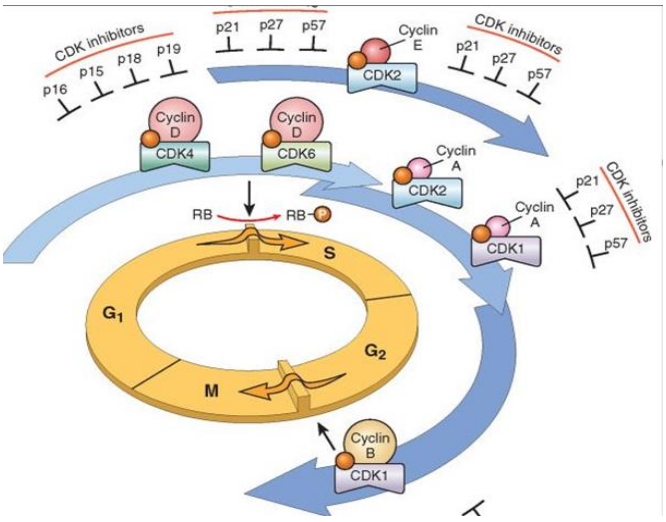


Figure 2: Role of p16 protein in cell cycle at G1-S phase.[27]

Infection of cervix with HR-HPV causes integration of viral genome to host genome followed by, synthesis and release of E7 protein by viral genome which act on host cells and causes degradation / functional inactivation of Rb protein at the G1/S phase, a rate limiting step of the cell cycle, resulting in early immortalization of epithelial cells, rapid proliferation of cells, dysplastic changes and finally malignant transformation. Degradation / functional inactivation of Rb protein causes increased expression of p16 in p16–Rb pathway as a feedback mechanism to stop the cell proliferation.[10,11]

In normal cervix, p16 marker expression reported is 2%. In CIN I, CIN II, CIN III and in invasive cervical cancer the p16 gene expression is 38%, 68%, 82% and 96% respectively. Thus, p16 acts as a surrogate marker for cervical cancer. However, there is no uniform method of interpretation of p16 expression in tissue sections across the globe. Thus there is lack of standardization for interpretation of p16 marker expression.[12,26]

As per LAST criteria, p16 nuclear expression with or without cytoplasmic expression is considered as positive staining. In 2012, LAST classified the p16 marker expression on tissue sections as block positive, ambiguous and negative expression.[28] Block positive pattern of staining means, the expression is strong, continuous, nuclear positivity with or without cytoplasmic staining, and the staining extending from basal layers upwards for at least 1/3rd thickness of the epithelium (basal & parabasal layers) which can be further graded as 1/3rd, 2/3rd and more than 2/3rd and laterally extending over a significant distance (diffuse >25% of cells with staining). Ambiguous staining means, strong and basal (strong, diffuse, continuous, involves lower 1/3rd without upward extension) or weak, diffuse and discontinuous staining, involving at least 2/3rd of the epithelium or strong, focal and discontinuous located at any level of the epithelium. Negative staining means total absence or weak or focal and discontinuous or only cytoplasmic staining.[28]

USES OF EVALUATION OF p16 BIOMARKER

1. p16 biomarker can be used as adjuvant for cervical cancer screening and useful in undetermined morphology. However the marker is not used as standalone marker in US, instead used in conjunction with other markers as Ki67.[29]
2. p16 and Ki67 markers stain increases the reproducibility and specificity. The sensitivity and specificity of p16Ki67 to detect CIN2+ reported is 90.9% and 79.5% respectively. With atypical squamous cells of undetermined significance (ASCUS) and LSIL, the sensitivity and specificity to detect CIN2+ is 87.5% and 66.4% respectively.[30]
3. p16 biomarker is used for triage method.[31]
4. p16 marker expression is surrogate marker for early detection of carcinoma cervix. p16 biomarker is positive in 90% and 76.9% in squamous cell carcinoma and adenocarcinoma respectively.[31] Diffuse p16 staining is reported in squamous cell carcinoma especially high grade.[30] The expression of p16 has role in the pathogenesis and progress of cancer. It can be used as a diagnostic marker.[32]
5. When follicular cervicitis is associated with CIN, it is difficult to diagnose histomorphologically because of reactive and regenerative epithelial changes. In addition, CIN+ associated with follicular cervicitis and other inflammation is described histomorphologically as “spongiotic CIN” and “thin CIN” and mimics reactive lesion. Cervical inflammation is also a cofactor in pathogenesis of cervical neoplasia. p16 biomarker is of help as the expression is weak and patchy in LSIL and reactive lesions as atrophy and squamous metaplasia, while p16 expression is strong and diffuse (block positive) in HSIL as per the LAST classification.[33]
6. As per LAST, CIN2 has equivocal morphology and biology; p16 positive CIN2 cases are associated with subsequent transformation to CIN3 unlike p16 negative CIN2 cases.[34]

p16 positive lesions most likely progress to higher grades and thus can be used for triaging women with low grade lesion.[12,13]

7. p16 expression is associated with better prognosis and disease free survival.[31]
8. p16 biomarker has increased diagnostic performance, interobserver reliability, reproducibility and accuracy / precision of reports by which the patients get correct treatment.[8,35]
9. The rate of recurrence and death were higher in low expression (=/<80%) group than high expression (>80%) group of p16 cases. Patients with high p16 expression had high five year overall survival and five year disease free survival compared with low expression.[36] Hence high grade, non-SCC and low p16 expression are the independent prognostic factors for cervical cancer regarding disease free survival (DFS). Five year specific survival and DFS in p16 positive vs negative expression cases were 63% vs 33% and 57% vs 34% respectively. Five year specific survival and DFS in high p16 expression vs low p16 expression cases were 62% vs 35.2% and 60% vs 31.2% respectively. Multivariate analysis has stated that low p16 expression is a poor predictor for DFS in cervical cancer treated with radiotherapy. p16 can be considered as an indicator for radiosensitivity.[36]
10. Due to p16 anti-cancer activity, the molecule can be exploited for development of targeted chemotherapy in cervical cancer.[36]
11. p16 test can be used as primary screening test and adapted to the computerized image analysis techniques.[8]

CONTROVERSIAL ISSUES REGARDING p16 BIOMARKER

1. p16 marker cannot be used as reliable prognostic tool as whole of the cervix cannot be evaluated especially for low grade lesions.[31]

2. The interpretation of p16 biomarker is not standardized and heterogeneous which depends on choice of primary antibody by which defining positive or negative result is difficult and not clear. Hence specific guidelines are required to prevent diagnostic and treatment errors.[29]
3. In one study the authors did not find significant correlation between p16 expression and tumor grade and size.[31]
4. However p16 is sporadically positive in some benign squamous and glandular lesions of cervix including squamous metaplasia. Hence one should be diligent enough to evaluate the expression of P16 in cervical biopsy.[8]

EXPRESSION of p16 BIOMARKER IN OTHER CANCERS

Expression of p16 biomarker is reported in gynecologic tumours related and unrelated to HPV (non-HPV related tumours), oropharangeal squamous cell carcinoma and carcinoma of breast, pancreas, colon, melanomas and head & neck region (related to smoking). p16 shows strong and diffuse expression in uterine serous carcinoma, high grade serous carcinoma of mullerian origin, malignant mixed mullerian tumours and undifferentiated carcinoma. All these tumours are HPV unrelated.[37] Positive expression of p16 is observed in some tumours as liposarcoma, gastric adenocarcinoma, Hodgkin & non-Hodgkin lymphomas, pulmonary adenocarcinoma, neuroendocrine carcinoma and subset of uterine carcinoma. The pathogenesis in these cases are HPV independent mechanisms probably gene deletion, point mutation, functional mutations or other mechanisms in pRb pathway as inactivation of pRb resulting in increased expression of p16. Therefore p16 is not 100% specific for proving HPV tumourigenesis.[37]

4.5 p16 GENE EXPRESSION

Alteration of p16 gene are reported in cervical cancer. They are methylation, deletion, point mutation, LOH (Loss of heterozygosity) and amplification.[10] In one study, deletion of p16 gene increased the resistance for Cisplatin and radiotherapy to cervical cancer cells.[36] One study have reported methylation of p16 gene in cervical cancer in 28.2% cases in tissue DNA and 10% cases in plasma DNA.[7,12]

4.6 LIQUID BIOPSY

Tumour diagnosis is conventionally done by radiological findings and invasive surgical biopsy. In surgical biopsy or tissue biopsy a small chunk of tissue is taken from the cancer tissue for histopathological examination and diagnosis. Of late non-invasive technique where blood sample, urine and body fluids are used to extract CTC and genetic material for cancer diagnosis and treatment which is called as "Liquid Biopsy".[38,39] In this technique the liquid sample is used to isolate CTC, ctDNA, cfDNA, RNA, exosomes and proteins which are shed by tumour cells into blood circulation, body fluids or urine in most of the cancers depending on the site of the cancer. This technique enables non-invasive profiling of solid tumours, the results of which can be comparable with that of tissue biopsy.[9, 40,41,42,43,44,45,46]

Tissue biopsy gives only spatially and temporary snap shot of genetic makeup of cancer tissue unlike liquid biopsy, where samples can be taken at repeated intervals and it reveals the dynamic and heterogeneity of the cancer tissue.[45]

Originally liquid biopsy was used to analyze CTC. At present it is mainly used to analyze ctDNA. However CTC and ctDNA are complementary technologies which can be used in parallel. As ctDNA is a potential surrogate for the entire tumour genome, it is many times referred as "Liquid Biopsy".[47,48]

HISTORY OF LIQUID BIOPSY

Liquid biopsy has evolved slowly through different phases. Scientists isolated tumour cells in blood for the first time in 1869.[49] In 1948 Mandel and Metais isolated ctDNA and RNA in blood in healthy individuals. The neoplastic characteristics of these genetic material was defined after 30 years by Leon et al and 10 years later by Stroun et al.[46,48] The CellSearch technique to isolate the CTC was approved by US FDA in 2004.[49] Tissue DNA test was available by 2012.[44] Exosome diagnostic laboratory launched Clinical Laboratory Improvement Amendments (CLIA) certified test for isolation of exosomes in 2015.[50] US FDA approved cobas EGFR Mutation test v2 in 2016 for the lung cancer.[39]

ADVANTAGES OF LIQUID BIOPSY

Liquid biopsy is simple, safe, reliable, cost effective, quick, convenient, minimally painful and defines the highly sensitive and specific biomarker for the disease.[40,48,51,52] Liquid biopsy is minimally invasive procedure and an alternative method in cases where tissue biopsy is not available, insufficient or difficult to obtain or cannot be obtained safely, site difficult to assess, primary tumour spreads to bone / brain / lung / other organs which are difficult locations. Liquid biopsy can be done when genetic analysis of archived tumour samples is not possible.[40,46,48,50,52,53,54] It is useful in advanced-stage and unresectable cancers.[46]

Tumour related mutations have been observed in healthy individuals and smokers indicating genetic aberrations which might be present at low frequencies even in the absence of cancer.[46] Liquid biopsy assess the risk of acquiring specific cancer in future and hence can be used for screening of cancer.[40,54]

Liquid biopsy helps in diagnosing cancer at early stage. It assures highly individualized

health care and will revolutionize cancer diagnosis and treatment.[38,39,46,54] With advanced stage of cancer, as in the case of bone metastases, some pancreatic cancers and deep pelvic masses, primary are detected.[46]

Liquid biopsy has marked diagnostic and clinical implications. ctDNA is highly informative and 83% of cancer cases shows ctDNA. It captures entire heterogeneity of the tumour as tumour genotype is highly unstable and changes with multiple factors. Liquid biopsy provides an accurate snapshot of the genomic landscape of the tumour. Serial samples in liquid biopsy monitor change in genome in real time, gives more information than tissue biopsy, helps in treatment decision in nearly two thirds of patients, stratify the patients to treat with FDA approved drugs or clinical trial and ensures that the treatment is relevant. Hence it provides right tailored treatment targeting the correct molecular aberration and proteins.[38,39,40,42,44,50,54] It detects new genetic alteration, emergence of new / rare mutations which causes resistance to the targeted treatment, development of heterogeneous subclonal population of tumour cells during the course of progression of cancer, clue regarding the stage and spread of cancer.[44,46,50,54] Liquid biopsy helps in identifying signaling pathways which causes tumour invasiveness and development of metastatic competence.[38] Tumour-associated aberrations can be lost or gained over the monitoring period or in response to drug. Presence or absence of a single genetic alteration in tumour DNA is currently employed for clinical decision making for a number of targeted agents (for example, EGFR mutations for gefitinib in non-small cell lung cancer (NSCLC), BRAF mutations for vemurafenib in melanoma, KRAS mutations for cetuximab or panitumumab in colorectal cancer, ALK rearrangements for crizotinib in NSCLC).[46]

It decodes both the spatial and temporal tumor heterogeneity. In addition, it helps in analysis

of intra- and inter-molecular alterations of tumour tissue, tumor dynamic follow-up, early assessment of therapeutic efficacy, follow-up after surgery, understanding of the biology of genomic, transcriptomic, and proteomic of tumour cells.[55,56,57,58]

Genetic changes detected in liquid biopsy closely mirrors those identified by traditional tissue biopsy.[39] Liquid biopsy is more effective and alternative to gold standard tissue biopsy. The mutations detected in liquid biopsy paralleled that in tissue biopsy as in EGFR, ALK and TP3 gene alterations. In some cancer cases liquid biopsy could capture mutations not detected in tissue biopsy especially as disease progressed. They reveal molecular signatures which are targeted for chemotherapy.[43] It correlates with tumour burden also.[46]

Significant correlation is reported between disease stage and the presence of tumour associated new genetic aberrations with resectable breast cancer, ovarian cancer, pancreatic cancer, colorectal cancer and oral squamous-cell carcinoma. In breast cancer, following mastectomy and follow up of cases showed that, the vascular invasion, metastasis of more than three lymph-node and high histological grade at diagnosis had persistent tumour-associated microsatellite DNA alterations as detected in plasma extracted DNA by PCR. However there are conflicting studies correlation between stage and levels of tumour-associated genetic aberrations which may be due to limited sample, improperly designed studies and technical differences.[46]

Persistence of tumour-associated genetic aberrations in ctDNA after surgery in cases of incomplete resection of breast cancer, lung cancer and oral squamous cell indicates residual disease.[46] Liquid biopsy detects minimal residual disease which is undetectable by

imaging.[51] Stratification of patients with minimal residual disease and to identify patients likely to relapse are reported. In addition identification of cases with dormant disease which cannot be detected by standard methods are reported. As DNA is cleared from circulation within 30 minutes, the presence of DNA might reflect persistent dormant cells cycling between replication and cell death. Tumour specific copy number aberrations persisted up to 12 years post diagnosis. Significant reduction and not eradication in tumour derived DNA following surgery is also reported.[46]

The genetic changes in ctDNA can be detected much earlier than clinical signs and radiological findings of cancer progression.[46,48,50] Imaging cannot be used for frequent monitoring. Imaging techniques have limited sensitivity of detection of micrometastases. Monitoring tumour-specific aberrations in the plasma of patients with colorectal cancer identify disease recurrence with almost 100% sensitivity and specificity. An association is reported between disease recurrence and the reappearance of certain tumour aberrations, including KRAS, APC and TP53 mutations as well as allelic imbalances.[46]

Liquid biopsies helps in early detection of treatment resistance and thus spare the patient from unnecessary treatment and toxicity of the drug.[38,42,46] Detection of the emergence of resistant clones, by the presence of tumour associated genetic aberrations in the blood, identifies treatment resistance up to 10 months before radiological methods.[46] 50% of NSCLC patients became resistant to tyrosine kinase inhibitor therapy through an epidermal growth factor.[52]

Thus liquid biopsy is useful in various stages of development of cancer as; to assess the risk

of cancer, screening of cancer, early detection of cancer, cancer diagnosis, therapy guidance, therapy management, predict response to treatment, monitoring therapy success, early detection of resistance to therapy, recurrent monitoring of disease progression and death, risk stratification and treatment is more personalized at microlevel. Hence biomarkers in liquid biopsy act as surrogate markers.[40,46] Longitudinal monitoring with broader molecular understanding is very much required for cancer treatment to be successful. Clinical applications of liquid biopsies have significantly improved in recent times.[52]

DISADVANTAGES OF LIQUID BIOPSY

Preanalytical factors as blood sampling, processing, storage, DNA extraction and quantification can strongly affect DNA yield. The amount of DNA isolated in the blood sample may be very low especially in some tumours as glioblastoma which can be due to blood brain barrier where ctDNA finds difficult to cross blood brain barrier and reach the blood circulation.[50] The utility of liquid biopsies is likely to be limited in resectable tumours. There is lack of harmonization of quantification methods as these different methods produce different results because these measurements target either total or only amplifiable DNA. Hence the method in liquid biopsy has to be standardized, reproducible, approved, validated and cost-effective before it enters the market.[39,46,48] Isolation of ctDNA are costly, time consuming and complex.[48] The liquid biopsy has concern of accuracy to be used in clinical practice. Not all results consistently support the application of ctDNA to the patient as increased concentrations of ctDNA have also been detected in physiological and non-cancerous pathological conditions. Confounding events might also contribute to the release of ctDNA, e.g., non-malignant diseases, heavy smoking, pregnancy, exercise, and heart dysfunction which has to be accounted. During the time periods between sampling and clinical application, there can be alteration in genetic composition.[39,46,48] However ctDNA

appear to be a better prognostic marker than CTC count. Some authors consider analyzing ctDNA is like finding needle in a haystack.[52]

The results of this molecular studies has low accuracy rate due to small amounts in samples and easily degradable property of genetic material which requires extremely sensitive and specific methods. Current clinical efficacy of liquid biopsy techniques is very limited and large-scale prospective studies are required to validate.[55,57]

FUTURE OF LIQUID BIOPSY

There is a big hurdle between theoretical robustness, laboratory data, translational experience and the real possibility of a clinical application which requires 'formal validation'. There should be standardized and approved sampling technique (blood collection, processing and storage), extraction of DNA, quantification, analysis and reporting. Validation should be in multicenter clinical studies with respect to disease free or overall survival. ctDNA testing have been so far developed for research or investigational purposes only and should proceed to CLIA certification before implementation in clinical trials.[38,42,46,51]

To localize tissue of origin especially the small and occult lesions, organ-specific metastatic signatures in CTC cells should be evolved which guide diagnostic / therapeutic strategies and decrease cancer mortality.[51] The sensitivity of the technique has to be increased by which one can detect the genetic changes even in low frequency cfDNA.[50]

Liquid biopsy will be elegant, promising, reliable, robust non-invasive platforms for the diagnosis, patient stratification and to monitor treatment response.[43,52] Hopefully in future

liquid biopsy helps in decision making in cancer treatment replacing extensive imaging and invasive biopsy procedure. In addition liquid biopsy may screen cancer before it is visible on imaging.[54] It will be novel life saver technique and boon for human community.[40] However cost for test needs to go down and sensitivity needs to rise. At the moment, liquid biopsies are mostly confined to basic- and clinical-research.[49] In future liquid biopsy may give invaluable information for research and clinical management in oncology. It will transform clinical practice, becomes integral part of precision medicine, revolutionize cancer care and will be hallmark of cancer care.[38]

ROLE OF LIQUID BIOPSY IN CERVICAL CANCER

Mutational variations in ctDNA in different phases of cervical cancer will help to monitor the tumor status and predict therapeutic responses. The gene mutations can be identified by digital droplet PCR (ddPCR) and the sensitivity for the PIK3CA gene was 88.9% and specificity was 100% in cervical cancer. The ddPCR also confirmed 100% sensitivity and specificity in the detection of the KRAS gene in cervical cancer. The mutation rates of ZFHX3, KMT2C, KMT2D, NSD1, and RNF213 genes have been reported to have high frequency in cervical cancer patients. Gene mutation can serve as a prognostic biomarker. Mutations in tumor suppressor genes are prevalent in all stages of cervical cancer. Chemotherapy and radiotherapy affect the allele frequency, which can be utilized for monitoring cancer. Tumor suppressor gene mutations reveal the appropriate treatment modalities in patients.[59]

Table 1 : FIGO STAGING OF CERVICAL CARCINOMA [2018] [60]

FIGO Staging	2018 FIGO Definition
I	Confined to the cervix
IA	≤5 mm depth
IA1	≤3 mm depth
IA2	>3 mm and ≤5 mm depth
IB	>5 mm depth
IB1	≤2 cm maximum diameter
IB2	>2 cm and ≤4 cm maximum diameter
IB3	>4 cm maximum diameter
II	Extension beyond the uterus but lower one-third of the vagina is not involved.
IIA1	Involvement of upper two-thirds of the vagina
IIA2	Upper two-thirds of the vagina and ≤4 cm
IIB	Invasion of parametrium
III	Involvement of lower vagina, ureters, lymph nodes
IIIA	Inferior one-third of the vagina
IIIB	Pelvic sidewall
IIIC	Involvement of para-aortic and pelvic lymph nodes.
IIIC1	Pelvic lymph node involvement
IIC2	Para-aortic lymph node involvement
IV	Adjacent and distant organs
IVA	Rectal or bladder involvement
IVB	Outside the pelvis involving distal organs

5. MATERIALS AND METHODS

5.1 STUDY DESIGN: Laboratory based exploratory study.

5.2 DURATION OF STUDY: From October 2017 to March 2022.

5.3 PLACE OF STUDY: Department of Pathology in collaboration with Department of Obstetrics and Gynecology, Sri Devaraj Urs Medical College attached to R.L Jalappa Hospital andResearch centre, Tamaka, Kolar.

5.4 SAMPLE SIZE:

The sample size was calculated by using the Formula Z2PQ/d2, considering the p16 expression (p) as 96%, an absolute error (d) of 5% and a confidence level of 95% the estimated sample size for the study was 60.[61] Considering 20% non-responders, a total of 70 cases was considered for the study.

5.5 INCLUSION & EXCLUSION CRITERIA

INCLUSION CRITERIA:

All cases of invasive SCC of cervix diagnosed clinically and confirmed by histopathology.

EXCLUSION CRITERIA:

Post-chemotherapy cases, post-radiotherapy cases, recurrence of the disease, cervical intraepithelial squamous neoplasia, glandular neoplasia, adenocarcinoma of cervix, any other primary malignancy in the patient and metastatic deposits (secondary deposits) in the cervix.

5.6 METHOD:

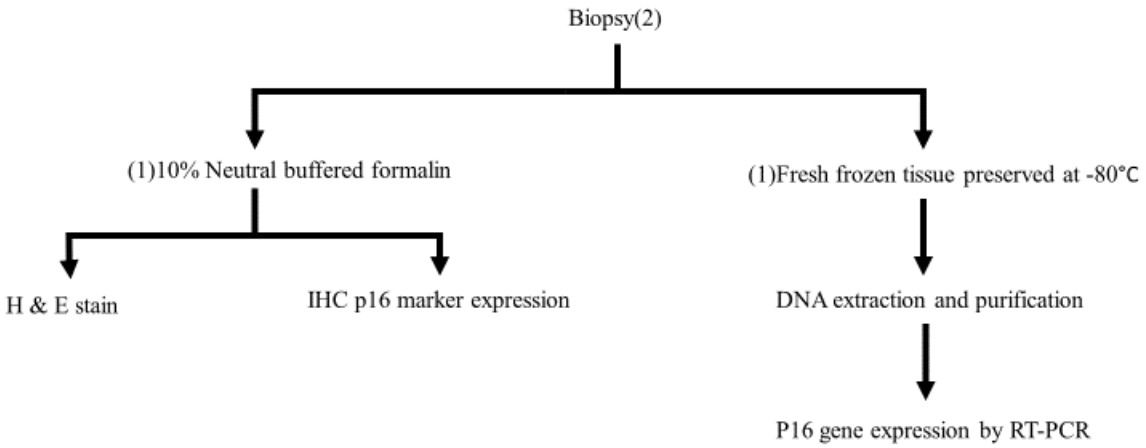
The approval for the study was taken by Central Ethics Committee, SDUAHER, Kolar (No.SDUAHER/KLR/CEC/04/2017-18). Informed consent was taken from all the patients and complete clinical details were noted. Following clinical diagnosis / Pap smear diagnosis of cervical cancer, two cervical biopsies were taken.

One tissue bit was placed in 10% neutral buffered formalin for routine tissue processing followed by hematoxylin and eosin staining and IHC staining for p16 marker. The hematoxylin and eosin stained tissue sections were used for histomorphological analysis and was classified as keratinized and non-keratinized types. Keratinized type was further classified as well differentiated SCC (WDSCC), moderately differentiated SCC (MDSCC), poorly differentiated SCC (PDSCC). Non-Keratinized type was further classified as non-keratinised Large Cell (NKLCSCC) and non-keratinised Small Cell (NKSCSCC) types.[21] The tissue sections were also used to do IHC staining to assess the expression of p16 marker (Figures: 3).

The other tissue bit was preserved at -80^{o}C immediately and used to extract DNA to analyze p16 gene expression by RT-PCR (Figures: 3).

Blood sample was collected from the same patients in K2 EDTA vacutainer, centrifuged at 1500 revolutions for 10 minutes and plasma was aliquoted in three aliquots and preserved at -80^{o}C. The plasma samples were used for estimation of p16 protein by ELISA test and p16 gene expression by RT-PCR (Figures: 4).

Figure 3: Processing of tissue samples



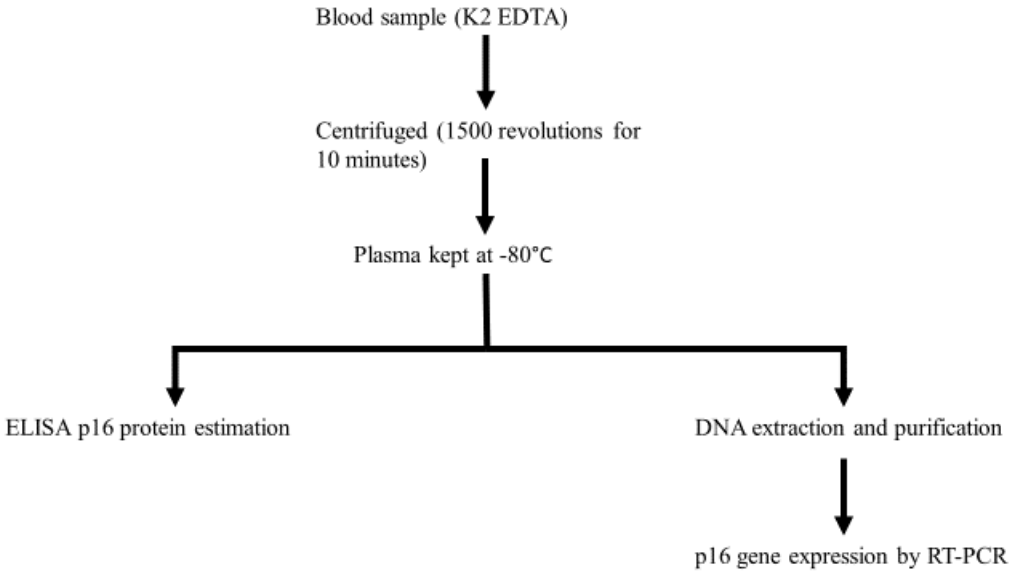


Figure 4: Processing of blood samples

5.7 STATISTICAL ANALYSIS

The data was entered in Microsoft excel data sheet and Analyzed using SPSS (Statistical Package for the Social Sciences) 22 version software. Categorical data was represented in the form of frequencies and proportions. Continuous data was represented as mean and standard deviation. Chi-square / Fisher Exact test was used to find the significance of difference between the categorical study parameters. Student 't' test / ANOVA was used to find the significance of difference between the continuous data. p value <0.05 was considered statistically significant. For association between expression of p16 by IHC and DNA extracts of cervical tissue with plasma p16 protein and DNA extracts, sensitivity, specificity, positive predictive value and negative predictive values were derived.

6. RESULTS - SOCIO-DEMOGRAPHIC DATA OF THE STUDY

Clinically diagnosed and histopathologically confirmed 70 freshly diagnosed cases of invasive SCC of cervix were considered for the study.

Table 2: Age distribution of cases

Age Range (years)	Cases
30-39	7 (10.0%)
40-49	19 (27.1%)
50-59	15 (21.4%)
60-69	18 (25.7%)
70-79	10 (14.2%)
80-89	1 (1.4%)
Total	**70 (100%)**
Total pre and perimenopausal cases	17 (24.2%)
Total postmenopausal cases	53 (75.7%)

The age distribution of the cases is shown in Table 2. The age range was 30-80 years of age with mean age of 54.2±12.0. Maximum cases were seen in age group of 40-49 years (27.1%) followed by 60-69 years (25.7%). 75.7% cases were postmenopausal and 24.2% cases were in pre and peri-menopausal group.

Table 3: Age at menopause among cases

Age in years at Menopause	No of cases
Pre & Peri-menopausal	17 (24.2%)
40-44	14 (20.0%)
45-49	25 (35.7%)
50-54	13 (18.5%)
55-59	1 (1.4%)
Total	**70 (100%)**

Table 3 shows the age at menopause among the cases where majority of females had attained menopause between 45-49 years (35.7%) of age group followed by 40-44 years (24.2%). The age at menopause range was 40-57 years with mean of 46.3±3.9 years.

Table 4: Age at which patients got married

Age at Marriage (Years)	No of cases (%)
12	3 (4.2%)
13	1 (1.4%)
14	12 (17.1%)
15	19 (27.1%)
16	11 (15.7%)
17	09 (12.8%)
18	11 (15.7%)
19	1 (1.4%)
20	1 (1.4%)
22	1 (1.4%)
23	1 (1.4%)
Total	**70 (100%)**

Table 4 shows age at which patients got married, maximum cases were noted at 15 years (27.1%) followed by 14 years (17.1%) of age. The age range was 12-23 years with mean of 15.7±2.1 years of age.

Table 5: Distribution of parity among cases

Para	No. of cases
Para 1	6 (8.5%)
Para 2	10 (14.2%)
Para 3	16 (22.8%)
Para 4	18 (25.7%)
Para ≥5	20 (28.5%)
Total	**70 (100%)**

Table 5 shows the distribution of parity among the cases where maximum cases had parity of ≥5 (28.5%) followed by 4 (25.7%). Parity ranged from 1-11with mean of 3.6±1.6.

Table 6: Clinical presentations of cases

Clinical Presentation	Total no of cases presented n=75 (%)	Pre & Peri-menopausal cases n=19 (%)	Post-menopausal cases n=56 (%)
Bleeding per vagina	50 (66.6%)	12 (62.9%)	44 (78.5%)
WDPV	49 (65.3%)	09 (47.3%)	40 (71.4%)
Others	44 (58.6%)	10 (52.5%)	34 (60.4%)
Pain Abdomen	35 (46.6%)	10 (52.5%)	25 (44.6%)
Post-coital bleeding	4 (5.3%)	1 (5.2%)	3 (5.3%)
Mass per vagina	2 (2.6%)	1 (5.2%)	1 (1.7%)
No symptoms	2 (2.6%)	2 (10.5%)	00

Table 6 shows the varied clinical presentation in cases. In both pre & peri-menopausal women and post-menopausal women bleeding per-vagina followed by white discharge per vagina were the common clinical presentation.

Table 7: Per-speculum findings in cases

Per-speculum findings	Total No. of cases	Pre & Peri-menopausal cases	Post-menopausal cases
Growth	47 (67.1%)	09 (52.9%)	38 (71.6%)
Bleeding	8 (11.4%)	2 (11.7%)	6 (11.3%)
Erosion	6 (8.5%)	3 (17.6%)	3 (5.6%)
Ulcer	4 (5.7%)	2 (11.7%)	2 (3.7%)
Unhealthy	3 (4.2%)	1 (5.8%)	2 (3.7%)
Mass	1 (1.4%)	00	1 (1.8%)
Stenosis	1 (1.4%)	00	1 (1.8%)
WDPV	00	00	00
Total	**70 (100%)**	**17 (100%)**	**53 (100%)**

Table 7 shows per-speculum findings of cases. Growth followed by erosion / bleeding were the common findings in both pre & peri-menopausal and post-menopausal women.

Table 8: Per-vaginal findings in cases

Per vaginal examination	Total No. of cases	Pre & Peri-menopausal cases	Post-menopausal cases
Friable Growth	42 (60.0%)	09 (52.9%)	33 (62.2%)
Induration	26 (37.1%)	7 (41.1%)	19 (35.8%)
Erosion	1 (1.4%)	00	1 (1.8%)
Stenosis	1 (1.4%)	1 (5.8%)	00
Total	**70 (100%)**	**17 (100%)**	**53 (100%)**

Table 8 shows per-vaginal findings of cases. Friable growth followed by induration were the common findings in both pre & peri-menopausal and post-menopausal women.

Table 9: Stage of the disease among cases

Stage of the disease [15]	Total No of Cases	Pre & Peri-menopausal cases	Post-menopausal cases
Stage I	5 (7.1%)	3 (17.6%)	2 (3.7%)
Stage II	23 (32.8%)	7 (41.1%)	16 (30.1%)
Stage III	28 (40.0%)	2 (11.7%)	26 (49.0%)
Stage IV	14 (20.0%)	5 (29.4%)	9 (16.9%)
Total	**70 (100%)**	**17 (100%)**	**53 (100%)**

Table 9 shows the stages of the disease among the cases. Maximum cases were seen in stage III (40%). However maximum cases in pre & peri-menopausal women were seen in stage II (41.1%) and in post-menopausal women it was seen in stage III (49.0%).

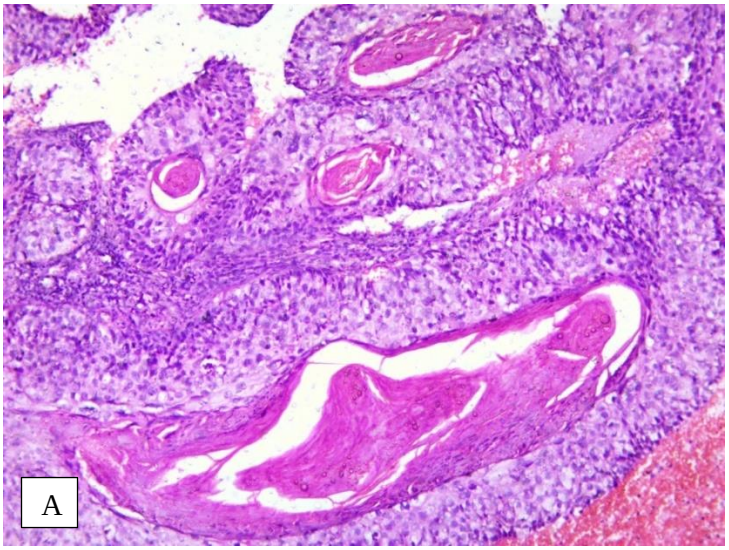
Table 10: Histopathology grades of the disease in cases

Grade of the disease	No of cases (%)	Pre & Peri-menopausal cases	Post-menopausal cases
WDSCC	38 (54.2%)	12 (70.5%)	26 (49.0%)
MDSCC	15 (21.4%)	2 (11.7%)	13 (24.5%)
PDSCC	10 (14.2%)	1 (5.8%)	09 (16.9%)
NKLCSCC	5 (7.1%)	2 (11.7%)	3 (5.6%)
NKSCSCC	2 (2.8%)	--	2 (3.7%)
Total	**70 (100%)**	**17 (100%)**	**53 (100%)**

Table 10 shows histological grade of the disease in cases. Keratinizing SCC was maximum, constituted 89.8% of cases and non-keratinizing 9.9%. Among keratinizing SCC, maximum cases were WDSCC (54.2%) followed by MDSCC (21.4%).

Figure 5: Histomorphology of well differentiated squamous cell carcinoma.

(A) H&E X100 (B) H&E X400



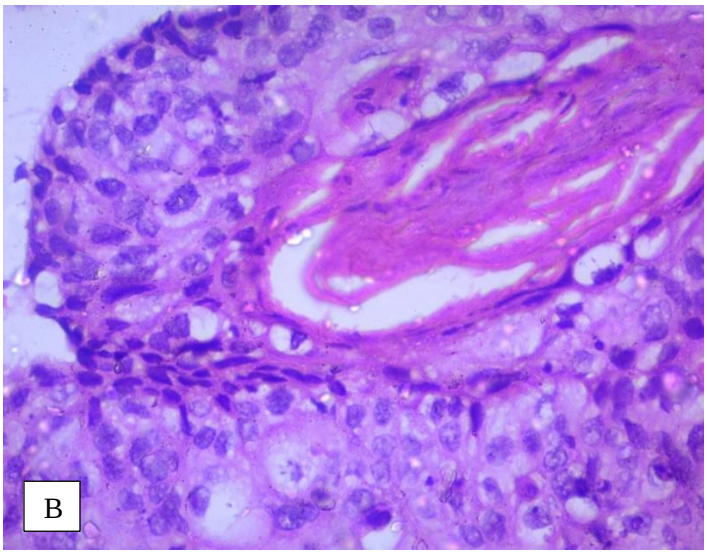


Figure 6: Histomorphology of moderately differentiated squamous cell carcinoma. (A) H&E X100 (B) H&E X400

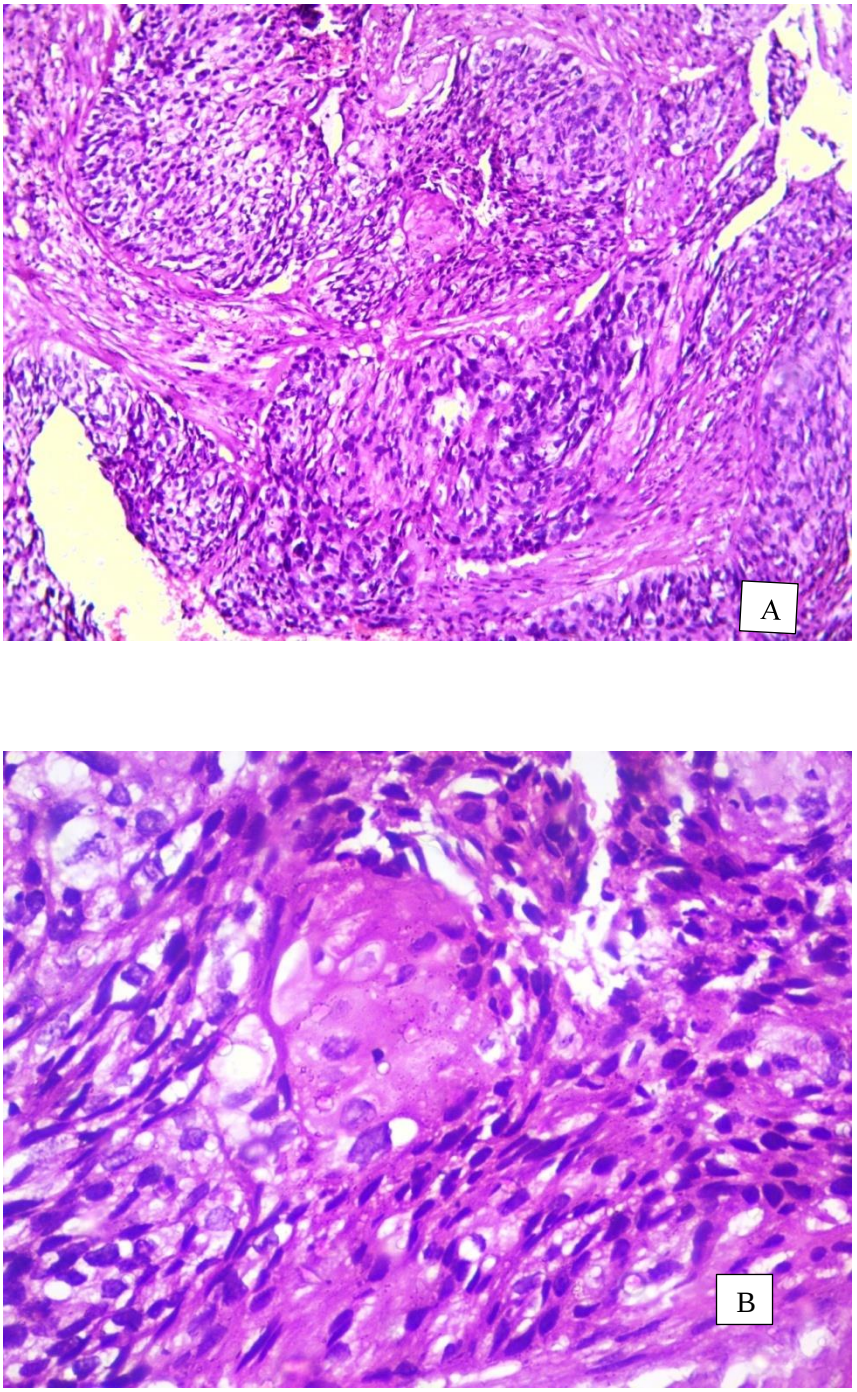
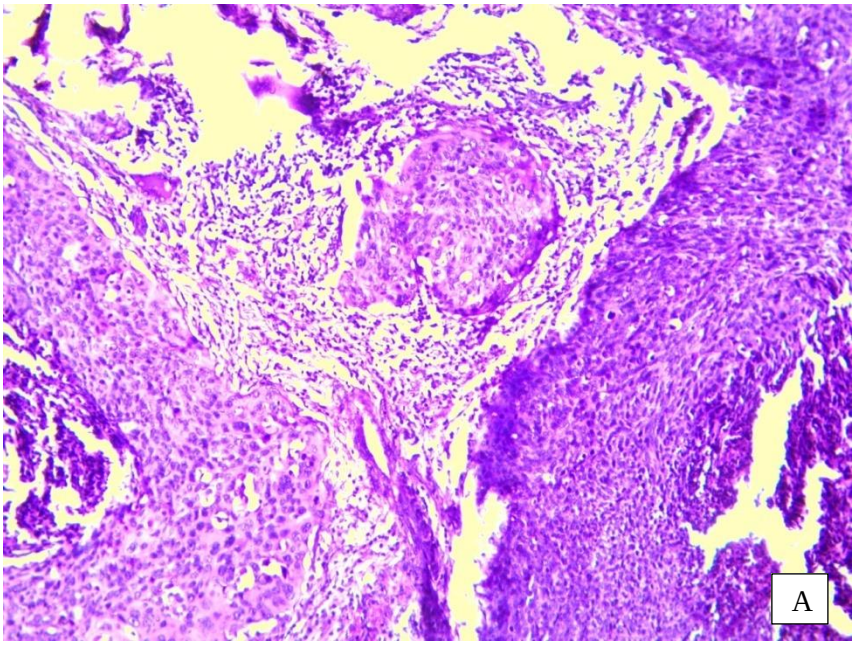


Figure 7: Histomorphology of poorly differentiated squamous cell carcinoma.

(A) H&E X100 (B) H&E X400



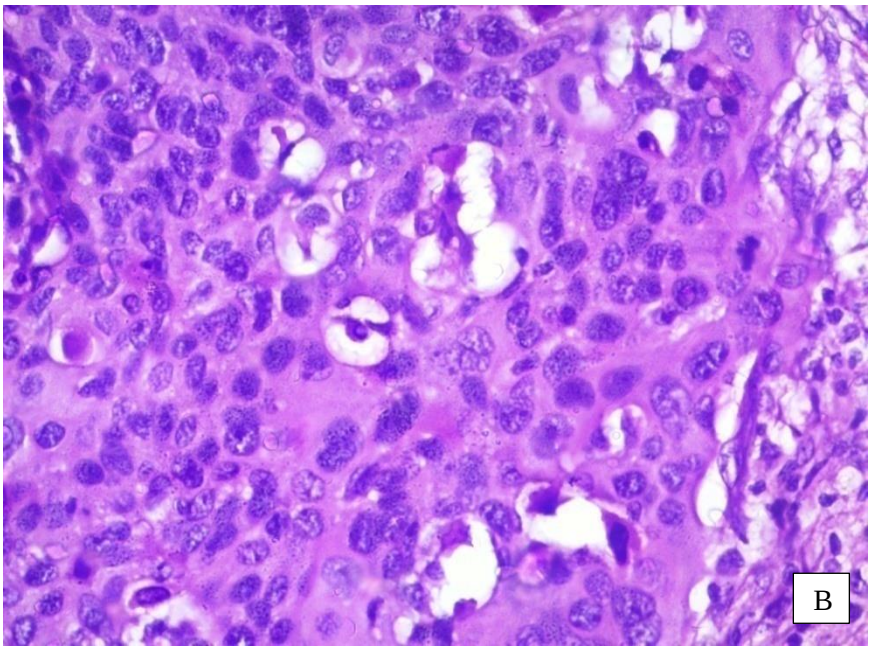


Figure 8: Histomorphology of non-keratinizing large cell squamous cell carcinoma.

(A) H&E X100 (B) H&E X400

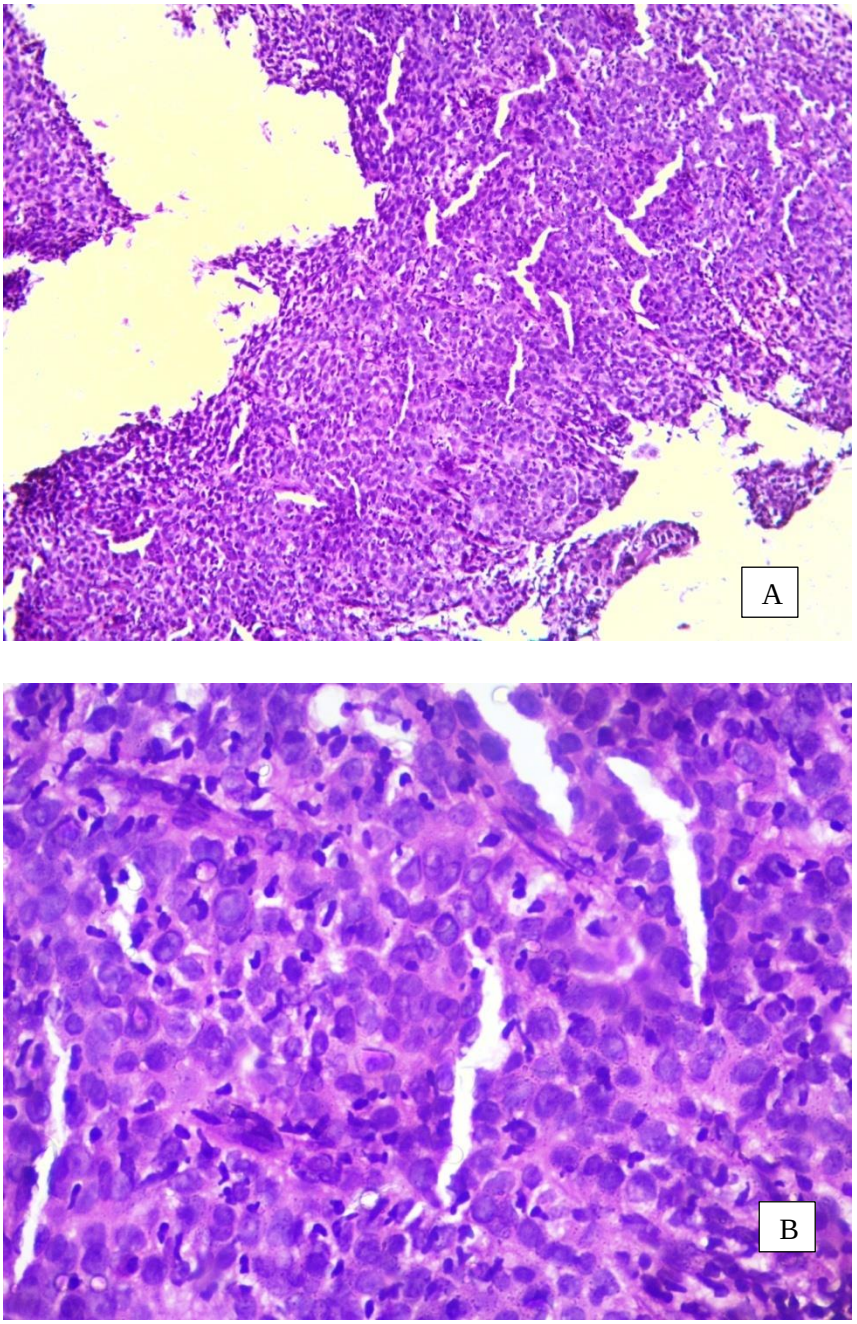
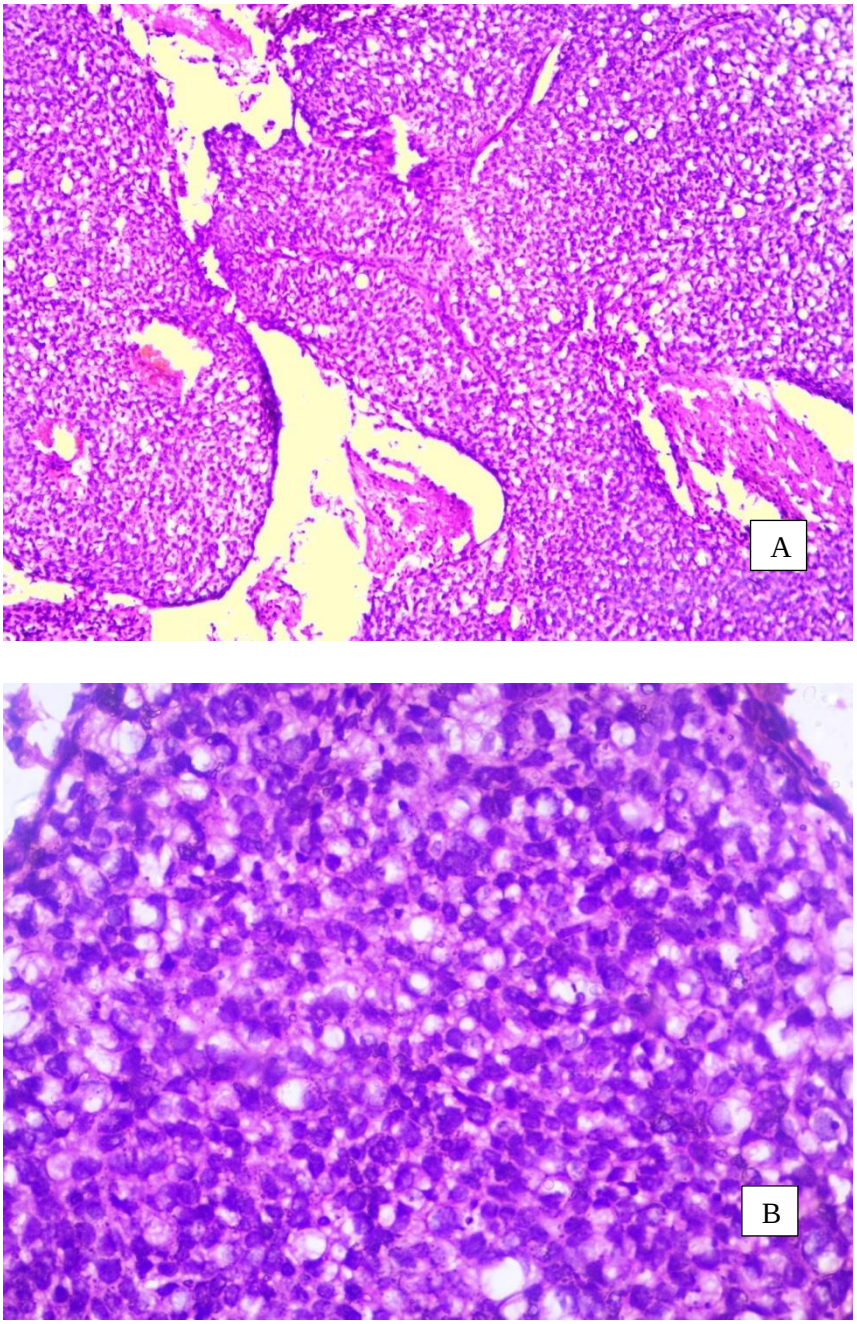


Figure 9: Histomorphology of non-keratinizing small cell squamous cell carcinoma.

(A) H&E X100. (B) H&E X400



7. CHAPTER 1

EVALUATION OF p16 EXPRESSION IN BIOPSY OF SQUAMOUS CELL CARCINOMA CERVIX BY IMMUNOHISTOCHEMISTRY METHOD.

7.1 Methodology:

Table 11: Antibody utilized for IHC:

Antigen	Clone	Species	Product	Dilution	Control	Stain
Anti-p16INK4	Monoclonal	Mouse	Biogenex	1:50 dilution	HSIL or SCC cervix	Nuclear Staining

IHC procedure:

Evaluation of p16 expression in tissue sections was done by IHC.

The procedure of IHC as per the protocol was:

1. The tissue sections of 3-4 µm thickness were floated on to organosilane coated slide and left on hot plate at 60^0C overnight.
2. Deparafinisation was done by using Xylene I and II for 15 minutes each.
3. Dexylenisation was done by using absolute alcohol I and II for 1 minute each.
4. Dealcoholisation was done by using 90% and 70% alcohol for 1 minute each.
5. Tissue sections were washed with distilled water.
6. Antigen retrieval technique was done by microwave power 10 for 6 minutes in Tris Ethylenediamine tetraacetic acid (EDTA) buffer at pH 9.0 for 3 cycles.
7. Sections were washed in distilled water for 3 minutes.
8. The tissue sections were transferred to Tris Buffered Saline (TBS) pH 7.0 for 5 minutes for 2 wash.
9. Peroxidase block was done for 10-15 minutes to block endogenous peroxidase enzyme.
10. Sections were washed in TBS for 3 minutes for 3 times.

11. Power block was done 10-15 minutes to block non-specific reaction with other tissue antigens.
12. The tissue sections were covered with targeted antibody (1:50 dilution) [Mouse monoclonal anti-p16INK4 clone G175-405 (Biogenex, code: AM540-5M, USA)] for 2 hours.
13. Washed with TBS for 3 times.
14. Super enhancer was given for 30 minutes to enhance the reaction between primary and secondary antibody. Wash done for 5 minutes.
15. TBS wash was done for 5 minutes for 3 times.
16. Tissue section were covered with super sensitive poly–HRP (horseradish peroxidase) (secondary antibody) for 30 minutes.
17. TBS wash was done for 5 minutes for 3 times.
18. Color development was done with working DAB (3, 3'-diaminobenzidine) solution for 5-8 minutes.
19. TBS wash was done for 5 minutes for 3 times.
20. Counter stain with Hematoxylin done 2 seconds.
21. Tap water wash was done for 5 minutes.
22. Tissue sections were dehydrated, cleared and mounted with DPX (Dibutylphthalate Polystyrene Xylene).
23. The procedure was done with positive (tissue section of HSIL and SCC cervix) and negative control (without primary antibody).

Immunohistochemical Analysis and Interpretation:

Nuclear staining with or without cytoplasmic staining was considered as positive staining and p16 marker expression was classified as per LAST criteria (2012) as block positivity,

ambiguous and negative.[14]

Block positive pattern: of staining means, the expression is strong, continuous, nuclear positivity with or without cytoplasmic staining, and the staining extending from basal layers upwards for at least 1/3rd thickness of the epithelium (basal & parabasal layers) which can be further graded as 1/3rd, 2/3rd and more than 2/3rd and laterally extending over a significant distance (diffuse >25% of cells with staining).

Ambiguous staining: means, strong and basal (strong, diffuse, continuous, involves lower 1/3rd without upward extension) or weak, diffuse and discontinuous staining, involving at least 2/3rd of the epithelium or strong, focal and discontinuous located at any level of the epithelium.

Negative staining: means total absence or weak or focal and discontinuous or only cytoplasmic staining.[18]

7.2 Statistical Analysis:

All data were entered in Microsoft Excel sheet. The data were analyzed using SPSS version 22. The categorical data was presented as frequency and proportions. Continuous data was presented as mean, standard deviation (SD) and confidence intervals. Significance of difference between the groups was estimated using standard 't' test and chi-square test. p value <0.05 was considered as statistically significant.

7.3 Results:

Seventy cases of SCC of cervix were considered for the study. Immunohistochemistry was done in all 70 cases for p16 biomarker. The following data was evaluated:

1. p16 expression as per LAST classification
2. Association of age distribution and p16 expression
3. Association of age at marriage and p16 expression
4. Association of age at menopause and p16 expression
5. Association of parity and p16 expression
6. Association of FIGO staging and p16 expression
7. Association of histological grading and p16 expression

Figure 10: IHC Block Positive expression of p16 marker. A: p16 IHC 100X. B. p16 IHC 400X

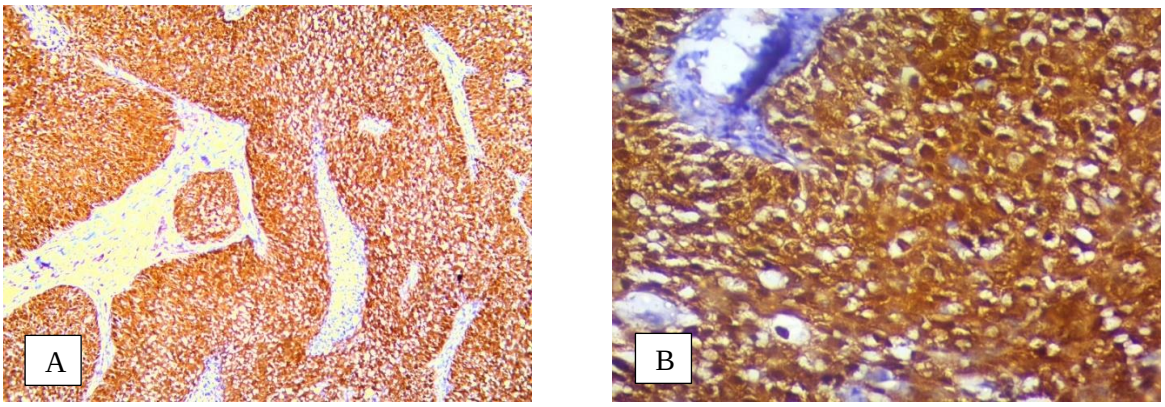


Figure 11: IHC Ambiguous expression of p16 marker. A: p16 IHC 100X. B. p16 IHC 400X

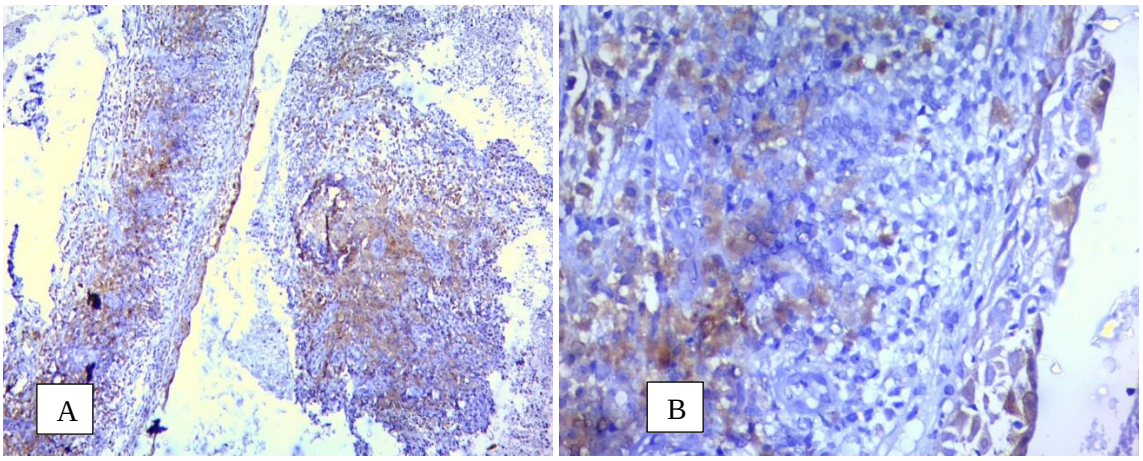


Figure 12: IHC Negative expression of p16 marker. A: p16 IHC 100X. B. p16 IHC 400X

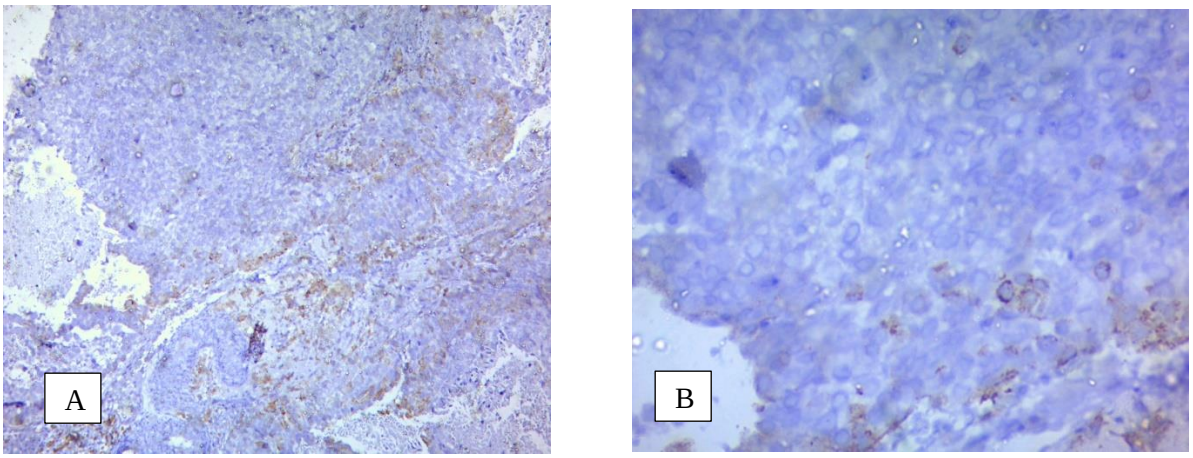


Table 12: IHC p16 expression as per LAST classification between pre & peri-menopausal and post-menopausal cases

p16 expression	No of Cases (%)	Pre & Peri-menopausal cases	Post-menopausal cases
Negative	3 (4.2%)	00	3 (5.6%)
Ambiguous	5 (7.1%)	1 (5.8%)	4 (7.5%)
Block Positive	62 (88.5%)	16 (94.1%)	46 (86.7%)
Total	70 (100%)	17 (100%)	53 (100%)

p value of p16 expression between pre & peri-menopausal and post-menopausal cases was 0.555

Out of 70 cases IHC p16 expression was block positive, ambiguous and negative in 62 (88.5%), 5 (7.1%) and 3 (4.2%) cases respectively. Block positivity was maximum in pre & peri-menopausal women (94.1%) compared to post-menopausal women (86.7%) which were not statistically significant (p=0.55) (Table 12).

Table 13: Association of age distribution and IHC p16 expression

Age range of cases	Expression of p16 (n)			
	Negative	Ambiguous	Block positivity	Total cases
30-39	0	0	7 (100%)	7 (100%)
40-49	0	0	19 (100%)	19 (100%)
50-59	0	0	15 (100%)	15 (100%)
60-69	2 (11.1%)	3 (16.6%)	13 (72.2%)	18 (100%)
70-79	1 (10%)	1 (10%)	8 (80%)	10 (100%)
80-89	0	1 (100%)	0	1 (100%)
Total cases	3 (4.2%)	5 (7.1%)	62 (88.5%)	70 (100%)

p value between age of cases and p16 expression was 0.008

Statistical significant association was observed between age distribution of cases and IHC p16 expression (p = 0.008) (Table 13). All cases (100% cases) between 30-59 years of age showed block positivity indicating cases with younger age showed increased IHC p16 positivity.

Table 14: Association of age at marriage and IHC p16 expression

Age at marriage	Expression of p16 (n)			
	Negative	Ambiguous	Block positivity	Total cases
12-14 years	1 (6.2%)	1 (6.2%)	14 (87.5%)	16 (100%)
15 to 18 years	2 (4.0%)	4 (8.0%)	44 (88.0%)	50 (100%)
>18 years	0	0	4 (100%)	4 (100%)
Total cases	3 (4.2%)	5 (7.1%)	62 (88.5%)	70 (100%)

p value between age at marriage and p16 expression was 0.951

There was no significant association between age at marriage of cases and IHC p16 expression (p = 0.951) (Table 14). The p16 expression was maximum among females with more than 18 years of age at marriage followed by those between 15 to 18 years.

Table 15: Association of age at menopause and IHC p16 expression

Age at menopause	Expression of p16 (n)			
	Negative	Ambiguous	Block positivity	Total cases
Pre & Peri-menopausal	0	1 (5.8%)	16 (94.1%)	17 (100%)
40-44	0	1 (6.6%)	14 (93.3%)	15 (100%)
45-49	2 (8.3%)	1 (4.1%)	21 (87.5%)	24 (100%)
50-54	1 (7.6%)	1 (7.6%)	11 (84.6%)	13 (100%)
55-59	0	1 (100%)	0 (0%)	1 (100%)
Total cases	3 (4.2%)	5 (7.1%)	62 (88.5%)	70 (100%)

p value between age at menopause of cases and p16 expression was 0.311

There was no statistically significant association between age at menopause of cases and IHC p16 expression (p = 0.311) (Table 15). 94.1% Pre & Peri-menopausal women showed block positivity. Among the post-menopausal women IHC p16 expression was maximum between 40-44 years of age.

Table 16: Association of parity and IHC p16 expression

Para of cases	Expression of p16 (n)			
	Negative	Ambiguous	Block positivity	Total cases
Para 1	0	0	6 (100%)	6 (100%)
Para 2	0	0	10 (100%)	10 (100%)
Para 3	0	1 (6.2%)	15 (93.7%)	16 (100%)
Para 4	1 (5.5%)	3 (16.0%)	14 (77.7%)	18 (100%)
Para ≥5	2 (10.0%)	1 (5.0%)	17 (85.0%)	20 (100%)
Total cases	3 (4.2%)	5 (7.1%)	62 (88.5%)	70 (100%)

p value between parity of cases and p16 expression was 0.554

There was no significant association (p value 0.554) between parity and IHC p16 expression. However, all cases (100% cases) with parity one and two showed block positive IHC p16 expressions (Table 16).

There was no significant association between clinical presentation (p=0.135) / per-speculum examination findings (p=0.217) / per-vaginal examination findings (p=0.982) and IHC p16 expression.

Table 17: Association of Stage of disease and IHC p16 expression

Stage of disease	Expression of p16 (n)			
	Negative	Ambiguous	Block positivity	Total cases
Stage I	0	0	5 (100%)	5 (100%)
Stage II	2 (8.6)	1 (4.3%)	20 (86.9%)	23 (100%)
Stage III	1 (3.5%)	1 (3.5%)	26 (92.8%)	28 (100%)
Stage IV	0	3 (21.4%)	11 (78.5%)	14 (100%)
Total cases	3 (4.2%)	5 (7.1%)	62 (88.5%)	70 (100%)

p value between stage of disease and p16 expression was 0.294

There was no significant association (p=0.29) between stage of the disease and IHC p16 expression (Table 17). However, all stage I cases (100% cases) showed block positive IHC p16 expression.

Table 18: Association of Histopathology grades and IHC p16 expression

Para of cases	Expression of p16 (n)			
	Negative	Ambiguous	Block positivity	Total cases
NKSCSCC	0	0	2 (100%)	2 (100%)
NKLCSCC	0	1 (20%)	4 (80%)	5 (100%)
PDSCC	0	1 (10%)	9 (90%)	10 (100%)
MDSCC	1 (6.6%)	0	14 (93.3%)	15 (100%)
WDSCC	2 (5.2%)	3 (7.8%)	33 (86.8%)	38 (100%)
Total cases	3 (4.2%)	5 (7.1%)	62 (88.5%)	70 (100%)

p value between parity of cases and p16 expression was 0.887

There was no significant association (p=0.887) between histopathological grade of the disease and IHC p16 expression of the disease (Table 18). However most of the cases of WDSCC (86.8%) and MDSCC (93.3%) showed block positive IHC p16 expression which was not statistically significant.

7.4 Discussion

Carcinoma cervix is the commonest cancer of female genital tract. HPV is proved as the etiological factor for cervical cancer. Infection of cervix with HR-HPV causes integration of viral genome to host genome followed by, synthesis and release of E7 protein by viral genome which act on host cells and causes degradation / functional inactivation of Rb protein at the G1/S phase, a rate limiting step of the cell cycle, resulting in early immortalization of epithelial cells, rapid proliferation of cells, dysplastic changes and finally malignant transformation. Degradation / functional inactivation of Rb protein causes increased expression of p16 in p16–Rb pathway as a feedback mechanism to stop the cell proliferation.[10,11]

p16 gene is a tumour suppressor gene, located on chromosome 9 band p21.3. It belongs to inhibitor kinase family (INK4 family) of CDK inhibitor proteins.[25,26] It normally functions as a tumour suppressor gene acting in association with CDK4/6 and Rb protein in cell cycle

at the rate limiting step G1/S phase.[10,11]

In normal cervix, IHC p16 marker expression reported is 2%. In CIN I, CIN II, CIN III and in invasive cervical cancer the IHC p16 gene expression is 38%, 68%, 82% and 96% respectively. Thus, p16 acts as a surrogate marker for cervical cancer. There is no uniform method of interpretation of p16 expression in tissue sections across the globe.[12,26] As per LAST criteria, IHC p16 nuclear expression with or without cytoplasmic expression is considered as positive staining. In 2012, LAST classified the p16 marker expression on tissue sections as block positive, ambiguous and negative expression.[28]

In the present study, IHC p16 expression as per LAST classification showed block positivity in 62 cases (88.5%), ambiguous in five cases (7.1%) and negative in three cases (4.2%). In a study by Sarwath et al, IHC p16 block positivity was seen in 92.2% of cases and negative in 7.8% with sensitivity, specificity, PPV and NPV of 79.2%, 46%, 83.9% and 27.2% respectively. Absence of IHC p16 expression in SCC of cervix may be due to absence of HPV infection, mutation in promoter region, epigenetic mechanism and hypermethylation.[31] Stoler et al classified IHC p16 expression as diffuse, focal and negative as in LAST criteria and stated that IHC p16 expression was diffuse in 100% invasive cancer.[35] Amaro-Filho has reported IHC p16 expression as diffuse, focal and negative in 85.5%, 9.9% and 4.6% cases respectively in SCC of cervix (Table 19).[62]

Table 19: Shows p16 IHC expression in SCC of cervix in the present study compared with other studies.

Sl.No	Author & Year	Negative	Ambiguous	Block positive
1	Sarwath H et al [31]	7.8%	-	92.2%
2	Stoler MH et all [35]	-	-	100%
3	Amaro-Filho et al [62]	4.6%	9.9%	85.5%,
4	Present Study	4.2%	7.1%	88.5%

In the present study, there was statistically significant association between age and IHC p16 expression where all cases (100%) between 30-59 years showed block positivity. Pre and peri-menopausal women showed maximum (94.1%) block positivity than post-menopausal women which was not statistically significant. Among the post-menopausal women, women between 40 and 44 years showed maximum (93.3%) block positivity which was not statistically significant. Sarwath et al in their study stated that there was significant correlation between IHC p16 expression and age group between 41–60 years. This was thought to be due to active transforming precancerous lesions in younger age group women. Hence IHC p16 was the appropriate surrogate marker to use it in early screening of cervical cancer.[31]

In the present study, parity and IHC p16 expression did not show statistically significant association. However, all cases (100%) with parity one and two showed block positivity. There was no statistically significant association between stage / histological grade of the disease and IHC p16 expression. However, all stage I cases (100%) showed block positivity and majority of cases of WDSCC (86.8%) and MDSCC (93.3%) showed block positivity. Fu et al in their study stated that IHC p16 expression was not found to have association with tumour stage, tumour size, histological grade, vascular invasion, CEA levels, SCC Ag levels and in non-squamous cell carcinoma. The independent prognostic factors for cervical cancer regarding disease free survival (DFS) is high grade SCC, non-SCC and low IHC p16 expression.[36] Sarwath et al stated that IHC p16 expression did not correlate with tumour grade and size of the tumour.[31]

In the present study there was no statistical association of IHC p16 expression with age at menopause (p=0.311), parity (p=0.554), clinical presentation (p=0.135), per-speculum

examination findings (p=0.217), per-vaginal examination findings (p=0.982), stage of disease (p=0.28) and histological grade (p=0.57).

Cervical cancer with IHC p16 expression has better prognosis.[36,63] High IHC p16 expression in cervical cancer is reported to have high five year overall survival and DFS, which is statistically significant. Five-year overall survival in high and low IHC p16 expression was 62.0% and 35.2% respectively. DFS in high and low IHC p16 expression was 60.0% and 31.2% respectively.[36,63] In the present study, the cases were not followed up for prognosis. IHC p16 is also an indicator for radiosensitivity. Due to the anti-cancer activity, IHC p16 can be exploited for development of targeted chemotherapy in cervical cancer.[36] IHC p16 marker can be used as primary screening test and adapted to the computerized image analysis techniques.[8]

The limitation of the present study was, we did not follow up the cases to assess the prognosis. However, IHC p16 block positivity was high in young females and was statistically significant. IHC p16 expression was maximum in pre / peri-menopausal females, post-menopausal females between 40-44 years of age, females with one/two parity and in stage I disease, though not statistically significant.

7.5 Conclusion

IHC p16 block positivity was observed in 88.5%, ambiguous in 7.1% and negative in 4.2%. The marker was expressed more in younger age and early stage of the disease by which IHC p16 biomarker can be used for screening or early diagnosis for better prognosis of SCC of cervix. This information can also be used as a concept for adjuvant targeted therapy as p16 protein has anti-cancer property.

8. CHAPTER 2

QUANTITATIVE ESTIMATION OF p16 PROTEIN IN PLASMA BY ELISA.

8.1 Methodology:

Plasma ELISA p16 protein estimation was performed in plasma samples of 70 cases and 70 age and sex matched controls. Plasma p16 protein was measured quantitatively by ELISA kit as per manufacturer's protocol. [Human p16 ELISA, ImmunoTag, Catalogue No: ITEH01637]. The principle was quantitative sandwich ELISA method. All the reagents and samples were brought to the room temperature. The number of strips required were selected. The coated wells had p16 antibody pre-coated microtitre plate. The wells were classified as;

- Blank well with 50 uL of standard diluent
- Standard well with 50 uL of standard + 50 uL Streptavidin HRP
- Sample well with 40 uL of sample + 10 uL of p16 Antibody + 50 uL Streptavidin HRP

1. All the wells were sealed and kept at 37°C for 60 mints.
2. The seal was removed, washed 5 times with wash buffer & soaked with 0.35 ml wash buffer for 30 secs to 1 minute.
3. 50 uL substrate A & 50 uL substrate B was added to all wells including blank well, mixed well, covered / sealed and incubated at 37°C for 10 minutes.
4. 50 uL of stop solution was added to each well including blank well (blue colour of solution changes to yellow colour), mixed well.

5. OD (optical density) was determined at 450 nm using microplate reader immediately within 10 minutes.

6. A standard curve was drawn with OD for each standard on the vertical (Y) axis and concentration on the horizontal (X) axis.

7. using the OD of samples, the concentration of the p16 protein was estimated and expressed as ng/ml.

8. Blank well acts as negative control. Standard well act as positive control, the OD values of this helps to derive the concentration of the test sample in the graph.

8.2 Statistical Analysis:

The values of both cases and controls were entered in the master chart (Microsoft excel sheet). The data was analyzed using SPSS 22 version software. Categorical data was represented in the form of frequencies and proportions. Continuous data was represented as mean and standard deviation. Chi-square / Fisher Exact test was used to find the significance of difference between the categorical study parameters. Association between p16 protein in plasma in cases and controls was done through sensitivity, specificity, positive predictive value and negative predictive value at different cut off values of p16 protein by estimating area under receiver operating characteristics (ROC) curve, z statistics and Youden index J. p value of <0.05 was considered as significant.

8.3 Results:

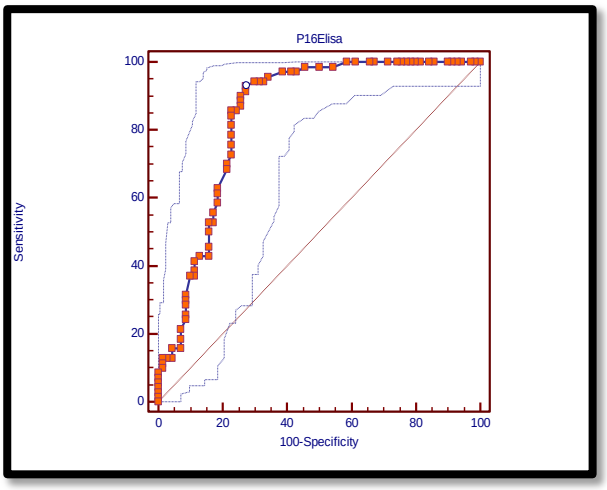
Seventy cases of SCC of cervix were considered for the study.

Table 20: Plasma p16 levels in different age groups in cases and controls in the present study.

Age Range (years)	Cases			Controls			P value
	No	Plasma P16 Range (ng/ml)	Mean Plasma P16 (ng/ml)	No	Plasma P16 Range (ng/ml)	Mean Plasma P16 (ng/ml)	
30-39	7	5.5 – 9.7	7.4±1.3	8	1.3 – 4.7	2.8±1.25	< 0.05
40-49	19	3.4 – 13.0	7.6±2.5	18	0.9 – 8.8	3.9±2.32	< 0.05
50-59	15	4.4 – 6.9	5.8±0.7	15	1.5 – 9.2	4.2±2.23	< 0.05
60-69	18	5.2 – 19.6	8.0±3.1	18	1.2 – 7.7	4.3±1.96	< 0.05
70-79	10	3.8 – 11.2	6.9±1.9	10	1.0 – 9.7	4.9±3.00	< 0.05
80-89	1	8.4	8.4	1	3.8	3.8	-------
Total	70	**3.4 – 19.6**	**7.2±2.3**	70	**0.9 – 9.7**	4.1±2.22	**< 0.05**

The p16 levels among cases ranged from 3.4 to 19.6 ng/ml with mean of 7.24 ng/ml (SD=2.35). The mean plasma p16 level in cases was maximum in a case of 80 years and minimum in age range of 50-59 years. Seventy age matched controls were considered for the study having age range between 30 to 80 years with mean age of 54.3±12.6. The plasma p16 level in controls ranged between 0.9 – 9.7 ng/ml with mean of 4.1±2.22 ng/ml. The mean plasma p16 level in controls was maximum between in age group of 70-79 years and minimum in age group of 30-39 years of age. The plasma p16 levels between cases and controls in different age groups was statistically significant with p value of <0.05.(Table 20)

Figure 13: Area under the ROC showing the sensitivity and specificity of ELISA plasma p16 protein



Area under the ROC curve (AUC[#][$])	0.843
Standard Error	0.0353
95% Confidence interval	0.772 to 0.899
z statistic	9.706
Significance level P (Area=0.5)	<0.0001

Figure 13 shows the area under ROC curve along with sensitivity and specificity between plasma p16 values of cases and controls indicating statistical significance with the p value of <0.0001.

Table 21: Showing the cut off of plasma ELISA p16 protein in cases with sensitivity, specificity, PPV and NPV

p16 protein in ng/ml	Sensitivity	95% CI	Specificity	95% CI	PPV	95% CI	NPV	95% CI
>1	100.00	94.9 - 100.0	2.86	0.3 - 9.9	50.7	42.1 - 59.3	100.0	15.8 - 100.0
>2	100.00	94.9 - 100.0	20.00	11.4 - 31.3	55.6	46.4 - 64.4	100.0	76.8 - 100.0
>3	100.00	94.9 - 100.0	32.86	22.1 - 45.1	59.8	50.4 - 68.8	100.0	85.2 - 100.0
>4.1	97.14	90.1 - 99.7	61.43	49.0 - 72.8	71.6	61.4 - 80.4	95.6	84.9 - 99.5
>4.8	**92.86**	**84.1 - 97.6**	**72.86**	**60.9 - 82.8**	**77.4**	**67.0 - 85.8**	**91.1**	**80.4 - 97.0**
>5	90.00	80.5 - 95.9	74.29	62.4 - 84.0	77.8	67.2 - 86.3	88.1	77.1 - 95.1
>6	70.00	57.9 - 80.4	78.57	67.1 - 87.5	76.6	64.3 - 86.2	72.4	60.9 - 82.0
>7	42.86	31.1 - 55.3	84.29	73.6 - 91.9	73.2	57.1 - 85.8	59.6	49.3 - 69.3
>8	25.71	16.0 - 37.6	91.43	82.3 - 96.8	75.0	53.3 - 90.2	55.2	45.7 - 64.4
>9.2	12.86	6.1 - 23.0	98.57	92.3 - 100.0	90.0	53.0 - 99.8	53.1	44.1 - 61.9
>9.4	11.43	5.1 - 21.3	98.57	92.3 - 100.0	88.9	48.9 - 99.8	52.7	43.8 - 61.5
>10.6	7.14	2.4 - 15.9	100.00	94.9 - 100.0	100.0	47.8 - 100.0	51.9	43.1 - 60.5
>11.1	5.71	1.6 - 14.0	100.00	94.9 - 100.0	100.0	39.8 - 100.0	51.5	42.8 - 60.1
>13	1.43	0.04 - 7.7	100.00	94.9 - 100.0	100.0	2.5 - 100.0	50.4	41.8 - 58.9
>19.6	0.00	0.0 - 5.1	100.00	94.9 - 100.0			50.0	41.4 - 58.6

Table 21 shows the sensitivity, specificity, positive predictive value and negative predictive value at 95% confidence interval at different cut off levels of plasma p16 protein in cases. At 95% confidence interval, cut off levels between 3.9 to 5 ng/ml levels had relatively high sensitivity and specificity. The specificity increased and sensitivity decreased with increase in plasma p16 levels.

Table 22: Validity of ELISA plasma p16 protein levels in differentiating Cases and Controls at different cut-off levels

Youden index J	0.6571
95% Confidence interval	0.5000 to 0.7429
Associated criterion	**>4.8**
95% Confidence interval	3.9 to 5

Table 23: Sensitivity, specificity, PPV, NPV and Diagnostic accuracy of ELISA plasma p16 protein at cut off levels of more than 4.8 ng/ml.

Parameter	Estimate	Lower - Upper 95% CIs [#]
Sensitivity	92.86%	(84.34, 96.91[1])
Specificity	72.86%	(61.46, 81.88[1])
PPV	77.38%	(67.35, 85.01[1])
NPV	91.07%	(80.74, 96.13[1])
Diagnostic Accuracy or Overall Positivity of p16	82.86%	(75.76, 88.2[1])

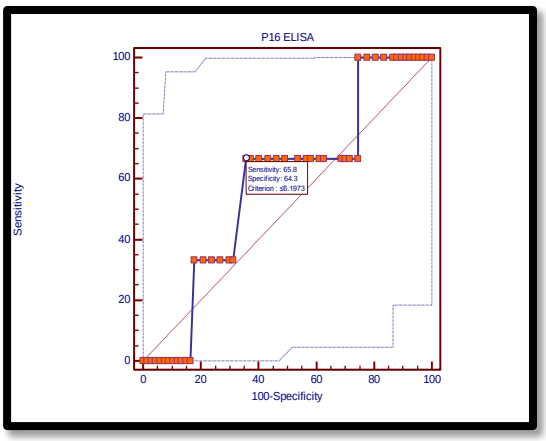
At cut off more than 4.8 ng/ml in cases, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy or overall positivity was 92.86%, 72.86%, 77.4%, 91.1% and 82.86% respectively (Table 22 & 23).

Table 24: Shows ELISA plasma p16 levels in different groups of IHC p16 expression as per LAST criteria

IHC p16 expression	No of Cases	Plasma p16 protein range (ng/ml)	Mean plasma p16 protein (ng/ml)	p value
Negative	3 (4.2%)	5.6 – 8.1	6.6±1.30	0.598
Ambiguous	5 (7.1%)	6.4 – 11.2	**8.2±1.88**	
Block Positive	62 (88.5%)	3.4 – 19.6	**7.1±2.42**	
Total	70 (100%)	3.4 – 19.6	7.24±2.35	

Among 70 cases, IHC p16 biomarker expression showed block positive, ambiguous and negative in 62 (88.5%), 5 (7.1%) and 3 (4.2%) cases respectively. Regarding ELISA plasma p16 levels in different groups of IHC p16 expression, the levels were maximum in ambiguous group (8.2±1.88) followed by block positivity (7.1±2.42) and then negative cases (6.6±1.30) with p value of 0.598, which was not statistically significant (Table 24).

Figure 14: ROC curve showing validity of p16 protein in predicting negative IHC p16 expression.



rea under the ROC curve (AUC)	0.582
Standard Error	0.176
95% Confidence interval	0.458 to 0.699
z statistic	0.467
Significance level P (Area=0.5)	**0.6407**

Table 25: Validity of ELISA plasma p16 protein levels in predicting negative IHC p16 expression

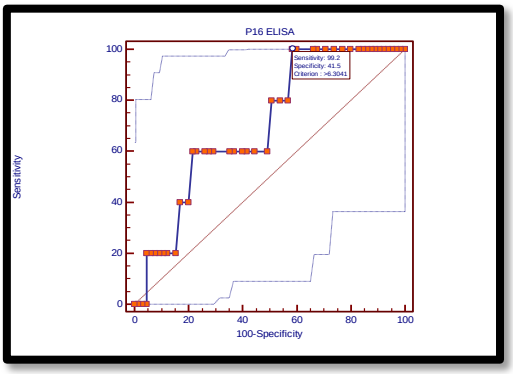
Youden index J	0.3085
95% Confidence interval	0.1940 to 0.3433
Associated criterion	**≤6.2**
95% Confidence interval	**5.1 to 6.2**

Table 26: Sensitivity, specificity, PPV, NPV and Diagnostic accuracy at cut off levels of ≤6.2 ng/ml of ELISA plasma p16 protein in predicting negative IHC p16 expression.

Parameter	Estimate	Lower - Upper 95% Cis
Sensitivity	66.67%	(20.77, 93.85)
Specificity	64.18%	(52.22, 74.6)
Positive Predictive Value	76.92%	(2.135, 24.14)
Negative Predictive Value	97.73%	(88.19, 99.6)
Diagnostic Accuracy	64.29%	(52.59, 74.5)

Validity of ELISA plasma p16 levels in predicting negative expression of IHC p16 is showed in Figure 14 & Table 25. At 95% confident interval the range of p16 protein levels was 5.1 to 6.2 ng/ml and mean was ≤6.2 ng/ml. Sensitivity, specificity, PPV, NPV and Diagnostic accuracy at cut off levels of ≤6.2 ng/ml of ELISA plasma p16 protein in predicting negative IHC p16 expression was 66.67%, 64.18%, 76.92%, 97.73% and 64.29% respectively (Table 26).

Figure 15: ROC curve showing validity of p16 protein in predicting ambiguous IHC p16 expression.



Area under the ROC curve (AUC)	0.702
Standard Error	0.109
95% Confidence interval	0.580 to 0.805
z statistic	1.843
Significance level P (Area=0.5)	**0.0653**

Table 27: Validity of ELISA plasma p16 protein levels in predicting ambiguous IHC p16 expression

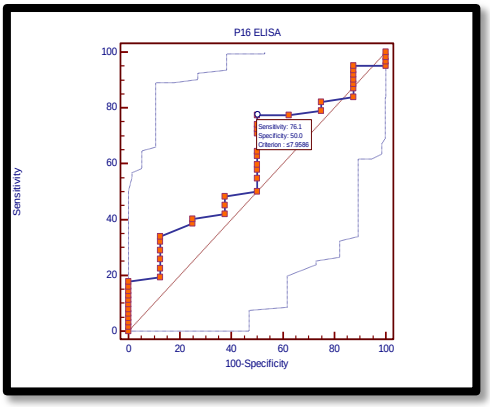
Youden index J	0.4154
95% Confidence interval	0.2769 to 0.5538
Associated criterion	**>6.3**
95% Confidence interval	**6 to 6.6**

Table 28: Sensitivity, specificity, PPV, NPV and Diagnostic accuracy at cut off levels of >6.3 ng/ml of ELISA plasma p16 protein in predicting ambiguous IHC p16 expression.

Parameter	Estimate	Lower - Upper 95% CIs
Sensitivity	100%	(56.55, 100)
Specificity	41.54%	(30.36, 53.66)
Positive Predictive Value	11.63%	(5.07, 24.48)
Negative Predictive Value	100%	(87.54, 100)
Diagnostic Accuracy	45.71%	(34.57, 57.3)

Validity of ELISA plasma p16 levels in predicting ambiguous expression of IHC p16 is showed in Figure 15 & Table 27. At 95% confident interval the range of p16 protein levels was 6.0 to 6.6 ng/ml and mean was >6.3 ng/ml. Sensitivity, specificity, PPV, NPV and Diagnostic accuracy at cut off levels of >6.3 ng/ml of ELISA plasma p16 protein in predicting ambiguous IHC p16 expression was 100%, 41.54%, 11.63%, 100% and 45.71% (Table 28).

Figure 16: ROC curve showing validity of p16 protein in predicting block positive IHC p16 expression.



Area under the ROC curve (AUC)	0.599
Standard Error	0.104
95% Confidence interval	0.475 to 0.714
z statistic	0.950
Significance level P (Area=0.5)	**0.3420**

Table 29: Validity of ELISA plasma p16 protein levels in predicting block positive IHC p16 expression

Youden index J	0.2742
95% Confidence interval	0.1331 to 0.4227
Associated criterion	**≤8**
95% Confidence interval	**5.5 to 9.7**

Table 30: Sensitivity, specificity, PPV, NPV and Diagnostic accuracy at cut off levels of ≤8 ng/ml of ELISA plasma p16 protein in predicting block positive IHC p16 expression.

Parameter	Estimate	Lower - Upper 95% CIs
Sensitivity	77.42%	(65.59, 86.04)
Specificity	50%	(21.52, 78.48)
Positive Predictive Value	92.31%	(81.83, 96.97)
Negative Predictive Value	22.22%	(9.001, 45.22)
Diagnostic Accuracy	74.29%	(62.97, 83.07)

Validity of ELISA plasma p16 levels in predicting block positivity expression of IHC p16 is showed in Figure 16 & Table 29. At 95% confident interval the range of p16 protein levels was 5.5 to 9.7 ng/ml and mean was ≤8 ng/ml. Sensitivity, specificity, PPV, NPV and Diagnostic accuracy at cut off levels of ≤8 ng/ml of ELISA plasma p16 protein in

predicting ambiguous IHC p16 expression was 77.42%, 50%, 92.31%, 22.22% and 74.29% (Table 30).

Table 31: Shows IHC p16 expression and ELISA plasma p16 levels in different age groups

Age Range (years)	No of Cases	IHC P16 expression			P value of IHC p16	ELISA Plasma P16		P value of ELISA plasma p16
		Negative	Ambiguous	Block		Range (ng/ml)	Mean (ng/ml)	
30-39	7 (100%)	0 (0.0%)	0 (0.0%)	**7 (100%)**	**0.008**	5.5 – 9.7	7.4±1.3	**0.139**
40-49	**19 (100%)**	0 (0.0%)	0 (0.0%)	**19 (100%)**		3.4 – 13.0	7.6±2.5	
50-59	15 (100%)	0 (0.0%)	0 (0.0%)	**15 (100%)**		4.4 – 6.9	5.8±0.7	
60-69	**18 (100%)**	2 (11.1%)	3 (16.6%)	13 (72.2%)		5.2 – 19.6	**8.0±3.1**	
70-79	10 (100%)	1 (10,.0)	1 (10.0%)	8 (80.0%)		3.8 – 11.2	6.9±1.9	
80-89	1 (100%)	0 (0.0%)	1 (100%)	0 (0.0%)		8.4	**8.4**	
Total	70 (100%)	3 (4.2%)	5 (7.1%)	62(88.5%)		3.4 – 19.6	7.2±2.35	

Youngest age among cases was 30 years and oldest was 78 years with mean of 54.3±12.0 years. The p value between age of cases and IHC p16 expression was 0.008, which was statistically significant. All cases between 30-59 years of age showed block positivity for p16 IHC (i.e. 100%). The ELISA plasma p16 levels ranged from 3.4 to 19.6 ng/ml with mean of 7.24 ng/ml (SD±2.35). Plasma p16 levels was maximum in a case of 80 years and minimum in age range of 50-59 years. The p value between age of cases and ELISA plasma p16 protein was 0.139, which was not statistically significant (Table 31).

Table 32: Shows IHC p16 expression and ELISA plasma p16 levels in different stages of the disease

Stage of the disease	No of Cases	IHC P16 expression			P value of IHC p16	ELISA Plasma P16		P value of ELISA plasma p16
		Negative	Ambiguous	Block		Range (ng/ml)	Mean (ng/ml)	
Stage I	5 (100%)	0 (0.0%)	0 (0.0%)	**5 (100%)**	**0.294**	3.4 – 9.4	6.86±2.4	**0.068**
Stage II	23 (100%)	2 (8.6%)	1 (4.3%)	20(86.9%)		3.8 – 19.6	7.03±3.08	
Stage III	28 (100%)	1 (3.5%)	1 (3.5%)	**26 (92.8%)**		4.8 – 10.6	6.74±1.29	
Stage IV	14 (100%)	0 (0.0%)	3 (21.4%)	11 (78.5%)		5.1 – 13.0	**8.71±2.26**	
Total	70 (100%)	3 (4.2%)	5 (7.1%)	62 (88.5%)		3.4 – 19.6	7.24±2.35	

There was no statistically significant association (p=0.294) between stage of the disease and IHC p16 expression. However, all stage I cases (100% cases) showed block positive p16 expression. Regarding ELISA plasma p16 levels in different stages of the disease, maximum levels of p16 protein was recorded in stage IV of the disease. The p value was 0.068 between the stages and ELISA plasma p16 protein levels (Table 32). The p value between IHC p16 expression and ELISA p16 values in stage II, III and IV was 0.975, 0.917 and 0.652 respectively.

Table 33: Shows IHC p16 expression and ELISA plasma p16 levels in various Histological Grades

Grade of the disease	No of cases	IHC P16 expression			P value of IHC p16	ELISA Plasma P16		P value of ELISA plasma p16
		Negative	Ambiguous	Block		Range (ng/ml)	Mean (ng/ml)	
WDSCC	38 (100%)	2 (5.2%)	3 (7.8%)	33 (86.8%)	**0.887**	3.4 – 13.0	**7.1±1.97**	**0.018**
MDSCC	15 (100%)	1 (6.6%)	0 (0.0%)	**14 (93.3%)**		5.1 – 11.4	6.7±1.56	
PDSCC	10 (100%)	0 (0.0%)	1 (10.0%)	**9 (90.0%)**		4.4 – 11.2	6.7±2.01	
NKLCSCC	5 (100%)	0 (0.0%)	1 (20.0%)	4 (80.0%)		7.7 – 19.6	**10.6±5.08**	
NKSCSCC	2 (100%)	0 (0.0%)	0 (0.0%)	2 (100%)		7.0 – 7.4	**7.2±0.28**	
Total	70 (100%)	3 (4.2%)	5 (7.1%)	62 (88.5%)		3.4 – 19.6	7.24±2.35	

There was no statistically significant association (p=0.887) between histopathological grade and IHC p16 expression of the disease. 86.8% of the cases of WDSCC (n=33) and 93.3% of MDSCC cases (n=14) showed block positive expression p16 IHC which was not statistically significant. Regarding, ELISA plasma p16 levels in different histological grades of the disease, maximum was recorded in WDSCC and minimum in PDSCC with gradual decrease in values from WDSCC to PDSCC. The p value was 0.018, which was statistically significant (Table 33). The p value between IHC p16 and ELISA p16 in WDSCC, MDSCC and PDSCC was 0.682, 0.406 and 0.008 respectively.

8.4 Discussion:

p16 is a surrogate biomarker in cervical cancer. p16 biomarker can be demonstrated in tissues and in cells by IHC or immunocytochemistry techniques respectively in cervical epithelial dysplastic and tumour cells. Expression of p16 protein in precancerous lesion

suggests possibility of progression to malignancy. Studies have shown positive correlation of p16 marker expression with HSIL and SCC of cervix.[9,10,11] Studies have shown that estimation of p16 protein by ELISA on lysed samples of cervical cells had positive correlation with HSIL. The levels were low in LSIL cases and normal cervix. The rate of detection of cervical dysplasia by ELISA p16 protein and HR-HPV DNA were similar in cervical samples.[12]

In a study by Balasubramaniam et al, the sensitivity and specificity of ELISA p16 protein test in cervical samples was similar to cytology, both having low sensitivity and high specificity compared to Hybrid capture2 test. ELISA p16 protein test showed low sensitivity in detecting small lesions using cervical specimens as lesions shed only a few abnormal cells. The rate of positivity with cutoff of 8 U/ml was 90% for ≥CIN3, 77% for CIN2 and 53% for CIN1. The prevalence of screening was 10.4% with ELISA p16 protein test with cutoff of 8U/ml. The sensitivity and specificity reported for ≥CIN3 at cutoff of ≥8 U/ml was 50.9% and 90.4% respectively and at cutoff of ≥6 U/ml was 64.1% and 77.5% respectively. The sensitivity and specificity reported for ≥CIN2 at cutoff of ≥8 U/ml was 39.9% and 90.7% respectively and at cutoff of ≥6 U/ml was 50.1% and 77.7% respectively.[12]

In a study by Mao et al in cervical samples, the sensitivity and specificity of ELISA p16 protein at cutoff ≥8 U/ml for CIN3 was 90.0% and 46% respectively versus 85% and 35.4% respectively for Hybrid capture2 test. The sensitivity of ELISA p16 protein at cutoff of ≥6 U/ml was 95.0%. The cutoff between 6-12 U/ml had relatively high sensitivity and specificity by which the test can be considered as positive. The cutoff of ≥8 U/ml was

reported as the reasonable choice. Increased size of the lesion was associated with increased p16 protein levels.[13]

In a study by Wu et al in cervical samples, it was reported that ELISA p16 protein had increased specificity in detection of CIN compared to HPV as screening test. The p16 protein estimated was 32.6 U/ml, 38.7 U/ml, 63.4 U/ml and 210 U/ml in normal, CIN1, CIN2/3 and invasive cervical cancer respectively which shows progressive increase in levels of p16 protein with increase in degree of dysplasia. However, the values of p16 protein in each group was quite high compared to other similar studies as the cases were HIV positive women and in HIV positive women the viral load of HPV will be increased compared to non-HIV women. In CIN2+ cases, 78.6% showed positive for p16 protein at cutoff level of 9 U/ml with sensitivity, specificity, positive predictive value and negative predictive value of 89.0%, 22.9%, 13.6% and 93.8% respectively. The sensitivity and specificity varied in different cutoff levels of p16 protein as; at cutoff 7 U/ml, 90.6% and 18.2%, at 8 U/ml 89.8% and 20.6%, at 9 U/ml 89.0% and 22.9%, at 10 U/ml 85.8% and 26.9% respectively.[64]

Huangfu et al in their study reported that auto-antibodies against P16 protein (tumour associated antigen) are released in cases of cervical cancer and is found to have highest levels in serum in stage I of cervical cancer with sensitivity of >90% and specificity of 20.3%. Hence p16 auto-antibody can be used as one of the parameter for early diagnosis and assess prognosis.[65]

In the present study, the blood samples were collected from histologically proved invasive SCC cases and the separated plasma was subjected for estimation for p16 protein by ELISA method. The p16 protein levels among cases ranged from 3.4 to 19.6 ng/ml with

mean of 7.24±2.35 ng/ml. There was statistically significant association of plasma p16 protein levels between cases and controls at different age groups (<0.05) (Table 20). The cut off levels of plasma p16 protein between 3.9 to 5 ng/ml levels in cases had relatively high sensitivity and specificity by which the test can be considered as positive. At cut off more than 4.8 ng/ml in cases, the sensitivity, specificity, PPV, NPV and diagnostic accuracy or overall positivity was 92.86%, 72.86%, 77.4%, 91.1% and 82.86% respectively. Hence cut off at more than 4.8 ng/ml can be considered as reasonable choice for diagnosis of SCC of cervix. The specificity increased with increase in plasma p16 levels in cases. Hence testing p16 protein in SCC of cervix in liquid samples as blood in the present study is the concept of "Liquid Biopsy".

LAST criteria in 2012 defines p16 immunoreactivity as block positive, ambiguous and negative considering p16 biomarker expression in the nucleus with or without cytoplasmic staining.

LAST gives standard guidelines for precise utility of p16 biomarker, decreases interobserver's variation and increases accuracy.[12,18,30,41] In the present study, there was no statistically significant association between IHC p16 expression and ELISA plasma p16 levels. ELISA plasma p16 levels were maximum in ambiguous and block positive cases compared to negative cases. Plasma p16 levels of 5.1 to 6.2 ng/ml, 6.0 to 6.6 ng/ml and 5.5 to 9.7 ng/ml predicts negative, ambiguous and block positivity of IHC p16 expression in corresponding tissue biopsy. The concept of this study was thinking in the angle of liquid biopsy to find the association between tissue IHC p16 expression and plasma ELISA p16 levels. In addition, plasma p16 estimation is molecular evaluation, which will be an objective estimation.

In both cases and controls maximum plasma p16 protein levels were observed in older age group and minimum levels in younger age group. This may be probably due to, majority of cases in older age group in present study had disease of higher stage compared to younger age group. In controls the higher plasma p16 levels in older age group may be probably due to senescence changes. There was significant association between age distribution among cases and IHC p16 expression with younger age (30 to 59 years) showing 100% block positivity probably because of evolving phase of the disease. However, association between age distribution and plasma ELISA p16 levels was not significant (Table 31).

There was no significant association between stage of the disease with IHC p16 expression and ELISA plasma p16 levels. However, all stage I cases showed block positivity IHC p16 expression and maximum cases of block positivity were in stage III followed by stage II indicating increased expression at higher stage of the disease. ELISA plasma p16 levels was maximum in stage IV followed by stage II indicating ELISA plasma p16 increases with stage of the disease (Table 32).

There was no significant association between histological grade and IHC p16 expression. However, there was significant association between ELISA plasma p16 levels and histological grade, the levels were maximum in WDSCC, followed by MDSCC and PDSCC (Table 33).

The ELISA p16 protein test is a molecular evaluation than the morphological evaluation and hence estimation of this biomarker will be more objective with increased reproducibility. It will be a better indicator of molecular changes associated with carcinogenesis. The test procedure can be improved using enhanced version of ELISA which may offer improved sensitivity in screening. Combined with Hybrid capture2 test,

the sensitivity and specificity can be increased. This protein assay biomarker if developed and validated can be more objective, faster and more affordable with less infrastructure and trained technical personnel. It can be a promising potential screening test especially in low resource settings and point of care test (POCT) with low false positivity.[12,13,64] This study points towards the concept of liquid biopsy which is a minimally invasive technique having potential to revolutionize the treatment of cancer, evaluate the progress and relapse of the disease unlike biopsy and cytology techniques.[58]

The limitation of this study was, all the cases were SCC of cervix and CIN as HSIL or LSIL was not considered for the study as we thought of proving the hypothesis in frank cases of SCC of cervix and then consider in CIN. The plasma p16 levels in this study showed statistically significant association between cases and controls. The cut off level of p16 protein level more than 4.8 ng/ml showed statistical acceptable level for diagnosis of disease. The study can be taken forward by conducting it in larger study population and the procedure has to be standardized to prove the hypothesis of the concept of liquid biopsy. The test procedure has to be validated and accredited with laboratory accredited bodies by which the test can be used for screening and follow up of the disease as POCT. As far as our knowledge goes, the concept of this study thinking in the angle of liquid biopsy is first of its kind in English literature to find the association between tissue IHC p16 expression and ELISA plasma p16 levels.

8.5 Conclusion:

Plasma p16 protein estimation by ELISA can be considered as test for diagnosis of SCC of cervix with further standardization of procedure. ELISA plasma p16 protein level ranged from 3.4 – 19.6 ng/ml with a mean of 7.2±2.35 ng/ml in SCC of cervix. The p16 protein

levels ≥4.8 ng/ml can be considered as the test to be positive. In clinically suspected cases of cervical cancer, levels ≥4.8 ng/ml can be considered for the diagnosis as POCT. The ELISA plasma p16 protein levels of 5.1 to 6.2 ng/ml, 6.0 to 6.6 ng/ml and 5.5 to 9.7 ng/ml predicts negative, ambiguous and block positivity of IHC p16 expression respectively in corresponding tissue biopsy. Plasma ELISA p16 levels were maximum in WDSCC, followed by MDSCC and PDSCC. The plasma ELISA p16 levels were high in higher disease stage. Estimation of plasma p16 levels is an objective evaluation. With basic lab facilities and well trained technical personal it can be considered for screening and diagnosis of SCC of cervix.

9. CHAPTER 3

DETECTION OF p16 GENE AMPLIFICATION IN TISSUE DNA BY REAL TIME-PCR.

9.1 Methodology:

DNA extraction from the tissue: The procedure is for two days.

1. First day the tissue biopsy is taken along with 20 µl ELB (Erythrocytes lysis buffer) + 10 µl Proteinase K.
2. Vortex spin was done and refrigerated (- 4ºC) for 10-15 minutes.
3. Again, vortex spin was done and kept in water bath at 55 ºC for 2 hours.
4. Every 2 hours Proteinase K 10 µl was added for 3 cycles (Total of 30 µl of Proteinase K was be added).
5. After 3rd cycle, it is kept in water bath at 55 ºC overnight.
6. On second day, 500 µl 5 M NaCl / 3 M Na acetate (Ph 5.7) + Equal amount of Isopropyl alcohol was added and kept in refrigerator for 30 minutes.
7. It is spinned at 14,000 rpm at 4ºC for 10 minutes, supernatant was removed and pellet retained. 400 µl 80% Ethanol was added to wash pellet and spinned at 14,000 rpm at 4ºC for 10 minutes.
8. Supernatant was removed and pellet retained. 40-50 µl TE (Tris EDTA) was added and spinned at 14,000 rpm at 4 ºC for 10 minutes.
9. Then DNA was suspended in the TE.

Purification of DNA:

1. 20 µl of sample DNA was mixed with 300 µl of 3 molar sodium acetate and 1.5 ml of 100% ice cold ethanol.
2. Kept at minus 20 ºC for one hour, then centrifuged at 12000 rpm/minute at 4 ºC for 30 minutes.
3. Supernatant was removed.
4. Pellet was washed twice with 75% alcohol.
5. Supernatant was removed.
6. Pellet was air dried to remove the ethanol completely.
7. 50 µl of nuclear free water was added to the pellet.

Validation of p16 primers:

1. The working solution of the primers (Sigma Life Sciences) was made by mixing 5 µl of stock solution and 45 µl of nuclear free water so that 1.0 µl had approximately 300 copies.
2. The two primer sets were run in qRT-PCR for standardization of primers at annealing temperature from 50.0 ºC to 60 ºC.
3. The annealing temperature of 58.3 ºC was standardized for one set of primer.
4. The primers were run in qRT-PCR in dilutions from 1:1, 1:10 to 1:100000 in duplicates with blank and Ct values of each was noted.
5. The average Ct (cycle threshold) value between the duplicates was derived.

6. A linearity graph was drawn with average Ct values of dilutions and Log copy number was derived (Figure 17).

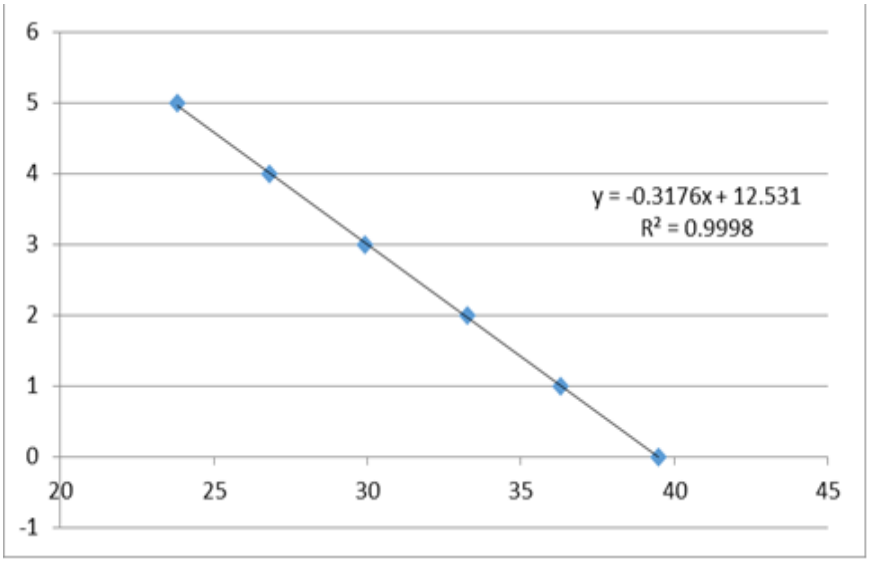
Table 34: The quantity of primers taken for the validation of primers.

TB green	12.5 µl
Forward primer	1.0 µl
Reverse primer	1.0 µl
Template	5.0 µl
Nuclear free water	8.5 µl

Table 35: Shows the dilutions done for primers and average Ct value taken

Sl no.	Dilutions	Conc.	Copies	Ct 1	Ct 2	Avg Ct	Log copy no.
1	1	300 ng	99000	23.1	24.56	23.83	4.995635195
2	10	30 ng	9900	27.02	26.59	26.805	3.995635195
3	100	3 ng	990	29.87	29.95	29.91	2.995635195
4	1000	0.3 ng	99	33.27	33.27	33.27	1.995635195
5	10000	0.03 ng	9.9	36.13	36.45	36.29	0.995635195
6	100000	0.003 ng	0.99	39.58	39.43	39.505	-0.004364805

Figure 17: The linearity graph derived out of average Ct values of dilutions



Estimation of observed Ct value:

1. The primers were run in qRT-PCR with known samples as controls, which showed single peak in melt curve analysis at Ct value of 30.13.
2. Then the purified DNA of test samples were run in qRT-PCR along with positive and negative controls.
3. Of 70 samples, only 43 samples worked with single peak in melt curve analysis with Ct values.
4. The Ct values of 43 were noted (observed Ct values).
5. The samples with Ct value of less than 30 was considered for further analysis, which was 22 samples.

Estimation of expected Ct value:

1. The quantitative estimation of DNA of 22 samples were done by Qubit method (ng/µl) to calculate expected Ct value (1.0 µl approximately had 330 copies of DNA).
2. The copy number for 5 µl of 22 samples was calculated (multiplied) as we had taken 5 µl template (test sample).
3. The log copy number of 22 samples was calculated from the Microsoft excel and expected Ct value was calculated using a formula.

Expression of p16 gene:

The difference between expected Ct value and observed Ct value showed the p16 gene expression

9.2 Statistical Analysis:

The data was entered in Microsoft excel data sheet and analyzed using SPSS 22 version software. Categorical data was represented in the form of frequencies and proportions. Continuous data was represented as mean and standard deviation. Chi-square / Fisher Exact test was used to find the significance of difference between the categorical study parameters. Student T test / ANOVA was used to find the significance of difference between the continuous data. p value <0.05 was considered statistically significant.

9.3 Results:

Seventy cases were considered for the study. Of 70 samples processed, 43 samples showed single peak in melt curve analysis by qRT-PCR. Of 43 samples, 22 samples had Ct value of less than 30.

Table 36: Expected Ct value of 22 cases

Sample No.	QUBIT (ng/ul)	1ng = 330copies (copies/ul)	qRT-PCR (y) (copies/5ul)	log copy number for 5ul	Expected Ct value (x)
4	130	42900	214500	4.632457292	22.6686798
5	80.8	26664	133320	4.425925301	23.31896944
6	89.4	29502	147510	4.469851459	23.1806629
8	202	66660	333300	4.823865309	22.06600972
9	366	120780	603900	5.081995025	21.25325872
10	386	127380	636900	5.105101245	21.18050614
12	54.2	17886	89430	4.252513226	23.86497723
13	218	71940	359700	4.856970433	21.96177444
14	144	47520	237600	4.676876432	22.52882104
15	142	46860	234300	4.670802284	22.54794619
16	105	34650	173250	4.539703239	22.96072656
17	38.8	12804	64020	4.107345665	24.32205394
21	52	17160	85800	4.234517284	23.92163952
22	582	192060	960300	5.283436925	20.61899582
25	268	88440	442200	4.946648734	21.67941203
27	150	49500	247500	4.694605199	22.47299999
28	51.6	17028	85140	4.231163642	23.93219885
29	1380	455400	2277000	5.658393026	19.43840356
7	25.2	8316	41580	3.919914481	24.9122025
18	186	61380	306900	4.788026884	22.17885111
19	886	292380	1461900	5.465947662	20.04433984
20	352	116160	580800	5.065056603	21.30659129

Table 36 shows the method of deriving expected Ct value in tumour tissue DNA samples from the DNA quantification by Qubit method, calculating the copy numbers, converting it into log numbers and then expected Ct value is derived.

Table 37: Expression of p16 gene derived from expected and observed Ct values in 22 cases

Sample no	Expected Ct value (x)	observed Ct value	difference exp-obs	
4	22.6686798	27.76	-5.091320203	Deletion
5	23.31896944	22.58	0.738969443	No change
6	23.1806629	21.14	2.0406629	Amplification
8	22.06600972	23.16	-1.093990282	Deletion
9	21.25325872	26.14	-4.886741277	Deletion
10	21.18050614	26.81	-5.629493857	Deletion
12	23.86497723	21.26	2.604977233	Amplification
13	21.96177444	27.38	-5.41822556	Deletion
14	22.52882104	21.95	0.578821044	Nochange
15	22.54794619	21.56	0.987946195	No change
16	22.96072656	20.82	2.140726564	Amplification
17	24.32205394	20.86	3.462053936	Amplification
21	23.92163952	20.48	3.441639522	Amplification
22	20.61899582	23.29	-2.671004184	Deletion
25	21.67941203	20.1	1.579412033	Amplification
27	22.47299999	26.58	-4.10700001	Deletion
28	23.93219885	23.95	-0.017801152	No change
29	19.43840356	21.9	-2.461596444	Deletion
7	24.9122025	19.69	5.222202503	Amplification
18	22.17885111	23.51	-1.331148893	Deletion
19	20.04433984	20.05	-0.005660158	No change
20	21.30659129	25.97	-4.663408714	Deletion

The p16 gene expression is derived by difference between expected and observed Ct values. Of 22 samples, 7 cases showed p16 gene amplification, 10 cases p16 gene deletion and in 5 there was no change in p16 gene expression (Table 37).

Table 38: Association of tumour grades with p16 gene expression

Sl.no	Tumour grade	P16 gene expression		
		Amplification	Deletion	No change
1	WDSCC	4	7	3
2	MDSCC	-	2	1
3	PDSCC	1	1	1
4	NKLCSCC	1	-	-
5	NKSCSCC	1	-	-
Total		7	10	5

Table 38 shows the p16 gene expression of amplification, deletion and no change is distributed arbitrarily among the different histological grades of the disease.

Table 39: Association of tumour stage with p16 gene expression

Sl. no	Tumour Stage	P16 gene expression		
		Amplification	Deletion	No change
1	I	1	1	1
2	II	1	5	2
3	III	2	3	1
4	IV	3	1	1
Total		7	10	5

Table 39 shows the p16 gene expression of amplification, deletion and no change is distributed arbitrarily among the different stages of the disease.

Table 40: Comparison of p16 gene expression in tissue DNA and IHC p16 expression in tissue sections

Sl.No	Sample No.	p16 gene expression in tissue DNA	p16 IHC expression in tissue sections
1	4	Deletion	Block positive
2	5	No change	Block positive
3	6	Amplification	Block positive
4	7	Amplification	Block positive
5	8	Deletion	Block positive
6	9	Deletion	**Negative**
7	10	Deletion	Block positive
8	12	Amplification	**Ambiguous**
9	13	Deletion	Block positive
10	14	No change	Block positive
11	15	No change	Block positive
12	16	Amplification	Block positive
13	17	Amplification	Block positive
14	18	Deletion	Block positive
15	19	No change	Block positive
16	20	Deletion	Block positive
17	21	Amplification	Block positive
18	22	Deletion	Block positive
19	25	Amplification	Block positive
20	27	Deletion	Block positive
21	28	No change	Block positive
22	29	Deletion	Block positive

The p16 gene expression in tissue DNA (22 cases) shows amplification in 7 cases, no change in 5 cases and deletion in 10 cases. Among these 22 cases, block positivity of IHC p16 expression was noted in 20 cases, ambiguous and negative in one case each (Table 40).

Table 41: Association of p16 gene expression in tissue DNA with DNA quantity

P16 gene expression in tissue DNA	Number of cases	Mean quantity of tissue DNA (ng/µl)
Amplification	7	90.37 (±83.1)
No change	5	260.88 (±351.7)
Deletion	10	**395.20 (±372.7)**
Total	22	267.68 (±321.4)

p value: 0.158

Table 41 shows that the mean DNA quantity was maximum in cases with p16 gene deletion expression followed by no change and then amplification expression. The mean tissue DNA quantity was 267.68 ng/µl.

9.4 Discussion:

p16 protein is a regulator of cell cycle and a tumour suppressor protein. It forms complex with CDK4 and CDK6 and activate pRb which results in arrest of cell cycle at G1-S phase of cell cycle. In HPV related cancers, as in cervical cancer, the HR-HPV infects the tissue and releases E7 protein which inactivates pRb protein resulting in increased synthesis and accumulation of p16 protein in cells due to negative feedback mechanism and results in increased cell proliferation. In non-HPV cancer, p16 gene can be inactivated due to deletion, mutation, hypermethylation or loss of heterozygosity resulting in increased CDK activity, inactivation of pRb and increase in cell proliferation.[12,13,64]

Alteration of p16 gene are reported in cervical cancer. They are methylation, deletion, point mutation, LOH and amplification.[66] In one study, deletion of p16 gene increased the resistance for Cisplatin and radiotherapy to cervical cancer cells.[36]

In a study by Yang et al, methylation of p16 gene was detected in tissue DNA of cervical cancer in 28.2% (24/85) cases. 18 cases were SCC and 6 adenocarcinoma (p = 0.678). Methylation of p16 gene was not detected in normal cervical tissue. Cases with methylation

were older by age than non-methylation cases. Of 24 cases, cases with early stage were 13 and late stage 11 with p value of 0.492. Of 24 cases, WDSCC cases were 12 cases and MDSCC+PDSCC were 12 cases with p value of 0.060. Methylation of p16 gene was not associated with any of clinical parameters. However it was detected in CIN cases. Hence it was concluded that methylation of p16 gene is an intermediate event in carcinogenesis of cervical cancer. [7]

A study by Romagosa et all has stated that over-expression of p16 gene is noted in some types of tumors, such as cervical cancer, head and neck cancer and perianal lesions and was used as a diagnostic tool. It is directly associated with infection by high-risk genotypes of HPV. Over-expression of p16 gene in HPV related tumors is an unsuccessful attempt of p16 gene to stop cell proliferation in p16-Rb pathway. Over-expression of p16 gene in non-HPV cancers are due to Rb gene deregulation, loss of Rb gene or LOH.[66]

In the present study, DNA was extracted from tissue biopsy of SCC of cervix. The study was done to find out the p16 gene amplification in tissue DNA by RT-PCR. The extracted DNA was purified and subjected to qRT-PCR for observed Ct value. The samples with less than 30 Ct value was subjected to DNA quantification by Qubit method and with calculation, expected Ct value was derived. The difference between the expected and observed values showed the expression of p16 gene. Out of 70 samples only 40 samples worked with single peak in melt curve analysis of qRT-PCR. This may be due to pre-analytical error as sampling error, error in preservation of samples, sample consisting of mainly necrotic/hemorrhagic material and fibrous tissue than tumour tissue.

In the present study, the p16 gene expression was varied, 7 cases showed p16 gene amplification, 10 cases showed deletion of p16 gene and 5 cases, no change in expression of p16 gene. The mean tissue DNA quantity was 267.68 ng/µl. The tissue DNA quantity

among the cases with p16 gene deletion was maximum (395.20±372.7) compared to cases with amplification and no change (Table 41).

The limitation of this study was, all the cases were SCC of cervix. CIN as HSIL or LSIL was not considered for the study as we thought of proving the hypothesis in frank cases of SCC of cervix. The study indicates genetic instability, increased amount of DNA in tumour cells with p16 gene deletion, probably heterogeneity in tumour tissue and/or genetic expression in our patient population.

9.5 Conclusion:

p16 gene expression in tumour tissue of squamous cell carcinoma of cervix showed varied expression indicating genetic instability and probably it was due to heterogeneity in tumour tissue.

10. CHAPTER 4

DEECTION OF p16 GENE AMPLIFICATION IN THE DNA EXTRACTS OF PLASMA BY REAL TIME-PCR.

10.1 Methodology:

DNA Extraction from plasma:

1. 560 µl of prepared Buffer AVL containing carrier RNA was taken into a 1.5 ml microcentrifuge tube. (QIAamp DNA Mini Kit, QIAGEN, Germany, Catalog number: 51304)
2. 140 µl plasma was added to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mixed by pulse-vortexing for 15 secs.
3. Incubated at room temperature (15–25°C) for 10 min.
4. Briefly the tube was centrifuged to remove drops from the inside of the lid.
5. 560 µl of ethanol (96–100%) was added to the sample, and mixed by pulse-vortexing for 15 secs. After mixing, briefly the tube was centrifuged to remove drops from inside the lid.
6. 630 µl of the solution was transferred from step 5 to the QIAamp Mini column (in a 2 ml collection tube) carefully without wetting the rim. The cap was closed, and centrifuged at 8000 rpm for 1 min. QIAamp Mini column was placed into a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
7. Carefully the QIAamp Mini column was opened, and step 6 was repeated.
8. Carefully the QIAamp Mini column was opened, and 500 µl of Buffer AW1 was added. The cap was closed, and centrifuged at 8000 rpm for 1 min. The QIAamp Mini Column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

9. Carefully the QIAamp Mini column was opened, and 500 µl of Buffer AW2 was added. The cap was closed and centrifuged at full speed (14,000 rpm) for 3 min.
10. The QIAamp Mini column was placed in a new 2 ml collection tube, and the old collection tube with the filtrate was discarded. Centrifuged at full speed for 1 min. It is done to eliminate any chance of possible Buffer AW2
11. The QIAamp Mini column was placed in a clean 1.5 ml microcentrifuge tube. The old collection tube containing the filtrate was discarded. The QIAamp Mini column was opened and 60 µl of Buffer AVE was added to equilibrated to room temperature. The cap was closed, and incubated at room temperature for 1 min. Centrifuged at 8000 rpm for 1 min.

Estimation of observed Ct value:

1. The p16 primers were run in qRT-PCR with the 22 purified DNA of test (plasma) samples along with positive and negative controls.
2. All 22 samples worked with single peak in melt curve analysis with Ct values.
3. The Ct values of 22 samples were noted (observed Ct values).
4. All 22 samples had Ct value of less than 30.

Estimation of expected Ct value:

1. The quantitative estimation of DNA of 22 samples were done by Qubit method (ng/µl) to calculate expected Ct value.
2. The copy number for 5 µl of 22 samples was calculated (multiplied) as we had taken 5 µl template (test sample).

3. The log copy number of 22 samples was calculated from the Microsoft excel and expected Ct value was calculated using a formula.

Expression of p16 gene:

The difference between expected Ct value and observed Ct value gives the p16 gene expression

10.2 Statistical Analysis:

The data was entered in Microsoft excel data sheet and analyzed using SPSS 22 version software. Categorical data was represented in the form of frequencies and proportions. Continuous data was represented as mean and standard deviation. Chi-square / Fisher Exact test was used to find the significance of difference between the categorical study parameters. Student 't' test / ANOVA was used to find the significance of difference between the continuous data. p value <0.05 was considered statistically significant.

10.3 Results:

A total of 22 cases which showed single peak in melt curve analysis with Ct value of less than 30 in tumour tissue DNA was further analyzed with paired plasma DNA.

Table 42: Estimation of Expected Ct value in 22 plasma samples

Sl.No	Sample No.	Qubit (ng/ul)	1ng = 330copies (copies/ul)	qRT-PCR (y) (copies/5ul)	log copy number for 5ul	Expected Ct value (x) by formula
1	4	31.2	10296	51480	4.711638538	24.62015574
2	5	33.9	11187	55935	4.747683642	24.50666359
3	6	30.4	10032	50160	4.700357528	24.65567529
4	7	61.1	20163	100815	5.003525154	23.70111727
5	8	75.5	24915	124575	5.095430896	23.41174151
6	9	72.6	23958	119790	5.078420565	23.46530049
7	10	28.8	9504	47520	4.676876432	24.72960821
8	12	49.6	16368	81840	4.912965621	23.98625434
9	13	24.5	8085	40425	4.606650029	24.95072409
10	14	60	19800	99000	4.995635195	23.72595971
11	15	56	18480	92400	4.965671971	23.82030236
12	16	**8.3**	2739	13695	4.136562037	26.43085001
13	17	49.9	16467	82335	4.91558449	23.97800853
14	18	34.3	11319	56595	4.752778064	24.49062322
15	19	61.4	20262	101310	5.005652315	23.69441966
16	20	32.5	10725	53625	4.729367305	24.56433468
17	21	56.7	18711	93555	4.971067003	23.80331548
18	22	57.2	18876	94380	4.974879973	23.79130991
19	25	**110**	36300	181500	5.258876629	22.89711389
20	27	105	34650	173250	5.238673243	22.96072656
21	28	59	19470	97350	4.988335956	23.7489422
22	29	106	34980	174900	5.242789809	22.94776508

Table 42 shows the method of deriving expected Ct value in plasma DNA samples from the DNA quantification by Qubit method, calculating the copy numbers, converting it into log numbers and then expected Ct value is derived.

Table 43: Expression of p16 gene was derived from the difference between expected and observed Ct value

Sl.No	Sample No.	Expected Ct value (x) by formula	Observed Ct value	Difference exp-obs	P16 gene expression
1	4	24.62015574	28.62	-3.999844264	Deletion
2	5	24.50666359	25.69	-1.183336406	Deletion
3	6	24.65567529	26.22	-1.56432471	Deletion
4	7	23.70111727	27.46	-3.758882728	Deletion
5	8	23.41174151	29.18	-5.768258488	Deletion
6	9	23.46530049	29.1	-5.634699512	Deletion
7	10	24.72960821	29.82	-5.090391788	Deletion
8	12	23.98625434	29.65	-5.663745657	Deletion
9	13	24.95072409	29.39	-4.439275909	Deletion
10	14	23.72595971	29.31	-5.584040285	Deletion
11	15	23.82030236	30.01	-6.189697642	Deletion
12	16	26.43085001	27.52	-1.089149989	Deletion
13	17	23.97800853	29.05	-5.071991467	Deletion
14	18	24.49062322	28.33	-3.839376777	Deletion
15	19	23.69441966	28.61	-4.915580338	Deletion
16	20	24.56433468	30.65	-6.085665319	Deletion
17	21	23.80331548	28.86	-5.056684519	Deletion
18	22	23.79130991	27.74	-3.948690091	Deletion
19	25	22.89711389	28.61	-5.712886113	Deletion
20	27	22.96072656	28.58	-5.619273436	Deletion
21	28	23.7489422	28.85	-5.101057796	Deletion
22	29	22.94776508	28.68	-5.732234916	Deletion

The p16 gene expression is derived by difference between expected and observed Ct values. All the 22 cases showed deletion of p16 gene (Table 43).

Table 44: Comparison of p16 gene expression in DNA of tissue and plasma

Sl.No	Sample No.	P16 gene expression in tissue	P16 gene expression in plasma	Concordance/ Discordance
1	4	Deletion	Deletion	Concordance
2	5	No change	Deletion	Discordance
3	6	Amplification	Deletion	Discordance
4	7	Amplification	Deletion	Discordance
5	8	Deletion	Deletion	Concordance
6	9	Deletion	Deletion	Concordance
7	10	Deletion	Deletion	Concordance
8	12	Amplification	Deletion	Discordance
9	13	Deletion	Deletion	Concordance
10	14	No change	Deletion	Discordance
11	15	No change	Deletion	Discordance
12	16	Amplification	Deletion	Discordance
13	17	Amplification	Deletion	Discordance
14	18	Deletion	Deletion	Concordance
15	19	No change	Deletion	Discordance
16	20	Deletion	Deletion	Concordance
17	21	Amplification	Deletion	Discordance
18	22	Deletion	Deletion	Concordance
19	25	Amplification	Deletion	Discordance
20	27	Deletion	Deletion	Concordance
21	28	No change	Deletion	Discordance
22	29	Deletion	Deletion	Concordance

Table 44 shows the concordance / discordance of p16 gene expression between 22 paired tissue DNA samples and plasma DNA samples. Among 22 cases, there was concordance in 10 cases and discordance in 12 cases.

10.4 Discussion:

Tumour diagnosis is conventionally done by radiological findings and invasive surgical biopsy. Of late non-invasive technique where blood sample, urine and body fluids are used to extract CTC and genetic material for cancer diagnosis and treatment is called as "Liquid Biopsy".[38,39] Liquid biopsy has evolved slowly at different phases. Scientists isolated tumour cells in blood for the first time in 1869.[49] In 1948 Mandel and Metais isolated ctDNA and RNA in blood in healthy individuals. The neoplastic characteristics of these genetic material was defined after 30 years by Leon et al and 10 years later by Stroun et al.[46,48]

In this technique the liquid sample is used to isolate CTC, ctDNA, RNA, exosomes and proteins which are shed by tumour cells into blood circulation, body fluids or urine in most of the cancers depending on the site of the cancer. The components of liquid biopsy are shed into the body fluids by two mechanisms; active and/or passive mechanism. It is derived by primary tumour or metastatic tumour. The active mechanism is, some tumour cells spontaneously release small pieces of genetic material or DNA into circulation. The passive mechanism is ctDNA is released by tumour cell by apoptosis or necrosis. The ctDNA is also derived by rupture of CTCs in circulation. A single human cell contains 6 pg of DNA and there is an average of 17 ng of DNA per ml of plasma in advanced stage cancers. This technique enables non-invasive profiling of solid tumours, the results which can be comparable with that of tissue biopsy.[9,38-46,48] Tissue biopsy gives only spatially and temporary snap shot of genetic makeup of cancer tissue unlike liquid biopsy, where samples can be taken at repeated intervals and it reveals the dynamic and heterogeneity of the cancer tissue.[46]

Originally liquid biopsy was used to analyze CTC. At present it is mainly used to analyze ctDNA. However CTC and ctDNA are complementary technologies which can be used in

parallel. As ctDNA is a potential surrogate for the entire tumour genome, it is many times referred as “Liquid Biopsy”.[47,48]

Mutational variations in ctDNA in different phases of cervical cancer is reported from circulating tumor DNA in blood which help to monitor the tumor status and predict therapeutic responses. In cervical cancer the sensitivity for the PIK3CA gene was 88.9% and specificity was 100%. The ddPCR confirmed 100% sensitivity and specificity in the detection of the KRAS gene in cervical cancer. The mutation rates of ZFHX3, KMT2C, KMT2D, NSD1, and RNF213 genes have been reported to have high frequency in cervical cancer patients. Gene mutation can serve as a prognostic biomarker. Mutations in tumor suppressor genes are prevalent in all stages of cervical cancer. Chemotherapy and radiotherapy affect the allele frequency, which can be utilized for monitoring cancer through circulating tumor DNA in blood. Tumor suppressor gene mutations reveal the appropriate treatment modalities in patients.[59]

In a study by Yang et al, methylation of p16 gene was detected in plasma DNA of cervical cancer cases in 10% cases. In tissue DNA, methylation of p16 gene was noted in 28.2% cases. In paired cases of tissue and plasma samples, 39% (9/23) and 13% (3/23) cases showed p16 gene methylation respectively. None of the patients without methylation in cervical cancer tissues was found to have methylation in their plasma in paired samples. Hence they concluded that p16 gene methylation may be used as markers for cancer detection.[7]

In the present study, DNA was extracted from plasma sample of SCC of cervix. The study was done to find the p16 gene amplification in plasma sample DNA by RT-PCR. The extracted DNA was subjected to qRT-PCR for observed Ct value. All samples had Ct value less than 30. Then extracted DNA was subjected to DNA quantification by Qubit method and expected Ct value was derived by calculation. The difference between the expected and observed Ct values determined the expression of p16 gene.

In the present study, all 22 cases in plasma sample showed deletion of p16 gene. When compared with paired tissue DNA sample p16 expression, there was concordance in ten paired samples, all showing deletion of p16 gene. There was 12 discordance, 7 having p16 gene amplification and 5 no change in expression of p16 gene in tissue DNA. In addition, the tissue DNA quantity among the cases with p16 gene deletion was maximum (395.20±372.7) compared to cases with amplification and no change (Table 41) probably due to increased secretion of genetic material by tumour cells or increased necrosis / apoptosis of tumour cells with p16 gene deletion. This indicates probably tumour with p16 gene deletion is a fast proliferating or aggressive tumour. In the present study mean plasma and tissue DNA quantity was 54.7 ng/µl and 267.68 ng/µl respectively.

The limitation of this study was, all the cases were SCC of cervix and CIN as HSIL or LSIL was not considered for the study as we thought of proving the hypothesis in frank cases of SCC of cervix. The study indicates tumour heterogenicity / genetic instability in tissue samples and probably the tumour cells with p16 gene deletion expression secretes or releases DNA relatively more into the blood / plasma compared with clones of tumour cells with p16 gene expression of amplification or no change.

As far as our knowledge goes, this study is first of its kind in English literature, where p16 gene expression was observed in DNA extraction of paired tissue / plasma sample of SCC of cervix.

10.5 Conclusion:

The DNA analysis of plasma sample in SCC of cervix showed deletion of p16 gene indicating that the tumour cells with p16 gene deletion secrete or release DNA relatively more into the blood / plasma than the tumour cells with p16 gene expression of amplification and no change.

11. SUMMARY OF THE STUDY

- This is a cross sectional study
- Study period October 2017 to March 2022
- 70 freshly diagnosed cases of invasive SCC of cervix were considered for the study
- Age ranged 30-80 years with mean age of 54.2±12.0
- Age at marriage ranged 12-23 years with mean 15.7±2.1 years of age.
- Parity ranged from 1-11 with mean of 3.6±1.6.
- Common clinical presentation were bleeding and white discharge per vagina
- Growth followed by bleeding were common findings in all cases
- Maximum cases were seen in stage III (40%).
- Keratinizing SCC constituted 89.8% of cases and non-keratinizing 9.9%. Among keratinizing SCC maximum cases were WDSCC (54.2%) followed by MDSCC (21.4%)
- Of 70 cases, p16 expression was block positive, ambiguous and negative in 62 (88.5%), 5 (7.1%) and 3 (4.2%) cases respectively
- Statistically significant association was observed between age of cases and p16 expression. All cases (100% cases) between 30-59 years of age showed block positivity.
- There was no significant association between IHC p16 expression and age at marriage, age at menopause, parity, clinical presentation, stage of the disease, histopathological grade.
- All stage I cases and para 1-3 (100% cases) showed block positive p16 expression
- Most of the cases of WDSCC (86.8%) and MDSCC (93.3%) showed block positive p16 expression.

- The plasma p16 levels among cases ranged from 3.4 to 19.6 ng/ml with mean of 7.24±2.3 ng/ml and in controls ranged between 0.9–9.7 ng/ml with mean of 4.1±2.22 ng/ml, and was statistically significant.
- At 95% confidence interval, cut off p16 levels between 3.9 to 5 ng/ml in cases had relatively high sensitivity and specificity.
- At cut off more than 4.8 ng/ml in cases, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy or overall positivity was 92.86%, 72.86%, 77.38%, 91.01% and 82.86% respectively.
- Maximum levels of p16 protein levels were recorded in stage IV (mean value 8.71±2.26 ng/ml) followed by stage II of the disease (mean value 7.03±3.08 ng/ml), which was not statistically significant
- Maximum p16 protein levels were recorded in WDSCC (mean value 7.1±1.97 ng/ml) and minimum in MDSCC and PDSCC (mean value 6.7 ng/ml), however it was not statistically significant.
- Plasma ELISA p16 protein levels were maximum in ambiguous group (mean value 8.2±1.88 ng/ml) followed by block positive (mean value 7.1±2.42 ng/ml) and then negative cases (mean value 6.6±1.30 ng/ml) among IHC p16 expression with p value of 0.598.
- Validity of mean ELISA plasma p16 protein in predicting negative, ambiguous and block positive expression of IHC p16 in tissue biopsy was 5.1 to 6.2 ng/ml, 6.0 to 6.6 ng/ml and 5.5 to 9.7 ng/ml respectively.
- The mean DNA quantity in tissue was 267.68 ng/µl
- Of 70 samples of tissue DNA, 22 samples had single peak in melt curve analysis by qRT-PCR and Ct value of less than 30.

- Of 22 cases of tissue DNA, 7 cases had p16 gene amplification, 5 cases no change and 10 cases deletion.
- The mean DNA quantity in plasma was 54.7 ng/µl.
- All 22 cases showed p16 gene deletion in plasma samples.
- Of 22 cases considered for p16 gene analysis in plasma samples, only 10 cases showed concordance between tissue and plasma DNA p16 gene expression which was deletion.

12. CONCLUSION OF THE STUDY

- Plasma ELISA p16 protein estimation can be used for diagnosis of SCC cervix as POCT
- Plasma ELISA p16 protein estimation can predict the IHC p16 expression in the tissue
- Detection of plasma p16 gene expression can be considered for the diagnosis of SCC of cervix only in p16 gene deletion cases.
- Detection of plasma p16 gene expression cannot predict tissue p16 gene expression due to heterogenicity in tumour tissue and genetic instability.

13 LIMITATIONS OF THE STUDY:

- The study was done only in one center
- Only SCC of cervix was considered for the study.
- HSIL, LSIL and Adenocarcinoma was not considered for the study.
- Presence of the other primary cancer was excluded only by clinical presentation and clinical examination of the patient.

14. FUTURE OF THE STUDY

The study can be taken forward by conducting it in larger population.

Procedures of the tests has to be standardized / validated for the clinical utility.

15. BIBILIOGRAPHY

1. Chen Z, Pang N, Du R, Zhu Y, Fan L, Cai D et al. Elevated Expression of Programmed Death-1and Programmed Death Ligand-1Negatively Regulates Immune Response against Cervical Cancer Cells. Mediators Inflamm 2016;68:1-11.
2. Balasubramaniam G, Gaidhani HR, Khan A , Saoba S, Mahantshetty U , Maheshwari A Survival rate of cervical cancer from a study conducted in India. India J Med Sci 2021;73:203-11.
3. Ramesh C. Department of Epidemiology and Biostatistics, (Hospital Based Cancer Registry) Kidwai Memorial Institute of Oncology. http://www.kidwai.kar.nic.in/statistics.htm (24/06/2017)
4. Takiar R, Shrivastava A, Nadayil D. The impact of census 2011 data on cancer incidence rates in registries under NCRP – first observations. Cancer Registry Abstract (CRAB), The newsletter of NCRP 2011; XVI(1): 6-11.
5. Kalyani R, Das S, Bindra Singh MS, Kumar H. Cancer profile in the Department of Pathology of Sri Devaraj Urs Medical College, Kolar: a ten years study. Indian J Cancer 2010;47:160-5.
6. Goldblum J, Lamps L, McKenney J, Myers J. Rosai and Ackerman's Surgical Pathology.11th ed. Missouri, USA: Elsevier publishers(p) Ltd; 2017.p.1265-7.
7. Yang HJ,. Liu VWS, Wang Y, Chan KYK, Tsang PCK, Khoo US, Cheung ANY, Ngan HYS. Detection of hypermethylated genes in tumor and plasma of cervical cancer patients. Gynecologic Oncology 2004; 93: 435–440.
8. Sun M, Shen Y, Ren ML, Dong YM. Meta-analysis on the performance of p16/Ki-67 dual immunostaining in detecting high-grade cervical intraepithelial neoplasm. J Can Res Ther 2018; 14:S587-93.

9. Cree IA. Liquid biopsy for cancer patients: Principles and practice. Pathogenesis 2015; 2: 1-4.
10. Romagosa C, Simonetti S, Lo´ pez-Vicente L, Mazo A, Lleonart ME, Castellvi J, Ramon y Cajal S. p16Ink4a overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. Oncogene 2011; 30, 2087–2097
11. Lin J, Albers AE, Qin J, Kaufmann AM (2014) Prognostic Significance of Overexpressed p16INK4a in Patients with Cervical Cancer: A Meta-Analysis. PLoS ONE 2014; 9(9): e106384. doi:10.1371/journal.pone.0106384.
12. Balasubramanian A, Hughes J, Mao C, Ridde R, Herkert M, Kiviat NB, Koutsky LA. Evaluation of an ELISA for p16INK4a as a Screening Test for Cervical Cancer. Cancer Epidemiol Biomarkers Prev 2009; 18(11):3008-3017.
13. Mao C, Balasubramanian A, Yu M, Kiviat N, Ridder R, Reichert A, Herkert M, Doeberitz MVK, Koutsky LA. Evaluation of a new p16INK4a ELISA test and a high-risk HPV DNA test for cervical cancer screening: Results from proof-of-concept study. Int J Cancer 2007; 120: 2435–2438.
14. Wright TC, Ronnett BM, Ferenczy A, Kurman RJ, Elleson LH. Benign diseases of cervix. Blaustein's Pathology of Female Genital tract. 6th ed. New York,USA: Springer publishers(p) Ltd; 2011.p.155-91.
15. Deng H, Hillpot E, Yeboah P,Mondal S, Woodworth CD Susceptibility of epithelial cells cultured from different regions of human cervix to HPV16-induced immortalization Plos one 2018;13:199-204.
16. Jayakumar NKB Cervicitis: How often Is It Non-Specfic J clin Diagn Res 2015;9:11-2.
17. Henry MR, Russel DK, Luff RD, Prey MU, Wright Jr TC, Nayar R Betheseda system for reporting cervical cytology, 3rd ed. Switzerland: Springer publishers (p) Ltd; 2015.p.135-190.

18. Darragh TM,Colgan TJ,Thomas Cox JT,Heller DS, Henry MR ,Luff RD et al. The lower Anogenital squamous Terminology Standardization Project for HPV- Associated Lesions : Background and Consensus Recommendations from the college of American Pathologists and the American society for colposcopy and Cervical Pathology. Arch Pathol Lab Med 2012;136:1-33.
19. Massad LS, Einstein MH, Huh WK, Katki HA, Kinney WK, Schiffman M et al 2012 updated consensus guidelines for the management of abnormal cervical cancer screening tests and cancer precursors. Obstet Gynecol 2013;121:829-4.
20. Berek J. Berek & Novak's Gynecology.14thed.USA: Lippincott williams and wilkins(p) Ltd;2007.p.1343-48.
21. WHO Classification of Tumours Editorial Board: Female Genital Tumours, 5th ed. France: International agency for Research on Cancer (IARC) (p) Ltd; 2020. p.12-14.
22. Maclean AB. Microinvasive carcinoma of the cervix. J obstet Gynaecol 2010; 30: 433-4.
23. Witkiewicz AK, Wright TC ,Ferenczy A , Ronnett BM Blaustein's Pathology of female Genital tract 6th ed. New york: Springer (p) Ltd; 2011.p.253-303.
24. Sheppard KA, Fitz LJ, Lee JM, Benander C , George JA , Wooters J et al. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAPθ/CD3zeta signalosome and downstream signaling to PKCtheta. FEBS Lett 2004;574:37-41.
25. Liu wong J, Zheng BY, Li XD, Angstrom T, Lindstrom MS, Wallin KL. Predictive significance of the alterations of p16 and p14, p53 and proliferating cell nuclear antigen expression in the progression of cervical cancer. Modern Pathology 2005; 18: 629–637.
26. Murphy N, Ring M, Killaloa AG, Uhlmann V, O'Donovan M, Mulcahy F, et al. P16 as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and Thin-Prep smears. J Clin Pathol 2003;56:56-63.

27. Mitchell Rn. The cell as a unit of health & disease. In: Kumar V, Abbas K, Aster JC. Robbins & Cotran Pathology Basis of Diseases. 10th ed. Canada, Jeramy Baues; 2021.p 27.
28. Darragh TM, Colgan TJ, Cox JT, Heller DS, Henry MR, Luff RD, McCalmont T, Nayar R, Palefsky JM, Stoler MH, Wilkinson EJ, Zaino RJ, Wilbur DC. The Lower Anogenital Squamous Terminology Standardization Project for HPV-associated Lesions: Background and Consensus Recommendations from the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology. International Journal of Gynecological Pathology 2013; 32: 76–115.
29. Gonçalves JES, de Andrade CV, Russomano FB, Nuovo GJ, Amaro-Filho SM, Carvalho MOO, Nico AF. The role of p16 as putative biomarker for cervical neoplasia: A controversial issue? Medical Express (Sao Paulo, online) 2017 December; 4(6): M170601.
30. Ahmed SA, Obaseki DE, Mayun AA, Mohammed A, Rafindadi AH, Abdul MA. The Role of Biomarkers (p16INK4a and Ki-67) in Cervical Cancer Screening: An Appraisal. Ann Trop Pathol 2017; 8:1-4.
31. Sarwath H, Bansal D, Husain NE, Mohamed M, Sultan AA, Bedri S. Introduction of p16INK4a as a surrogate biomarker for HPV in women with invasive cervical cancer in Sudan. Infectious Agents and Cancer 2017; 12: 50.
32. Esmaeili HA, Pourlak T, Ghamari B, Shayan FK, Sanaat Z, Soltani GG, Fattahi S. Comparison of nuclear P16 immunostaining in atypical and normal endocervical glands: A descriptive analytical study. J Anal Res Clin Med 2018; 6(2): 66-71.
33. Sheppard K, Bond S, Manek S. Diagnostic Challenge of Cervical Intraepithelial Neoplasia with Concurrent Follicular Cervicitis: Histomorphological Features and P16 Immunostaining as a Diagnostic Adjunct. Adv Cytol Pathol 2018; 3(1): 00041.

34. Umphress B, Sanchez B, Paintal A, Nayar R, Maniar KP. Utility of CK7 versus p16 as a Prognostic Biomarker in CIN 2. Am J Surg Pathol 2018; 42(4): 479-484.
35. Stoler MH, Wright Jr TC, Ferenczy A, Moore JR, Fang O, Kapadia M, Ridder R. Routine Use of Adjunctive p16 Immunohistochemistry Improves Diagnostic Agreement of Cervical Biopsy Interpretation Results From the CERTAIN Study. Am J SurgPathol 2018; 42: 1001–1009.
36. Fu HC, Chuang IC, Yang YC, Chuang PC, Lin H, Ou YC, et al. Low P16INK4A Expression Associated with High Expression of Cancer Stem Cell Markers Predicts Poor Prognosis in Cervical Cancer after Radiotherapy. Int J Mol Sci 2018: 19: 2541.
37. Mahajan Aparna. Practical issues in the application of p16 immunohistochemistry in diagnostic pathology. Human Pathology 2016. doi:10.1016/j.humpath.2015.12.021.
38. Giuseppe Curigliano. Liquid biopsies: Tumour diagnosis and treatment monitoring in a blood test. European Society For Medical Oncology 2014 onsite newspaper.
39. Jof Enriquez. Roche Granted First FDA Approval For Liquid Biopsy Test Detecting Non-Small Cell Lung Cancer. Med Device online News feature 2016.
40. Liquid biopsy. Datar Genetics Blog. (http://blog.datarpgx.com/tag/liquidbiopsy) (Assessed on 25/03/2019)
41. Zill OA, Mortimer S, Banks KC, Nagy RJ, Chudova D, Jackson C, et al. Somatic genomic landscape of over 15,000 patients with advanced stage cancer from clinical next generation sequencing analysis of circulating tumor DNA. J Clin Oncol 2016; 34 (suppl; abstr LBA11501).
42. Liquid Biopsies and Cancer Care. Lab Tests Online AU 2015. (http://www.labtestsonline.org.au/news/pathologyupdate/liquidbiopsiesandcancercare) (Assessed on 20/04/2019)
43. One Patient, Two Cancer DNA Tests, Two Different Results. 2016.

(https://www.cancercommons.org/news/onepatienttwocancerdnateststwodifferentresults) (Assessed on 25/03/2019)

44. In Cancer DNA Tests, Tissue Biopsies and Liquid Biopsies Give Conflicting Results. The Atlantic. (https://www.theatlantic.com/health/archive/2016/12/cancerbiopsygenetictest/510656) (Assessed on 25/03/2019)
45. Hirahata T, Quraish R, Quraish A, Quraish S, Naz M, Razzaq MA. Liquid Biopsy: A Distinctive Approach to the Diagnosis and Prognosis of Cancer. Cancer Informatics 2022; 21: 1–7
46. Crowley E, Nicolantonio FD, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. Nature Reviews Clinical Oncology 2013. (www.nature.com/nrclinonc) (Assessed on 25/03/2019)
47. Pantel K, Alix-Panabières C. Real-time Liquid Biopsy in Cancer Patients: Fact or Fiction? Cancer Res 2013; 73(21): 6384–8.
48. Heitzer E, Ulz P, Geigl JB. Circulating Tumor DNA as a Liquid Biopsy for Cancer. Clinical Chemistry 2015; 61(1): 112–123.
49. Chi KR. The Tumour Trail Left in Blood. Nature 2016; 532: 269-71.
50. Kelly Baldwin. Liquid Biopsy May Help Guide Treatment Decisions for Advanced Solid Tumors: Blood Test Offers a Non-Invasive Alternative to Tissue Biopsy. American Society of Clinical Oncology Annual Meeting News 2017. (https://www.asco.org/aboutasco/presscenter/newsreleases/liquidbiopsymayhelpguidetreatmentdecisionsadvanced) (Assessed on 25/03/2019)
51. Alix-Panabieres C, Pantel K. Circulating Tumor Cells: Liquid Biopsy of Cancer. Clinical Chemistry 2013; 59(1): 110–118.

52. Brock G, Castellanos-Rizaldos E, Hu L, Coticchia C, Skog J. Liquid biopsy for cancer screening, patient stratification and monitoring. Transl Cancer Res 2015;4(3):280-290.
53. Blood Test for Genetic Changes in Tumors Shows Promise as Alternative to Tumor Biopsy. National Cancer Institute 2016. (https://www.cancer.gov/newsevents/cancercurrentsblog/2016/ascoliquidbiopsy) (Assessed on 25/03/2019)
54. Karachaliou N, Mayo-de-las-Casas C, Molina-Vila MA, Rosell R. Real-time liquid biopsies become a reality in cancer treatment. Ann Transl Med 2015;3(3):36.
55. Poulet G, Massias J, Taly V. Liquid Biopsy: General Concepts. Acta Cytologica 2019; 63:449-455.
56. Neto SJ, Batista IA, Costa JL, Melo SA. Liquid Biopsy beyond Circulating Tumor Cells and Cell-Free DNA. Acta Cytologica 2019; 63:479-488.
57. Russano M, Napolitano A, Ribelli G, Luliani M, Simonetti S, Citarella F, et al. Liquid biopsy and tumor heterogeneity in metastatic solid tumors: the potentiality of blood samples. Journal of Experimental & Clinical Cancer Research 2020; 39: 95.
58. Costa JL, Schmitt FC. Liquid Biopsy: A New Tool in Oncology. Acta Cytologica 2019; 63:448.
59. Lee SY, Chae DK, Lee SH, Lim Y, An J, Chae CH, et al. Efficient mutation screening for cervical cancers from circulating tumor DNA in blood. BMC Cancer 2020; 20: 694.
60. Salib MY, Russell JHB, Stewart VR, Sudderuddin SA, Barwick TD, Rockall AG et al 2018 FIGO Staging Classification for Cervical Cancer: Added Benefits of Imaging Radiographics 2020;40:1807-20.
61. Lesnikova I, Lidang M, Hamilton-Dutoit S, Koch J. P16 as a diagnostic marker of cervical neoplasia: a tissue microarray study of 796 archival specimens. Diagnostic Pathology 2009; 4: 22.

62. Amaro-Filho SM, Golub JE, Nuovo GJ, Cunha CB, Levi JE, Villa LL, et al. A Comparative Analysis of Clinical and Molecular Factors with the Stage of Cervical Cancer in a Brazilian Cohort. Plos One 2013; 8: 1-10.
63. Tainio K, Athanasiou A, Tikkinen KAO, Aaltonen R, Hernandes JC, Livson SG, et al. Clinical course of untreated cervical intraepithelial neoplasia grade 2 under active surveillance: systematic review and meta-analysis. BMJ 2018; 360:k499 | doi: 10.1136/bmj.k499.
64. Wu TJ, Smith-McCune K, Reuschenbach M, Doeberitz MK, Maloba M, Huchko MJ. Performance of p16INK4a ELISA as a primary cervical cancer screening test among a large cohort of HIV-infected women in western Kenya: a 2-year cross-sectional study. BMJ Open 2016;6:e012547. doi:10.1136/bmjopen-2016-012547.
65. Huangfu M, Liu L, Xu S, Li S, Jiang K, Sun B, et al. Detecting of p16 Autoantibody as a Potential Early Diagnostic Serum Biomarker in Patients with Cervical Cancer. Clin Lab 2016; 62(6): 1117-20.
66. Romagosa C, Simonetti S, Lo´ pez-Vicente L, Mazo A, Lleonart ME, Castellvi J, Ramon y Cajal S. p16Ink4a overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. Oncogene 2011; 30, 2087–2097.

17. ANNEXURE

17.1. Patient Information Sheet

STUDY TITLE: P16 as a circulating biomarker in invasive squamous cell carcinoma of cervix.

PLACE OF STUDY: Sri Devaraj Urs Medical College attached to R.L Jalappa Hospital and Research, Tamaka, Kolar.

Cervical cancer is the commonest cancer in women in this part of state. The patients usually present at late stage of the disease. It is the commonest cause of death in women due to cancer. The treatment in early stage is removal of uterus and in late stage radiotherapy and chemotherapy. The complications of cervical cancer are obstruction of ureter commonly resulting in renal failure and also spread to other organs.

The participants in this study are patient diagnosed as cervical cancer clinically and confirmed by histopathology. The main aim of the study is to see the association of P16 protein and gene alteration in cervical biopsy and in plasma and to determine whether only plasma can be used to find P16 protein and P16 gene alteration (Liquid biopsy). This information can be used for early diagnosis, follow-up following treatment and early detection of relapse.

You are requested to participate in a study conducted by the department of Pathology as a part of thesis. This study will be done on cervical biopsy specimens and blood samples of the patients. The cervical biopsy specimens will be collected as a part of gynecology investigations in clinically diagnosed or suspicious case of cervical carcinoma and also for DNA extraction by FISH / Real Time PCR. Following confirmation of diagnosis of invasive squamous cell carcinoma of cervix, blood sample of 3 ml will be collected to see the association of the findings in tissue to that in plasma (liquid biopsy).

This study will be approved by the institutional ethical committee. The information collected will be used only for thesis and publication. There is no compulsion to agree to participate. You are requested to sign or provide thumb impression only if you voluntarily agree to participate in the study. All

information collected from you will be kept confidential and will not be disclosed to any outsider. Your identity will not be revealed. No extra cost will be charged to you to participate in this study. Your care will not be affected if you are not willing to participate.

This information sheet document is intended to give you a general background of the study. Please read the information carefully and discuss with your family members. You can ask your queries related to study at any time during the study. If you are willing to participate in the study you will be asked to sign an informed consent form by which you are acknowledging that you wish to participate in the study and entire procedure will be explained to you by the study doctor. You are free to withdraw your consent to participate in the study any time without explanation and this will not change your future care.

The treatment will be as per the standard treatment protocol which will be surgery, radiotherapy or chemotherapy depending on the stage of the cancer. The complications of surgery may vary from mild non-healing of wound to injury to ureter. The complication of radiotherapy will be skin hyperpigmentation / ulcer. The complication of chemotherapy will be nausea, vomiting and loss of hair.

For any clarification, you are free to contact the investigator.

PRINCIPAL INVESTIGATOR: Dr. Kalyani. R.

Contact no: 9448402775 E mail ID: drkalyanir@rediffmail.com

17.2. Patient informed consent

Sl No.

NAME:

AGE:

ADDRESS/PHONE:

I, hereby agree to participate in this study and give consent for conducting study on my tissue and blood sample and nondisclosure of personal information as outlined in the information sheet.

I have been read out/explained in my local language i.e., in_____ and understood the purpose of this study and the confidential nature of the information that will be collected and used for the study. I had the opportunity to ask questions to the principle investigator regarding the various aspects of this study and my questions have been answered to my satisfaction.

SIGN/THUMB IMPRESSION:

NAME AND SIGNATURE OF WITNESS:

NAME AND SIGNATURE OF INVESTIGATOR:

17.3. Proforma

Name: Age:

Hospital No: Biopsy No: Case No:

Nature of specimen:

- Cervical Biopsy
- Hysterectomy specimen
- Blood sample

History of presenting illness with duration:

- White discharge per vagina
- Bleeding per vagina
- Post-coital bleeding
- Mass per vagina
- Abdominal pain
- Others, specify

Menstrual history & Marital Status:

- Age at menarche
- Menstrual cycle
- Age at marriage
- Gravida
- Parity
- Living (children)
- Age at menopause
- Others

Personal History:

- Smoking
- Alcohol
- Use of tobacco
- Use of Oral contraceptives
- Others

Family History

- Family history of cancer: First / second degree relative / others
  - Type of cancer:
- Family history of any syndrome

Clinical findings:

- Significant finding in General physical Examination:
- Per-Abdomen Findings::
- Per-Vaginal Findings:
- Per-Speculum Findings:
- Clinical Diagnosis:
- Clinical stage:

Gross Examination of Specimen:

Histopathological Diagnosis:

Histopathological variant:

Histopathological grade:

Histopathological stage (in hysterectomy specimens):

IHC p16 Expression: Block positive / Ambiguous / Negative

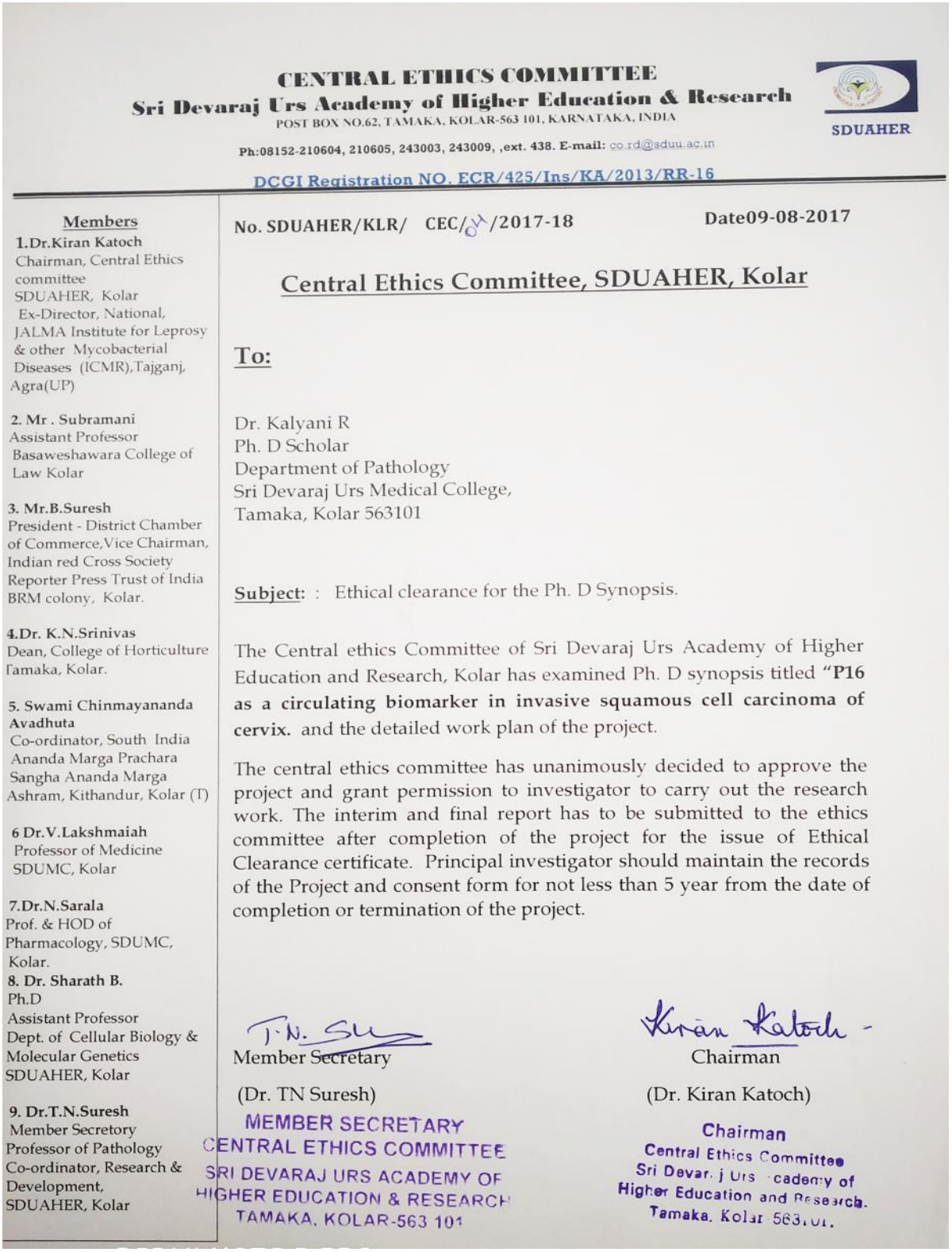
Plasma p16 Protein Estimation by ELISA (Quantitatively):

Expression of p16 gene in Tissue DNA:

Expression of p16 gene Plasma DNA:

Final Conclusion:

17.4. Central Ethics Committee letter



CENTRAL ETHICS COMMITTEE
Sri Devaraj Urs Academy of Higher Education & Research
POST BOX NO.62, TAMAKA, KOLAR-563 101, KARNATAKA, INDIA
Ph:08152-210604, 210605, 243003, 243009, ,ext. 438. E-mail: co.rd@sduu.ac.in
SDUAHER
DCGI Registration NO. ECR/425/Ins/KA/2013/RR-16

Members
1.Dr.Kiran Katoch
Chairman, Central Ethics committee
SDUAHER, Kolar
Ex-Director, National, JALMA Institute for Leprosy & other Mycobacterial Diseases (ICMR),Tajganj, Agra(UP)

2. Mr . Subramani
Assistant Professor
Basaweshawara College of Law Kolar

3. Mr.B.Suresh
President - District Chamber of Commerce,Vice Chairman, Indian red Cross Society
Reporter Press Trust of India
BRM colony, Kolar.

4.Dr. K.N.Srinivas
Dean, College of Horticulture Tamaka, Kolar.

5. Swami Chinmayananda Avadhuta
Co-ordinator, South India Ananda Marga Prachara Sangha Ananda Marga Ashram, Kithandur, Kolar (T)

6 Dr.V.Lakshmaiah
Professor of Medicine
SDUMC, Kolar

7.Dr.N.Sarala
Prof. & HOD of Pharmacology, SDUMC, Kolar.

8. Dr. Sharath B.
Ph.D
Assistant Professor
Dept. of Cellular Biology & Molecular Genetics
SDUAHER, Kolar

9. Dr.T.N.Suresh
Member Secretory
Professor of Pathology
Co-ordinator, Research & Development,
SDUAHER, Kolar

No. SDUAHER/KLR/ CEC/87/2017-18 Date09-08-2017

Central Ethics Committee, SDUAHER, Kolar

To:

Dr. Kalyani R
Ph. D Scholar
Department of Pathology
Sri Devaraj Urs Medical College,
Tamaka, Kolar 563101

Subject: : Ethical clearance for the Ph. D Synopsis.

The Central ethics Committee of Sri Devaraj Urs Academy of Higher Education and Research, Kolar has examined Ph. D synopsis titled "**P16 as a circulating biomarker in invasive squamous cell carcinoma of cervix.** and the detailed work plan of the project.

The central ethics committee has unanimously decided to approve the project and grant permission to investigator to carry out the research work. The interim and final report has to be submitted to the ethics committee after completion of the project for the issue of Ethical Clearance certificate. Principal investigator should maintain the records of the Project and consent form for not less than 5 year from the date of completion or termination of the project.

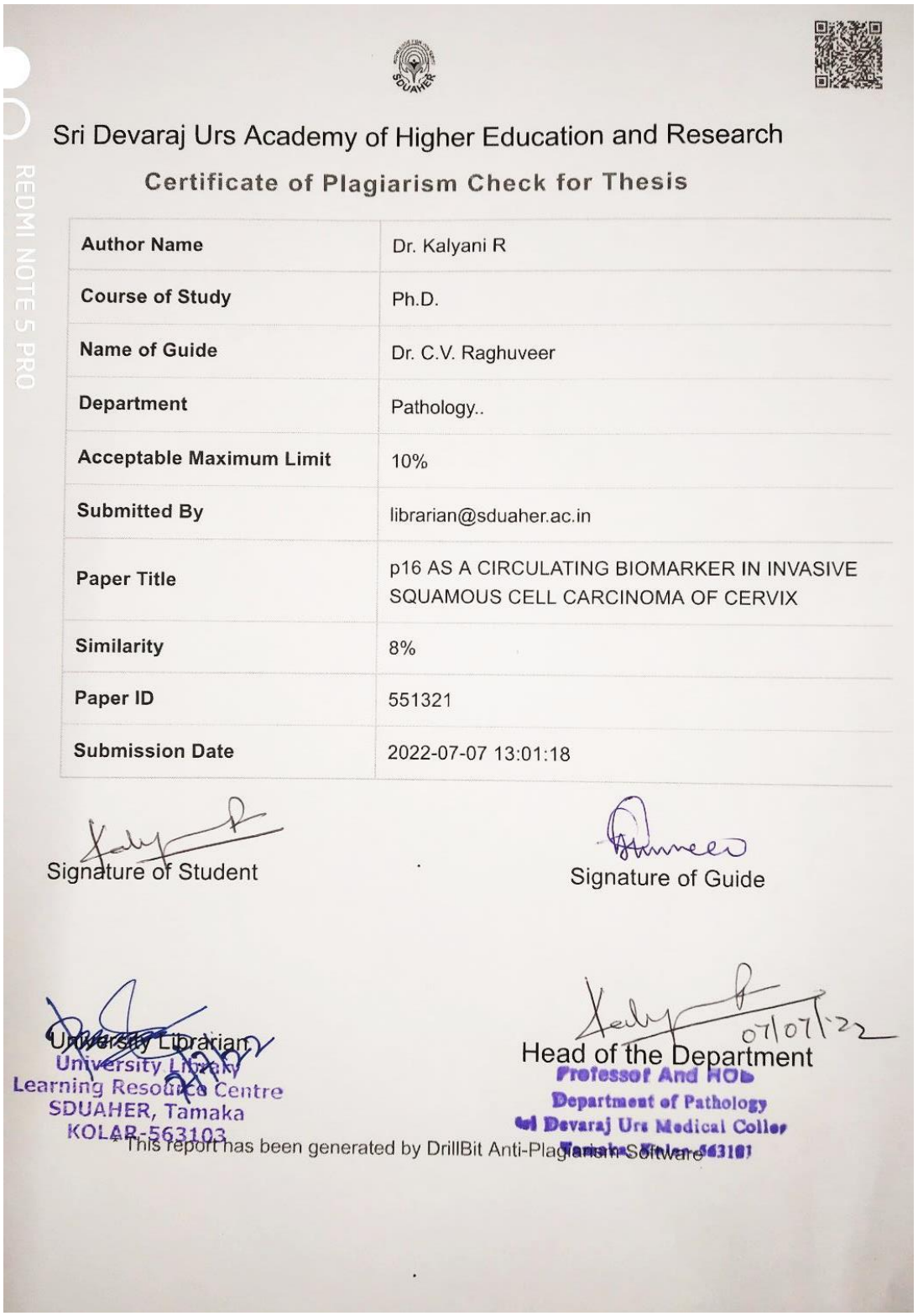
T.N. Su
Member Secretary
(Dr. TN Suresh)

MEMBER SECRETARY
CENTRAL ETHICS COMMITTEE
SRI DEVARAJ URS ACADEMY OF
HIGHER EDUCATION & RESEARCH
TAMAKA, KOLAR-563 101

Kiran Katoch
Chairman
(Dr. Kiran Katoch)

Chairman
Central Ethics Committee
Sri Devar. j Urs cademy of
Higher Education and Research.
Tamaka. Kolar 563101.

17.5. Plagiarism check certificate



Sri Devaraj Urs Academy of Higher Education and Research

Certificate of Plagiarism Check for Thesis

Author Name	Dr. Kalyani R
Course of Study	Ph.D.
Name of Guide	Dr. C.V. Raghuveer
Department	Pathology..
Acceptable Maximum Limit	10%
Submitted By	librarian@sduaher.ac.in
Paper Title	p16 AS A CIRCULATING BIOMARKER IN INVASIVE SQUAMOUS CELL CARCINOMA OF CERVIX
Similarity	8%
Paper ID	551321
Submission Date	2022-07-07 13:01:18

Signature of Student

Signature of Guide

University Librarian

University Library
Learning Resource Centre
SDUAHER, Tamaka
KOLAR-563103

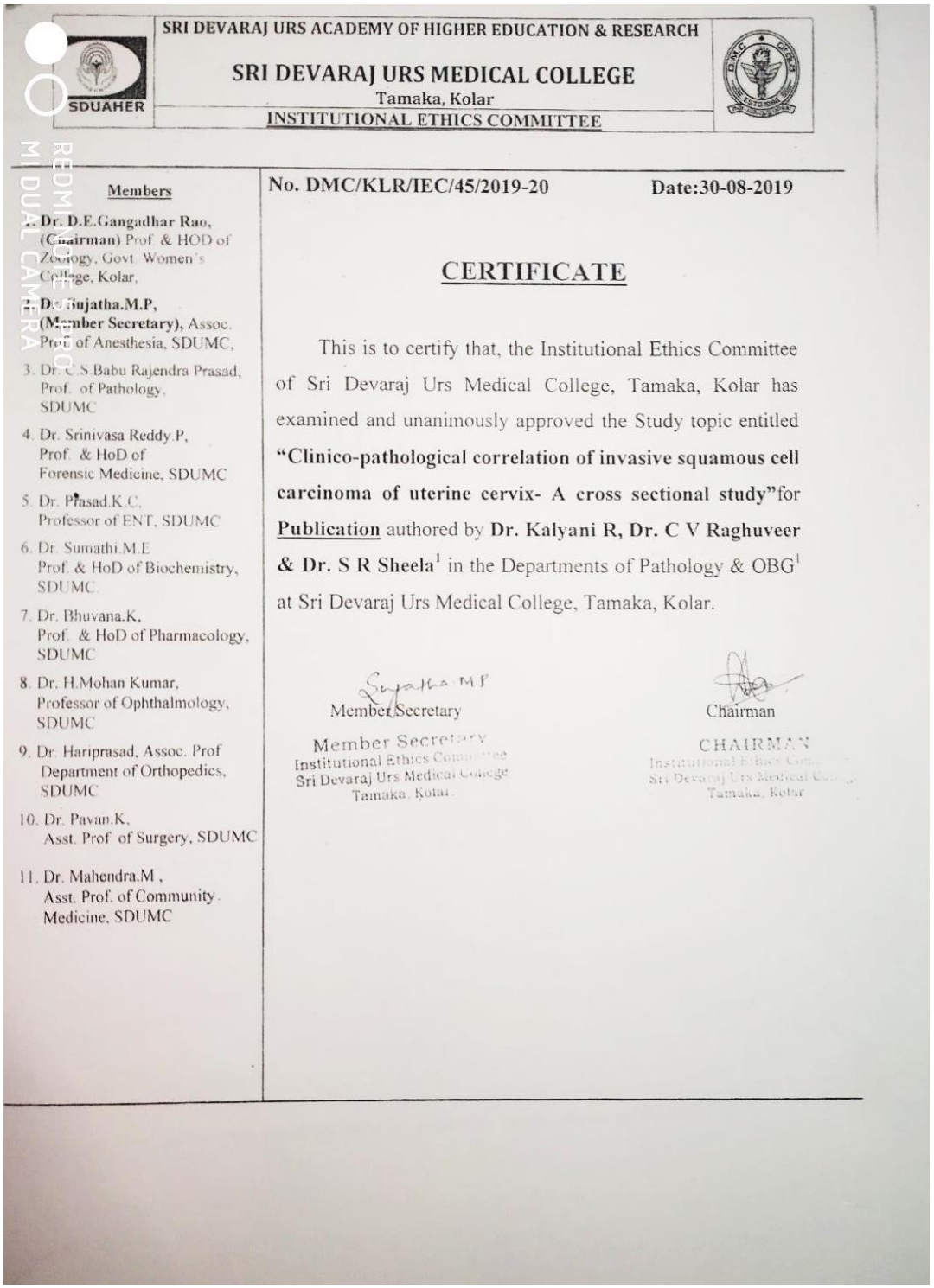
07/07/22

Head of the Department

Professor And HOD
Department of Pathology
Sri Devaraj Urs Medical College
Tamaka, Kolar-563101

This report has been generated by DrillBit Anti-Plagiarism Software

17.6. Ethical clearance certificate for publications



SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH

SRI DEVARAJ URS MEDICAL COLLEGE

Tamaka, Kolar

INSTITUTIONAL ETHICS COMMITTEE

Members

1. **Dr. D.E.Gangadhar Rao, (Chairman)** Prof. & HOD of Zoology, Govt. Women's College, Kolar,
2. **Dr. Sujatha.M.P, (Member Secretary),** Assoc. Prof. of Anesthesia, SDUMC,
3. Dr. C.S.Babu Rajendra Prasad, Prof. of Pathology, SDUMC
4. Dr. Srinivasa Reddy.P, Prof. & HoD of Forensic Medicine, SDUMC
5. Dr. Prasad.K.C, Professor of ENT, SDUMC
6. Dr. Sumathi.M.E Prof. & HoD of Biochemistry, SDUMC
7. Dr. Bhuvana.K, Prof. & HoD of Pharmacology, SDUMC
8. Dr. H.Mohan Kumar, Professor of Ophthalmology, SDUMC
9. Dr. Hariprasad, Assoc. Prof Department of Orthopedics, SDUMC
10. Dr. Pavan.K, Asst. Prof of Surgery, SDUMC
11. Dr. Mahendra.M , Asst. Prof. of Community Medicine, SDUMC

No. DMC/KLR/IEC/45/2019-20 **Date:30-08-2019**

CERTIFICATE

This is to certify that, the Institutional Ethics Committee of Sri Devaraj Urs Medical College, Tamaka, Kolar has examined and unanimously approved the Study topic entitled **"Clinico-pathological correlation of invasive squamous cell carcinoma of uterine cervix- A cross sectional study"** for **Publication** authored by **Dr. Kalyani R, Dr. C V Raghuveer & Dr. S R Sheela**[1] in the Departments of Pathology & OBG[1] at Sri Devaraj Urs Medical College, Tamaka, Kolar.

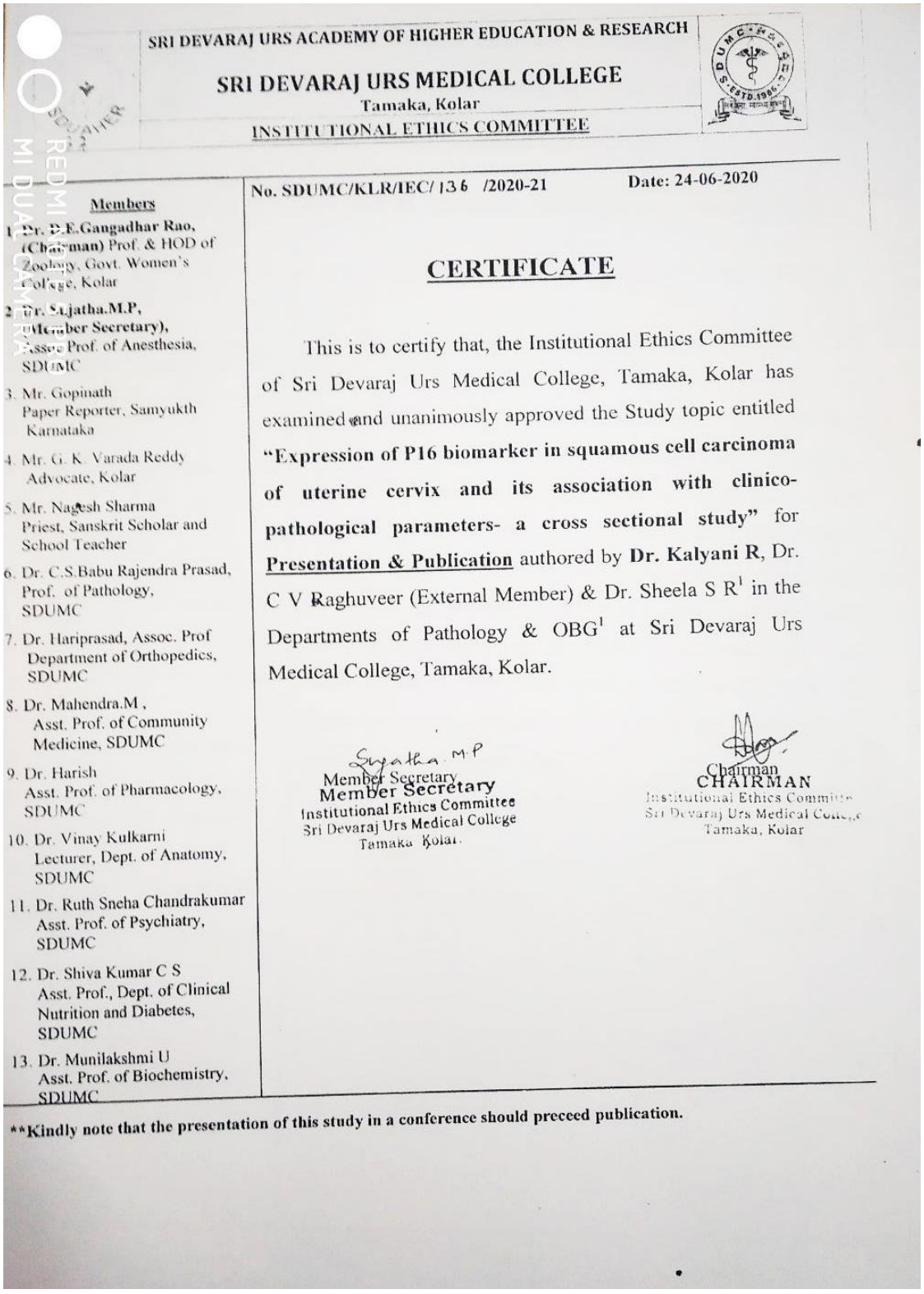
Sujatha M P

Member Secretary

Member Secretary
Institutional Ethics Committee
Sri Devaraj Urs Medical College
Tamaka, Kolar

Chairman

CHAIRMAN
Institutional Ethics Committee
Sri Devaraj Urs Medical College
Tamaka, Kolar



SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH

SRI DEVARAJ URS MEDICAL COLLEGE

Tamaka, Kolar

INSTITUTIONAL ETHICS COMMITTEE

Members

1. Dr. D.E.Gangadhar Rao, (Chairman) Prof. & HOD of Zoology, Govt. Women's College, Kolar
2. Dr. Sujatha.M.P, (Member Secretary), Assoc Prof. of Anesthesia, SDUMC
3. Mr. Gopinath Paper Reporter, Samyukth Karnataka
4. Mr. G. K. Varada Reddy Advocate, Kolar
5. Mr. Nagesh Sharma Priest, Sanskrit Scholar and School Teacher
6. Dr. C.S.Babu Rajendra Prasad, Prof. of Pathology, SDUMC
7. Dr. Hariprasad, Assoc. Prof Department of Orthopedics, SDUMC
8. Dr. Mahendra.M , Asst. Prof. of Community Medicine, SDUMC
9. Dr. Harish Asst. Prof. of Pharmacology, SDUMC
10. Dr. Vinay Kulkarni Lecturer, Dept. of Anatomy, SDUMC
11. Dr. Ruth Sneha Chandrakumar Asst. Prof. of Psychiatry, SDUMC
12. Dr. Shiva Kumar C S Asst. Prof., Dept. of Clinical Nutrition and Diabetes, SDUMC
13. Dr. Munilakshmi U Asst. Prof. of Biochemistry, SDUMC

No. SDUMC/KLR/IEC/136 /2020-21 Date: 24-06-2020

CERTIFICATE

This is to certify that, the Institutional Ethics Committee of Sri Devaraj Urs Medical College, Tamaka, Kolar has examined and unanimously approved the Study topic entitled **"Expression of P16 biomarker in squamous cell carcinoma of uterine cervix and its association with clinico-pathological parameters- a cross sectional study"** for **Presentation & Publication** authored by **Dr. Kalyani R**, Dr. C V Raghuveer (External Member) & Dr. Sheela S R[1] in the Departments of Pathology & OBG[1] at Sri Devaraj Urs Medical College, Tamaka, Kolar.

Sujatha. M.P
Member Secretary
Member Secretary
Institutional Ethics Committee
Sri Devaraj Urs Medical College
Tamaka, Kolar.

Chairman
CHAIRMAN
Institutional Ethics Committee
Sri Devaraj Urs Medical College
Tamaka, Kolar

**Kindly note that the presentation of this study in a conference should preceed publication.

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH

SRI DEVARAJ URS MEDICAL COLLEGE

Tamaka, Kolar

INSTITUTIONAL ETHICS COMMITTEE

Members

1. **Dr. D.E.Gangadhar Rao, (Chairman)** Prof. & HOD of Zoology, Govt. Women's College, Kolar
2. **Dr. Sujatha.M.P, (Member Secretary),** Assoc.Prof. of Anesthesia, SDUMC
3. Mr. Gopinath Paper Reporter, Samyukth Karnataka
4. Mr. G. K. Varada Reddy Advocate, Kolar
5. Mr. Nagesh Sharma Priest, Sanskrit Scholar and School Teacher
6. Dr. C.S.Babu Rajendra Prasad, Prof. of Pathology, SDUMC
7. Dr. Hariprasad, Assoc. Prof Department of Orthopedics, SDUMC
8. Dr. Mahendra.M , Asst. Prof. of Community Medicine, SDUMC
9. Dr. Harish Asst. Prof. of Pharmacology, SDUMC
10. Dr. Vinay Kulkarni Lecturer, Dept. of Anatomy, SDUMC
11. Dr. Ruth Sneha Chandrakumar Asst. Prof. of Psychiatry, SDUMC
12. Dr. Shiva Kumar C S Asst. Prof., Dept. of Clinical Nutrition and Dietetics, SDUMC
13. Dr. Munilakshmi U Asst. Prof. of Biochemistry, SDUMC

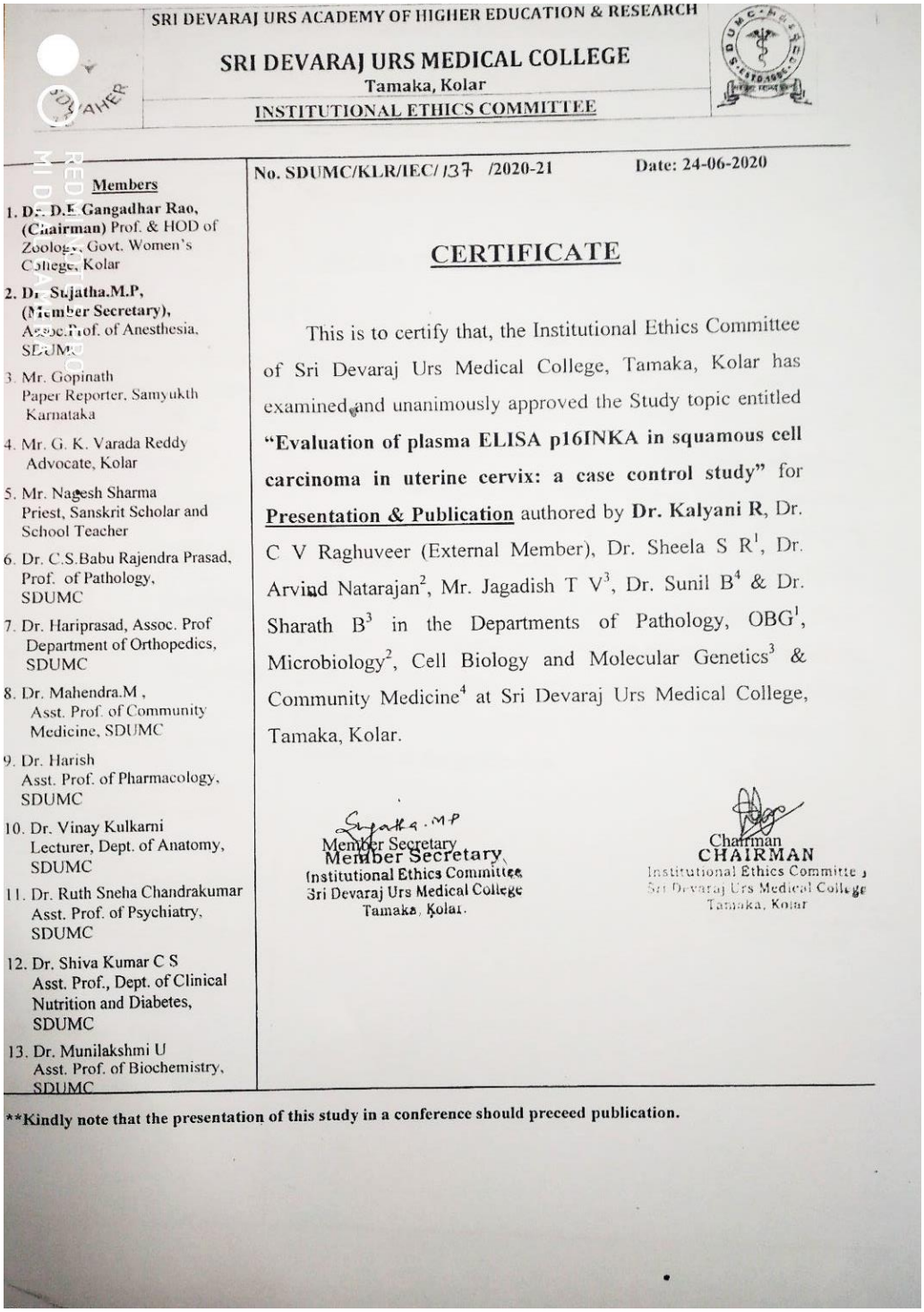
No. SDUMC/KLR/IEC/283/2020-21 Date: 29-09-2020

CERTIFICATE

This is to certify that, the Institutional Ethics Committee of Sri Devaraj Urs Medical College, Tamaka, Kolar has examined and unanimously approved the Study topic entitled **"Association of IHC p16INKa expression and ELISA plasma p16INK4a protein in squamous cell carcinoma of uterine cervix: A concept of liquid of liquid biopsy"** for **Presentation & Publication** authored by **Dr. Kalyani R** (Corresponding Author), Dr. C V Raghuveer, Dr. Sheela S R[1] & Dr. Sharath B[2] in the Departments of Pathology, OBG[1] & Cell Biology and Molecular Genetics[2] at Sri Devaraj Urs Medical College, Tamaka, Kolar.

Sujatha. MP
Member Secretary
Member Secretary
Institutional Ethics Committee
Sri Devaraj Urs Medical College
Tamaka, Kolar.

**Kindly note that the presentation of this study in a conference should preceed publication.



SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH

SRI DEVARAJ URS MEDICAL COLLEGE

Tamaka, Kolar

INSTITUTIONAL ETHICS COMMITTEE

Members

1. Dr. D.E Gangadhar Rao, (Chairman) Prof. & HOD of Zoology, Govt. Women's College, Kolar
2. Dr. Sujatha.M.P, (Member Secretary), Assoc.Prof. of Anesthesia, SDUMC
3. Mr. Gopinath Paper Reporter, Samyukth Karnataka
4. Mr. G. K. Varada Reddy Advocate, Kolar
5. Mr. Nagesh Sharma Priest, Sanskrit Scholar and School Teacher
6. Dr. C.S.Babu Rajendra Prasad, Prof. of Pathology, SDUMC
7. Dr. Hariprasad, Assoc. Prof Department of Orthopedics, SDUMC
8. Dr. Mahendra.M , Asst. Prof. of Community Medicine, SDUMC
9. Dr. Harish Asst. Prof. of Pharmacology, SDUMC
10. Dr. Vinay Kulkarni Lecturer, Dept. of Anatomy, SDUMC
11. Dr. Ruth Sneha Chandrakumar Asst. Prof. of Psychiatry, SDUMC
12. Dr. Shiva Kumar C S Asst. Prof., Dept. of Clinical Nutrition and Diabetes, SDUMC
13. Dr. Munilakshmi U Asst. Prof. of Biochemistry, SDUMC

No. SDUMC/KLR/IEC/137 /2020-21 Date: 24-06-2020

CERTIFICATE

This is to certify that, the Institutional Ethics Committee of Sri Devaraj Urs Medical College, Tamaka, Kolar has examined and unanimously approved the Study topic entitled **"Evaluation of plasma ELISA p16INKA in squamous cell carcinoma in uterine cervix: a case control study"** for **Presentation & Publication** authored by **Dr. Kalyani R**, Dr. C V Raghuveer (External Member), Dr. Sheela S R[1], Dr. Arvind Natarajan[2], Mr. Jagadish T V[3], Dr. Sunil B[4] & Dr. Sharath B[3] in the Departments of Pathology, OBG[1], Microbiology[2], Cell Biology and Molecular Genetics[3] & Community Medicine[4] at Sri Devaraj Urs Medical College, Tamaka, Kolar.

Sujatha. M.P
Member Secretary
Member Secretary
Institutional Ethics Committee
Sri Devaraj Urs Medical College
Tamaka, Kolar.

Chairman
CHAIRMAN
Institutional Ethics Committee
Sri Devaraj Urs Medical College
Tamaka, Kolar

**Kindly note that the presentation of this study in a conference should preceed publication.

17.7. Keys to Master Chart

Samp No: Sample number

CF1: White discharge per vagina

CF2: Bleeding per vagina

CF3: Post-coital bleeding

CF4: Mass per vagina

CF5: Abdominal pain

CF6: Others

AAMi: Age at marriage

Para: Para of women

AAMe: Age at menopause

PS Ex: Per speculum examination findings

PV Ex: Per vaginal examination findings

PR Ex: Per rectal examination findings

HP Type: Histological type

PI: Parametrium involved

WDSCC: Well differentiated squamous cell carcinoma

MDSCC: Moderately differentiated squamous cell carcinoma

PDSCC: Poorly differentiated squamous cell carcinoma

NKLCSCC: Non-keratinizing large cell squamous cell carcinoma

NKSCSCC: Non-keratinizing small cell squamous cell carcinoma

p16 LAST: Expression of IHC p16 as per LAST 2012 classification

p16 ELISA cases: Plasma p16 protein levels in cases in ng/ml

p16 Elisa controls: Plasma p16 protein levels in controls in ng/ml

T p16 gene: Expression of p16 gene in tissue DNA

P p16 gene: Expression of p16 gene in plasma DNA

Master Chart of Cases

	Samp. No	Hosital No	Biopsy No	Age	CF	AAMi	Para	AAMe	PS Ex	PV Ex	PR Ex	HP Type	Stage	P16 LAST	P16 ELISA cases	T p16 gene	P p16 gene
1	4	488831	2230/17	55	1,2,5	14	5	45	Bleeding	Erosion	P1	MDSCC	III	Block	5.9	Deletion	Deletion
2	5	484935	2111/17	30	1,5,6	12	3	--	Growth	Growth	--	MDSCC	I	Block	6.1	No Change	Deletion
3	6	493423	2362/17	50	1,2,5,6	13	2	40	Ulcer	Induration	P1	WDSCC	III	Block	6.4	Amplification	Deletion
4	7	492189	2238/17	55	2,5,6	12	7	45	WD	Induration	--	WDSCC	II	Block	6.3	Amplification	Deletion
5	8	495253	2290/17	50	1	15	3	40	Ulcer	Growth	--	PDSCC	II	Block	4.4	Deletion	Deletion
6	9	501045	2424/17	65	1,2	13	6	50	Growth	Induration	--	WDSCC	II	Negative	5.6	Deletion	Deletion
7	10	501457	2436/17	45	2,4,5,6	15	4	--	Growth	Growth	--	WDSCC	II	Block	4.3	Deletion	Deletion
8	11	501609	2435/17	60	1,2,6	14	4	40	Erosion	Growth	--	WDSCC	II	Block	5.7		
9	12	504242	2494/17	75	6	15	5	40	Stenosis	Induration	P1	PDSCC	IV	Ambiguity	11.2	Amplification	Deletion
10	13	511439	2602/17	45	1,2,6	14	4	43	Growth	Growth	--	MDSCC	II	Block	7.3	Deletion	Deletion
11	14	508851	2579/17	64	1,5,6	14	3	44	Erosion	Induration	--	PDSCC	II	Block	8.4	No change	Deletion
12	15	516355	2674/17	40	1,2,6	12	3	43	Growth	Growth	P1	WDSCC	IV	Block	13	No change	Deletion
13	16	516902	2706/17	52	2,6	12	5	45	Growth	Growth	P1	WDSCC	IV	Block	6.9	Amplification	Deletion
14	17	519043	2755/17	45	1,3,6	15	1	44	Growth	Growth	P1	NKSCSCC	III	Block	7.4	Amplification	Deletion
15	18	461060	2801/17	45	1	15	2	40	Growth	Induration	P1	WDSCC	III	Block	8.7	Deletion	Deletion
16	19	524131	2802/17	48	1,2,5	14	4	47	Growth	Growth	P1	WDSCC	III	Block	7.9	No change	Deletion
17	20	526667	2848/17	40	1,2,5	12	1	45	Unhealthy	Induration	--	WDSCC	I	Block	3.4	Deletion	Deletion
18	21	524454	2822/17	30	1,2	17	2	--	Erosion	Induration	--	WDSCC	I	Block	8.3	Amplification	Deletion
19	22	546618	77/18	45	1,2,3,4,5,6	15	4	42	Bleeding	Induration	P1	WDSCC	III	Block	10.6	Deletion	Deletion
20	23	534707	2706/17	80	--	14	4	--	Growth	Growth	P1	NKLCSCC	IV	Ambiguity	8.4		
21	24	538046	171/18	51	2	16	4	42	Unhealthy	Induration	--	PDSCC	II	Block	5.7		
22	25	541261	229/18	38	1,5	14	1	--	Growth	Stenosis	P1	NKLCSCC	IV	Block	9.7	Amplification	Deletion
23	26	318491	239/17	38	2,5	16	5	--	Growth	Growth	P1	MDSCC	IV	Block	7		
24	27	544289	299/18	65	1,2,5,6	17	4	48	Bleeding	Growth	P1	WDSCC	IV	Block	8.7	Deletion	Deletion
25	28	562211	653/8	40	2,6	14	2	--	Ulcer	Induration	--	WDSCC	II	Block	5	No change	Deletion
26	29	562317	655/18	66	--	18	4	--	Growth	Growth	--	WDSCC	II	Block	9.5	Deletion	Deletion
27	30	560608	647/18	78	1,6	16	6	48	Growth	Growth	--	WDSCC	II	Block	3.8		
28	31	560614	651/18	50	1,5,6	15	3	--	Erosion	Induration	--	PDSCC	II	Block	6		
29	32	561416	727/18	45	1,2,5,6	15	3	44	Growth	Growth	P1	NKSCSCC	IV	Block	7		
30	33	566511	766/18	60	1,2,5,6	16	3	50	Growth	Growth	P1	WDSCC	IV	Ambiguity	8.2		
31	34	566339	806/18	47	1,5,6	18	3	--	Growth	Growth	P1	MDSCC	IV	Block	11.4		
32	35	580025	1107/18	40	5	17	3	--	Ulcer	Induration	P1	WDSCC	IV	Block	11.1		
33	36	573697	1139/18	35	1,2,3,5	15	5	--	Erosion	Induration	--	WDSCC	I	Block	5.5		
34	37	579694	1193/18	45	2,6	15	4	--	Bleeding	Induration	--	WDSCC	I	Block	9.4		
35	38	580449	1227/18	45	1,2	15	5	42	Growth	Growth	P1	MDSCC	II	Block	6.2		
36	39	584508	1265/18	40	1,2,6	18	2	--	Growth	Growth	P1	WDSCC	II	Block	6.5		
37	40	587201	1285/18	35	1,2,5,6	17	2	--	Unhealthy	Growth	P1	WDSCC	III	Block	6.3		
38	41	592899	1416/18	70	1,2,5	16	3	45	Bleeding	Induration	P1	PDSCC	III	Block	6.9		

39	42	592979	1424/18	65	2,5	15	6	45	Mass	Induration	P1	MDSCC	II	Block	6.2		
40	43	592897	1425/18	60	2,5	15	4	45	Growth	Growth	P1	MDSCC	II	Negative	8.1		
41	44	594165	1439/18	65	1,5,6	16	1	50	Growth	Growth	P1	NKLCSCC	II	Block	19.6		
42	45	595556	1464/18	70	2	16	5	45	Growth	Growth	P1	WDSCC	II	Block	6		
43	46	595616	1465/18	55	1,2,5,6	15	4	45	Growth	Growth	P1	WDSCC	IV	Block	6.6		
44	47	598421	1527/18	60	1,2,5,6	19	1	53	Growth	Growth	P1	NKLCSCC	III	Block	7.7		
45	48	591499	1376/18	76	1,2,6	18	4	46	Growth	Induration	P1	MDSCC	II	Block	6.6		
46	49	600124	1585/18	70	1,2	16	5	50	Growth	Growth	P1	WDSCC	IV	Block	7.7		
47	50	597991	1343F/18	56	2	18	6	46	Growth	Growth	P1	WDSCC	III	Block	6.5		
48	51	601000	1604/18	70	1,2	18	4	50	Growth	Induration	P1	MDSCC	III	Block	8.2		
49	52	605342	1704/18	38	1,2,5,6	17	3	--	Growth	Growth	P1	WDSCC	III	Block	7.8		
50	53	605701	1703/18	55	1,2	15	4	42	Growth	Induration	P1	WDSCC	III	Block	5.8		
51	54	606116	1716/18	64	1,2,6	17	2	54	Ulcer	Induration	--	NKLCSCC	I	Block	7.7		
52	55	608576	1782/18	70	1,2,5,6	15	11	50	Growth	Induration	P1	WDSCC	III	Block	7.7		
53	56	608877	1718/18	45	1,2,6	15	2	--	Growth	Growth	P1	WDSCC	II	Block	8.3		
54	57	611282	1847/18	61	1,2,3	15	4	48	Growth	Growth	P1	WDSCC	II	Ambiguity	6.4		
55	58	611518	1863/18	53	1,2,5,6	16	5	47	Growth	Induration	P1	MDSCC	III	Block	5.9		
56	59	616252	1979/18	50	1,2,5,6	15	6	45	Growth	Induration	P1	WDSCC	III	Block	5.3		
57	60	615819	2051/18	58	1,2,5,6	16	2	48	Erosion	Induration	P1	PDSCC	III	Block	4.8		
58	61	620230	2077/18	38	2,5,6	12	1	--	Bleeding	Induration	P1	WDSCC	II	Block	7.5		
59	62	632255	2324/18	70	1,2,6	18	3	50	Bleeding	Induration	PI	MDSCC	IV	Block	5.1		
60	63	633514	2350/18	55	2,6	22	5	50	Growth	Growth	PI	WDSCC	III	Block	4.9		
61	64	634167	2359/18	65	2,5,6	14	5	53	Growth	Growth	PI	WDSCC	III	Block	7.4		
62	65	634967	2372/18	60	1,6	18	3	45	Growth	Growth		MDSCC	II	Block	6.8		
63	66	634944	2377/18	65	2,5,6	23	3	51	Growth	Growth	PI	PDSCC	III	Block	8		
64	67	635273	2396/18	60	1,2,6	17	3	50	Growth	Growth	PI	MDSCC	III	Block	5.2		
65	68	638088	2451/18	60	2,5	14	2	45	Bleeding	Induration		WDSCC	II	Block	8		
66	69	639583	2488/18	49	1,2,5,6	18	2	46	Growth	Growth	PI	MDSCC	III	Block	5.8		
67	70	644058	2581/18	72	1,2,6	16	5	47	Growth	Growth	PI	WDSCC	III	Negative	6.2		
68	71	650745	2727/18	55	1,2,6	17	3	49	Growth	Growth	PI	PDSCC	III	Block	5.6		
69	72	651263	2735/18	65	1,2,5,6	17	4	57	Growth	Growth	PI	WDSCC	III	Ambiguity	6.8		
70	73	651733	2760/18	50	2,6	18	3	45	Growth	Growth	PI	WDSCC	III	Block	6.9		

Master Chart of Controls

Sl.No	Case no	Hosp No	Age	P16 Elisa Controls
1	56	677397	46	7.1
2	64	678030	65	4.8
3	65	677961	34	3.6
4	66	677878	60	7.6
5	67	584350	65	6.2
6	69	675714	60	3.4
7	34	674871	30	1.6
8	36	675014	37	2.8
9	37	675012	45	8.2
10	38	674770	60	7.3
11	41	675275	33	3.6
12	42	675251	31	1.3
13	43	670180	42	3.9
14	51	654791	70	7.1
15	5	672484	40	0.9
16	10	660601	42	8.8
17	24	674185	35	3.5
18	27	674851	46	1.2
19	30	435503	38	1.4
20	33	674873	42	3.6
21	71	659044	45	2.5
22	72	399927	50	8.5
23	126	637890	75	6.7
24	125	458901	70	1
25	124	267901	75	8.5
26	75	377898	48	6.5
27	85	670123	65	6
28	127	821402	56	3.2
29	128	635003	63	3
30	129	756943	60	2.3
31	130	821235	65	2.7
32	131	821044	62	2.4
33	132	820991	65	3.8
34	133	821018	55	3.2
35	134	819590	50	4.3

36	135	743980	40	4.1
37	136	820949	80	3.8
38	137	820953	70	9.7
39	141	820675	55	4.4
40	138	820943	76	3.5
41	139	820945	45	3.1
42	140	819590	50	3
43	142	563611	65	3.2
44	143	818203	78	5
45	144	716149	56	9.2
46	145	821397	65	5.4
47	146	806130	60	5.3
48	147	599204	47	3.4
49	154	818307	60	3.3
50	153	644465	60	7.7
51	152	818945	55	3
52	151	820236	41	2.8
53	150	819546	45	3.4
54	149	820518	55	4.1
55	148	822079	57	4.3
56	155	821741	65	3.5
57	156	808477	70	1.9
58	157	822233	54	1.9
59	158	822284	50	2
60	159	817257	61	1.2
61	160	822640	58	1.5
62	161	820056	75	1.6
63	167	823103	46	1.6
64	166	797271	42	1.7
65	165	822317	70	4.3
66	164	812097	49	3.3
67	163	822331	50	6.2
68	162	822330	53	4.6
69	54	675935	40	4.8
70	29	674804	33	4.7

14. FUTURE OF THE STUDY

The study can be taken forward by conducting it in larger population.

Procedures of the tests has to be standardized / validated for the clinical utility.