DISTRIBUTION OF HUMAN PAPILLOMA VIRUS GENOTYPES IN WOMEN WITH OR WITHOUT CERVICAL CANCER IN AND AROUND KOLAR DISTRICT

By

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REG.NO. 18PY1006

THESIS

Submitted to

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION AND RESEARCH

Kolar, Karnataka

For Awarding the Degree of

DOCTOR OF PHILOSOPHY

IN

MEDICAL MICROBIOLOGY

Under

Faculty of Medicine

Under the Guidance of

Dr. Sheela S.R, Professor

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Tamaka, Kolar, Karnataka 563103

DECEMBER 2022

DECLARATION BY CANDIDATE

I, **Prathyusha Manipathruni,** hereby declare that thesis titled: **"Distribution of Human Papilloma Virus genotypes in women with or without cervical cancer in and around Kolar district"** is original research work carried out by me for the award of **Doctor of Philosophy** in Medical Microbiology.

This study was carried out under the supervision of **Dr Sheela.S.R,** Professor, Department of Obstetrics And Gynecology, Sri Devaraj Urs Medical College, A Constituent Institute of Sri Devaraj Urs Academy of Higher Education and Research.

No part of this has formed the basis for the award of any degree of fellowship previously elsewhere.

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This is to certify the original research work contained in the thesis entitled: **Distribution of Human Papilloma Virus genotypes in women with or without cervical cancer in and around Kolar district",** in the subject Microbiology is carried out by **Prathyusha Manipathruni** (Reg No: **18PY1006**) for the requirement of the award of degree **Doctor of Philosophy** under Faculty of Medicine.

Study was carried out under the supervision of **Dr Sheela.S.R,** Professor ,Department of Obstetrics And Gynecology, Sri Devaraj Urs Medical College, A Constituent Institute of Sri Devaraj Urs Academy of Higher Education and Research

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PRIOR PERMISSION TO START OF STUDY

The Institutional Ethics Committee of Sri Devaraj Urs Medical College, Tamaka, Kolar has examined and unanimously approved the study entitled **"Distribution of Human Papilloma Virus genotypes in women with or without cervical cancer in and around kolar district"** being investigated by Manipatruni prathyusha, Dr. P.M. Beena, Dr. Sheela.S.R[1] & Dr. CSBR. Prasad[2] in the Department of Microbiology, OBG[1] & Pathology[2] Sri Devaraj Urs Medical College, Tamaka, Kolar. **Permission is granted by the Ethics Committee to start the study.**

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LIST OF ABBRIVIATIONS

ASCUS	Atypical squamous cell of undetermined significance
bp	Base pair
BPV	Bovine papillomavirus
CIN	Cervical intraepithelial neoplasia
CDC	Centers for Disease Control and Prevention
CC	Cervical Cancer
CRPV	Cottontail rabbit papillomavirus
DNA	Deoxyribonucleic acid
E6/E7	Early protein
FIGO	International Federation of Gynecology and Obstetrics
FDA	Food and Drug Administration
HPV	Human papillomavirus
H and E	Hematoxylin & Eosin
HR-HPV	High risk Human papillomavirus
HSIL	High grade squamous intraepithelial lesions
HNSCC	Head and neck squamous cell carcinoma

HC2	Hybrid capture 2
HIV	Human immunodeficiency virus
ICC	Invasive cervical carcinoma
IFN-α	Interferon-α
IL-6	Interleukin-6
IARC	International Agency for Research in Cancer
KSCC	Keratinizing squamous cell carcinoma
LR-HPV	Low risk Human papillomavirus
L1/L2	Late protein
LSIL	Low grade squamous intraepithelial lesions
LEEP	Loop electrosurgical excision procedure
MDSCC	Moderately differentiating squamous cell carcinoma
NILM	Negative For Intraepithelial Lesion/ Malignancy
Ocs	Oral contraceptives
ORF	Open reading frame
PAP	Papanicolaou
PAS	Periodic Acid Schiff

PCR	Polymerase chain reaction
PV	Papillomavirus
RFLP	Restriction fragment length polymorphism
SCC	Squamous cell carcinoma
TNF-α	Tissue necrosing factor-α
URR	Upstream regulatory region
VLP	Virus like particles
WHO	World health organization

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Distribution of Human Papilloma Virus genotypes in women with or without Cervical Cancer in and around Kolar district

Abstract

Cervical cancer (CC) is mostly caused by the sexually transmitted Human papillomavirus (HPV). It is the fourth most frequent malignancy in women worldwide and is assessed to have 6, 04, 000 new cases and 3, 42,000 fatalities in 2020. In India, it is the 2nd frequent common cancer in females, with an estimated 123,907 cases reported and 60,078 deaths in 2020. HPV, a heterogeneous DNA virus having icosahedral symmetry belongs to the family papillomaviridae exhibits tropism towards epithelial cells and infects skin and mucosa. HPV consist of more than 200 genotypes, categorised as low, intermediate or high risk based on the extent of the oncogenic potential among which more than 40 genotypes of HPV can easily spread through the genital tract. Fourteen HPV genotypes (HPV 16,18,31,33,35,39,45,51,52,56,58,59,66 & 68) are considered pathogenic or "High-risk" (HR) and persistent infection with these HR HPV contributes to the cancer progression. The genome of HPV has three regions: the early region(E), the late region(L) & the long control region (LCR). The early region encodes for early genes E1, E2, E4, E5, E6 & E7 which are involved in viral replication, transcription regulation, and oncogenesis. E6 and E7 have altering functions and play a vital role in the development of cancer. The oncoprotein E6 binds to the tumor suppressor gene p53 and other pro-apoptotic proteins and increases the efficacy of transformation in combination with E7.E7 attach to the retinoblastoma protein (Rb), which is correspondingly a tumor suppresser and brings about transformation of the cell independently of E6 and also helps in evasion of immune surveillance. The fact that few females with HPV infection advance to cervical cancer exhibits other risk factors which help in this process. According to epidemiological evidence, use of oral contraceptive, parity, having a lot of sex partners, and smoking may all contribute to the growth of cervical cancer.

In order to manage and prevent cervical cancer, genotyping and identification of HPV are essential tools due to the widespread genetic variety of the virus and its clinical consequences. No HPV screening has ever been done in the Kolar region. There is a scarcity of knowledge regarding the common genotypes. Knowledge of genotype prevalence in various populations is crucial for forecasting the efficiency of the existing vaccination and developing new vaccine strategies, especially in light of the partial cross protection provided by the existing vaccines. This work aims to close this gap and provide information for future perspectives.

Against this background, it is obvious that there is an immediate requirement to evaluate the related risk factors and study the prevalence of the HPV genotype in this district. Additionally, to standard cytology testing and histopathology testing on cervical tissues, a cost-effective genotyping technique that is standardized and clinically validated for diagnostic usage is needed for routine Co testing.

It is a cross sectional descriptive study. A total of 235 patients were included in the study. cytobrush samples had been collected from all the patients and were subjected to DNA isolation for HPV followed by PCR and sequencing

The overall incidence of HPV infection was 33.1% in our study. HPV 16 was most predominant 73.8% followed by HPV-18 (16.6%) and HPV-56 (9.5%) in our study. Age group >40 years, parity>3, age at marriage <20years and social habits like chewing tobacco was reported to be strongly linked to HPV infection. In present study the association of Hr HPV with Premalignant and Malignant Cytology showed a significant increase in severity of disease showing HPV as an major risk factor in disease progression (p value 0.03*)

In conclusion study offers first report about the genotype distribution amongst females with cancer cervix and without cancer cases in Kolar region which would help planning an appropriate strategy for disease monitoring. This research also provides the basic information

for future studies in the region. This study found genotypes (HPV 56) which were not included in any HPV vaccinations which would be helpful in devising new vaccine strategies in future. Our results and many studies advise that, regular cervical screening in adult women is vital, in the decrease of HPV linked cervical malignancy. Even though the HPV vaccines are accessible in market, they protect against major types of HPV only but cervical cancer can occur by the other genotypes of HPV as well, we still want to depend on early detection of infection, by various screening approaches. Thus, to find patients who at high risk for developing CC, infection with HPV should also be tested molecularly.

Introduction

INTRODUCTION

Cervical cancer (CC) is mostly caused by the sexually transmitted Human papillomavirus (HPV). It is the 4th most frequent malignancy in women worldwide and is assessed to have 6, 04, 000 new cases and 3, 42, 000 fatalities in 2020.[1] In India, it is the 2nd commonest common cancer in females, with an estimated 123,907 cases reported and 60,078 deaths in 2020.[2]

Infected individuals make up about 75% of the sexually active population. Not all females infected with high-risk groups develop precancerous lesions. The CC risk is increased by cofactors such smoking, many sexual partners, long time corticosteroid use, having poor personal cleanliness, and having poor nutritional condition.[3]

It is essential for greater life expectancy and lower recurrence rates that cervical premalignant lesions are recognized early and treated. In India, the occurrence of CC is still high due to a deficiency of a systematic screening program, and the information available.[4] Men may contract HPV and then pass it on to women, so it doesn't just impact women.[5-8]

Epitheliotropic double-stranded DNA viruses make up the huge and varied group known as human papillomaviruses (HPVs).[9] There are 225 different forms of HPVs and only 40 unique HPV genotypes, that can be grouped into 3 groups on their capacity to cause cancer.5 There are three categories of HPV: high-risk: HPV 16, 18,31,33,35,39, 45,51,52,56,58,59,68,73, and 82; low-risk: HPV6, 11,40,42,43,44,54,61, 70, 72; and intermediate-risk: HPV26, 53, and 66. Interestingly, not all HPV subtypes cause cervical cancer. However, genital types HPV 6 and HPV 11 are minimal risk, whereas HPV 16 &18 pose high risk.[10,11]

Although there are various CC prevention strategies, the HPV vaccines are now the most successful ones. HPV genotyping enables the identification of the predominant HPV types in a given region. This is crucial for choosing the right vaccine and assessing vaccination coverage and efficacy.[12]

HPV genotypes are sparse and inconclusive. HPV genotyping is crucial for monitoring the efficiency of HPV vaccination program, following up on cases of abnormal Papanicolaou (Pap) smears, preventing long-term infection that might result in cervical precancerous, and treating cancer at an early stage. Understanding the genotype distribution across various populations is crucial for predicting the efficiency of the existing vaccination and developing new vaccine strategies because the current vaccines only provide limited cross protection.[13]

Contrasted with other malignancies CC is a vaccine-preventable disease. Three FDA-approved HPV vaccines are at hand for boys and girls aged 9 to 26. Gardasil (2006) is a quadrivalent vaccination that gives protection from HPV 6, 11, 16,18, in addition being bivalent against HPV 16 and 18. Gardasil 9 gives defense against HPV genotypes 6, 11,16,18,31,33,45,52, and 58, and the FDA authorized one more dose of the vaccination in 2014.[14,15]

Although lot of developing countries have already included the HPV vaccine in their national immunization programs, but India is still lagging behind. [16] After the immunization program is put in place, its effectiveness must be checked in addition to make further decisions. Among the clearly observable outcomes of HPV vaccination is a marked decrease in the prevalence of particular strains of HPV. Therefore, estimating the prevalence of the HPV genotype is essential for both the execution of the right vaccine and post-vaccination monitoring.[17]

India has an estimated incidence of 18.7 per 100,000 people and an age-adjusted death rate of 11.7%, despite being one of the major countries with the maximum prevalence of CC. Data on the frequency, type distribution, and risk factors linked to HPV infection is lacking in many Indian states, including Karnataka.[18,19]

No HPV screening has ever been done in the Kolar region, and there is a paucity of knowledge regarding the common genotypes. knowledge of genotype distribution in various populations is crucial for forecasting the efficiency of the existing vaccination and developing new vaccine strategies, especially in light of the partial cross protection provided by the existing vaccines. This work aims to close this gap and provide information for future perspectives.

Against this background, it is obvious that there is an immediate requirement to evaluate the related risk factors and study the prevalence of the HPV genotype in this district. Additionally to standard cytology testing and histopathology testing on cervical tissues, a cost-effective genotyping technique that is standardized and clinically validated for diagnostic usage is needed for routine Co testing.

Review of Literature

REVIEW OF LITERATURE

Introduction to papilloma virus:

Papillomaviruses (PVs) are small non enveloped, Epitheliotropic double-stranded DNAs viruses capable of causing benign lesions and malignancies of skin and mucous membranes. This group of viruses infects both animals and humans. PVs have been recognized in various vertebrate species including primates, rabbits, dogs, and cattle other than humans. They are species specific to their host range; therefore, PV of one kind cannot produce natural infections in other species (vertebrate host).[20, 21]

Papilloma virus History:

Research on papillomaviruses began in 1930s. The revelation that cotton rabbit papillomavirus (CRPV) could not only produce benign lesions in rabbits but that some of these lesions also proceeded to squamous cell cancer was the first indication of papillomavirus (PV) work.[22]

Rabbit oral papillomavirus (ROPV), a non-oncogenic virus that formed spontaneously regressing oral papillomas in domestic rabbits, was the second papillomavirus to be discovered.[23]

These investigations confirmed the host specificity of papillomaviruses. During this time, other papillomaviruses, including the canine (COPV) and bovine oral papilloma virus (BOPV)varieties, were also identified. Owing their inability to be grown in monolayer cultures, studies on papillomaviruses have been restricted ever since.

The recent advances in molecular biology have given HPV research new directions. Several medically significant human papillomavirus (HPV) subtypes were discovered in the 1970s.

Those who have cutaneous warts showed evidence of HPV genotypes 1 and 2.[24] Epidermodysplasia Verruciformis (EV) patients have shown evidence of distinct types of HPV their lesions .[25]Squamous cell carcinoma has advanced in some lesions in EV patients. This was the initial proof that HPV and cancer in humans were related. HPV 6 and 11 were the first genital HPVs to be discovered.[26] Females who had genital warts were shown to carry these HPV subtypes. The recognition of two significant HPV varieties, HPV 16 & 18, has demonstrated the part of HPV in cervical cancer.[27,28] While both these genotypes have been known in females with cancer cervix, the growth of PCR primers to amplify HPV DNA from cervical tissue has transformed the true significance of the role of HPV in cervical cancer.[29]

Numerous studies have therefore determined that HPV infection as a important risk factor for the occurrence of cervical cancer.[30]

Morphology and molecular structure of Human papillomavirus(HPV)

Morphology

HPV are small, non - enveloped, icosahedral viruses with diameter of about 52–55 nm. Viral particles consist of a double-stranded DNA molecule that is made up of about 8,000 base pairs (bp). A protein capsid made up of 72 pentamer capsomers coats the virus and contains the structural proteins late L1 (size 55 kDa; 80% of the total viral protein) and L2 (70 kDa).[31] Production of virus-like particles (VLPs) is only possible by L1 expression

along with L2. The intact virion has a density of 1.34 g / ml in cesium Chloride and sedimentation coefficient (S20, W) 300

Molecular structure of the Virus

8 kbp of closed double-stranded DNA that carries the early genes and late genes of HPV genome in separate regions. Protein precursors engaged in viral DNA replication, transcription and cell transformation are coded by Early genes. The major viral capsid protein (L1) and the minor capsid protein (L2) are determined by late genes. There is upstream regulation between the two regions region (URR), also identified as the long control region. This non-coding region contains Promoters and elements associated with DNA replication and transcription. Every HPV type has not only a promoter for E6, common to all HPVs, but also one or more specific ones Promoters in URR.[33,34]

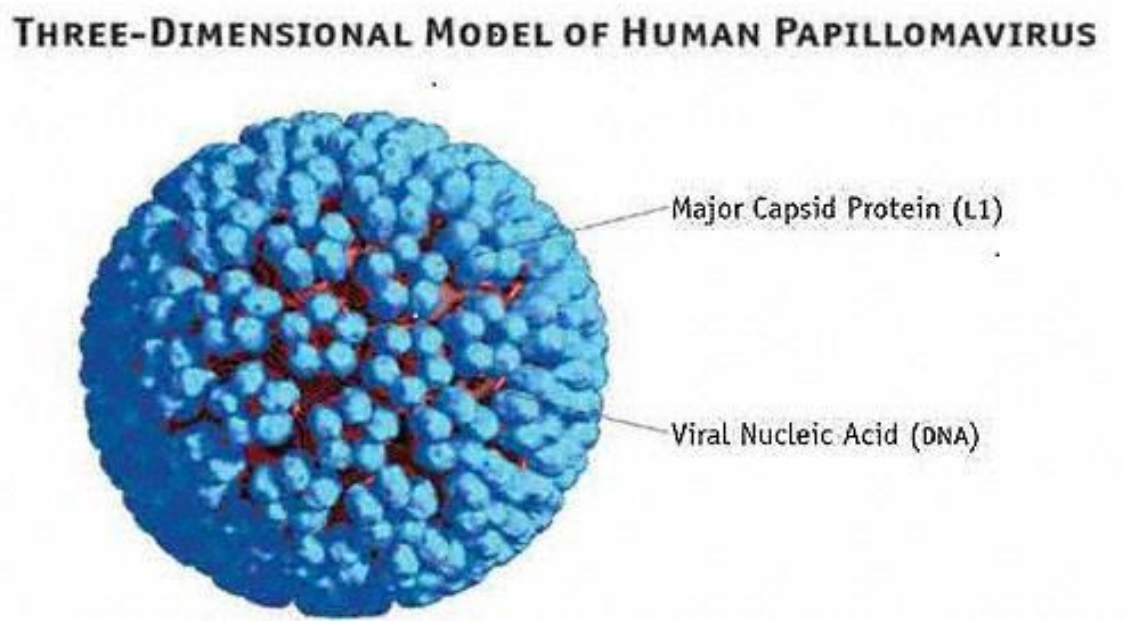


Fig 1: Structure of Human papilloma Virus

HPV Genome and proteins:

Approximately 8,000 base pairs of double-stranded DNA make up the HPV genetic material.[35] HPV genome composed of three main domains that is a one kilobyte upstream regulatory region, an early region comprising six genes (E1,E2,E4,E5,E6, E7), and also a late region with two genes (L1 and L2), which correspond to major and minor capsid proteins.[36]

The HPV genome has Early (E) genes and Late (L) genes. The area of early genes codes for transcriptional regulatory proteins and DNA replication whereas the area of late gene encodes major capsid and minor capsid proteins.

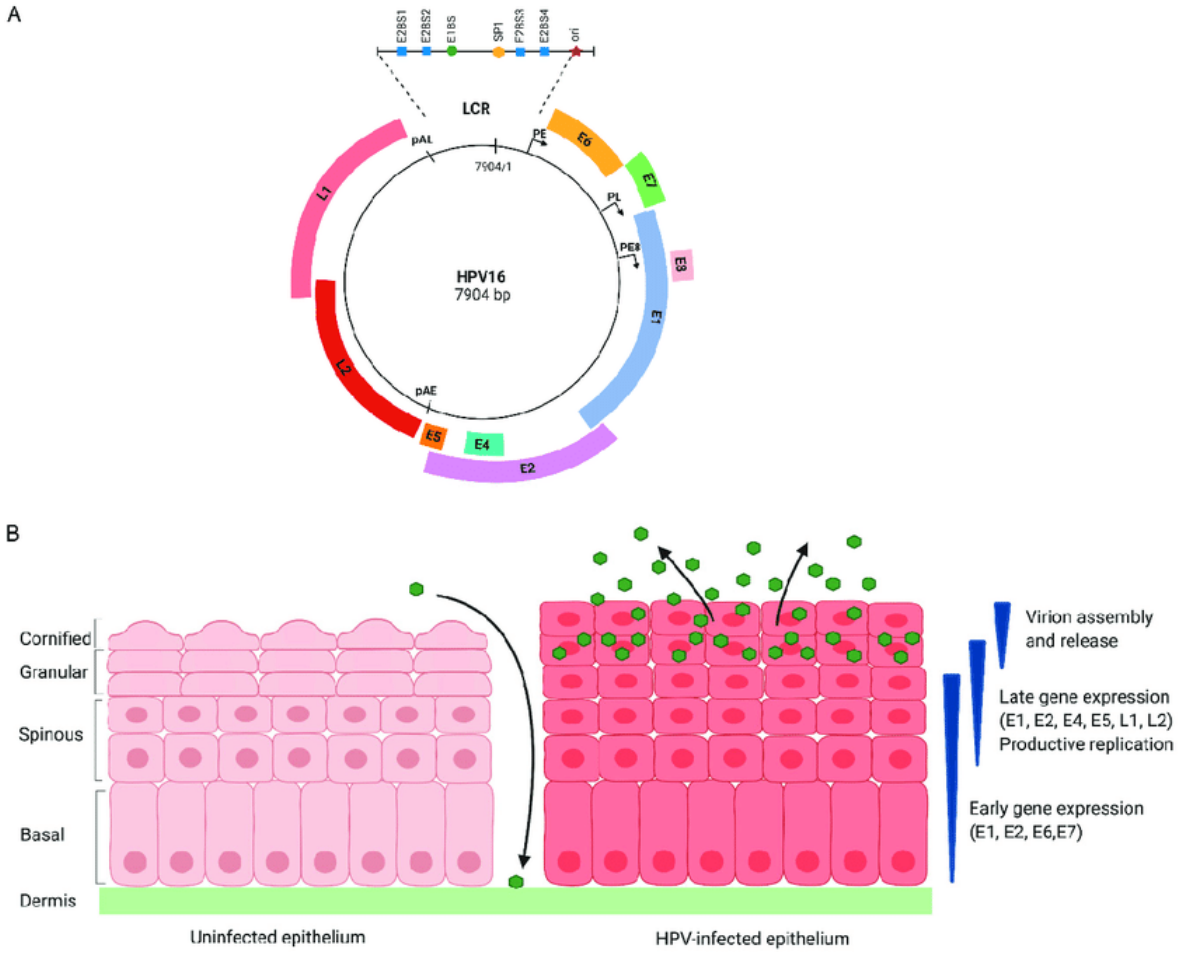


Fig 2: The HPV genome and epithelial expression

E1 protein: This protein helps in replication of DNA and maintains the copies of viruses in the infected cell.[37,38]

E2 protein: Along with the E1 protein, E2 also takes role in DNA replication. Additionally, this protein is crucial for cellular transformation, the start and prevention of apoptosis, the control of transcription, and the modification of HPV's capacity for immortalization and transformation.[37,39,40]

E4 protein: Due to its ability to change the cellular keratin framework and the growth of cornified envelope, this protein aids in the release or spread of viruses.[41]

E5 protein: It participates in DNA replication of virus & also cellular transformation.This protein also prevents the host's immune system from recognizing the infected cell.[37,38,39]

E6/E7 proteins: The major proteins in charge of HPV-mediated malignant cell changes are E6 and E7. They stop infected cells from going through the normal cell cycle & controlling it. The E6 protein interacts to the p53 protein, allowing unchecked replication of HPV 16/18 infected cells and its proteolytic destruction .[37,39,42]. The pRb protein is bound by the E7 protein, which degrades it and causes the cell to lose control of cell cycle.[43.] The onco-proteins E6/E7 expression is regulated by protein E2, host cell protein YY1 and pro-inflammatory cytokines[40]

L1 and L2 proteins: The structural proteins L1 /L2 are encoded in the genome's late region. These proteins contain a capsid protein and guard the viral DNA inside; they are synthesized in the top layer of the epithelium. [44]. In addition, L1 protein can be assembled into empty capsid-like structures and its immunogenicity is similar to infectious virions. Thus, current vaccines used to prevent HPV infection include L1 protein as the major

component[45,46]. Viral components must be transported into the nucleus by the L2 protein in order for them to enter cells and bind to DNA. L2 protein induces production a wide range of neutralizing antibodies against various types of HPV. These antibodies are more cross-reactive between HPV genotypes compared to L1-induced antibodies. The L2 protein is therefore considered as a vital constituent of the upcoming vaccines.[47]

Classification of Human papillomavirus (HPV)

HPVs are grouped between 5 of the 37 genera: alpha,beta,gamma,mu, and nu . Most alpha PVs shows predilection for genital epithelial cells, so they are called genital-mucosal types. HPV types which have a strong oncogenic potential to cause cancer in humans are classified in IARC Group 1 and are frequently referred to as high-risk types and belong to species 5, 6, 7, 9 and 11. The two utmost common high-risk types, HPV-16 and 18, belongs to species types 9 and 7 respectively. While HPV6 is a member of species 10 and is linked with most genital warts.[48]

Members of alpha species 4 (HPV2, 27 and 57), beta, gamma, mu and nu viruses are primarily infectious to non-genital skin. Unlike other HPVs, some are beta HPVsepidermodysplasic verruciformis (EV) -specific, cause lesions mainly in patients with EV.[49,50] If the genome diversity of any type of HPV is 1 to 2 percent, then it is considered Variant of this particular type of HPV. Even very closely related variants may have different ones pathogenicpotentials. For example, HPV type 16 has different variants; Asian American (AA) or African (AF) variants of HPV16 represent three times higher risk of CC compared to European onesvariants (EUV). Similarly, non-EUV HPV18s are more oncogenic to cervical tissues thanEUV.[51-54]

Anogenital infections are linked to over 50 genotypes. The International Agency for Research on Cancer (IARC, 2012) divides HPV into 3 groups based on the relationship with CC. Group 1 HPVs (high risk) are strongly associated with CC. HPV belonging to group 2A (probably carcinogenic) and group 2B (possibly carcinogenic) have low oncogenic potential for humans. 12 HPV, i.e. HPV 16,18,31,33,35,39,45,51,52,56,58 and 59 belong to IARC group 1, while HPV68 is the only member of the IARC group 2A. These 13 types are responsible for 98.7 percent of CCs worldwide. Less frequently than other high-risk kinds, IARC members group 2B (HPV 26,30,34,53,66,67,69,70,73,82,85 & 97) were shown to be related with CC.[55-58]

More than 70% of all CC & precancerous lesions worldwide are caused by just two high-risk strains of HPV: 16 and 18. [59]

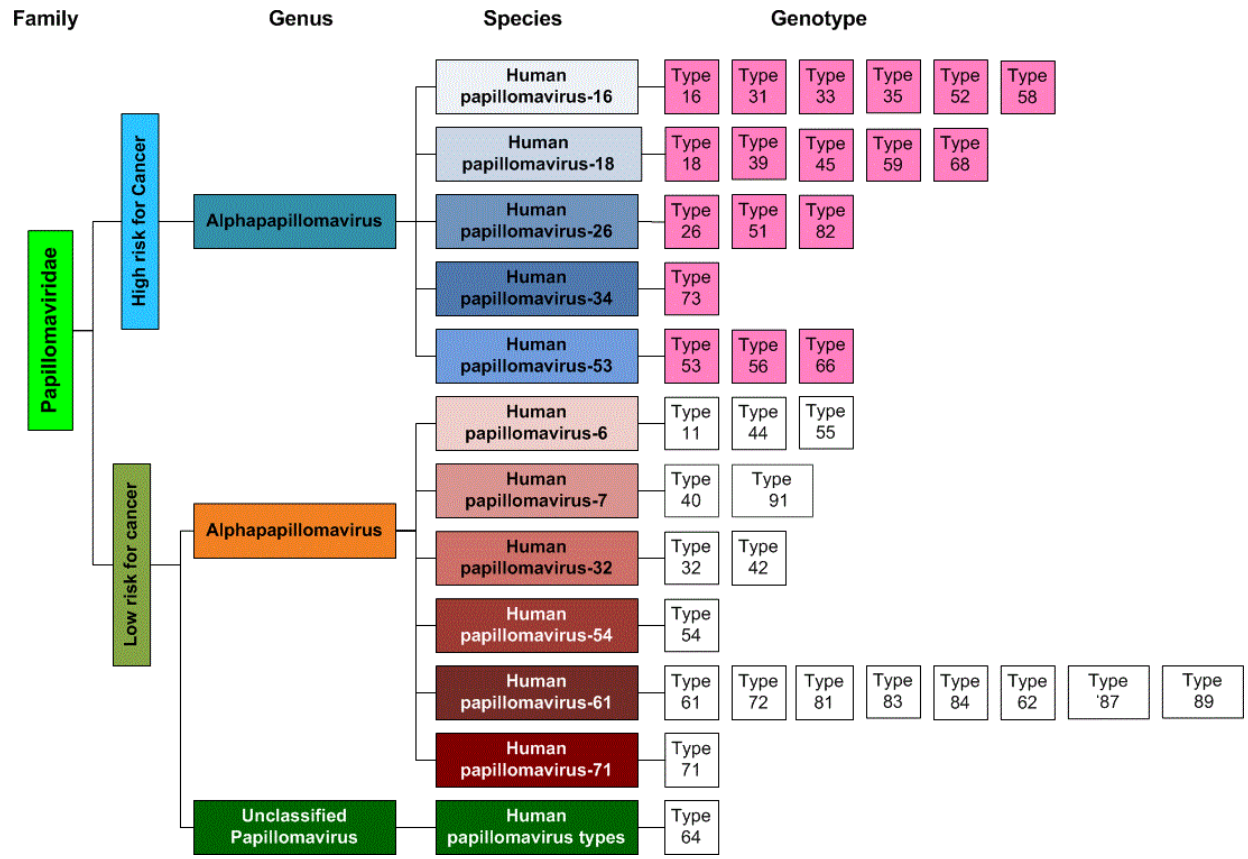


Fig 3 : HPV classification based on the L1 gene nucleotide sequence

HPV Life cycle:

The virus is believed to infect the epithelial stem cells in ectocervix's basal layer to begin a persistent lesion.[61,62] Disrupted host epithelial barriers lead to the virus access and infection of the basal epithelial layers and the subsequent development of productive HPV lesion. HPV is thought to reach the basal epithelium through naturally thin basal epithelial layers located in the transformation zone or through micro-abrasion in epithelium created during sexual intercourse.[63]

HPV16 binds to heparan sulfate proteoglycans (HSPGs) on the Epithelial cells (EPCs) or the basement membrane or through laminin-332 on the extra cellular matrix (ECM). The conformational change in the virion structure results in the fusion of the HPV16 capsid with the annexin A2 heterotetramer (A2t), a specific L2 receptor.

This event generates a signal for the virus to enter host cells through clathrin, caveolin, lipid raft, flotilin, cholesterol, and dynaminin-dependent endocytosis. [64-66]. Once the virus enters the basal epithelial cells, uncoating happens because of the breaking of intra capsomeric disulfide bonds due to reduced environment of the cell.

As a result, the DNA of the Virus is released and transported to the nucleus[67]. Infection of the dividing cell results in the establishment of a nuclear episomal form with a low copy number (100 virions) for varying periods of time.[68]The capability of HPV to establish a persistent lesion and the viral protein's expression determines cancer progression at a particular epithelial site. It is likely thought that the viral DNA is kept in episomal form by expression of the early viral proteins E1 and E2. Infected cells develop faster and expand laterally when the viral oncogenes E5, E6, and E7 are expressed simultaneously. This stops daughter cells from exiting the cell cycle.[69,70]

HPV gene expression is closely connected to the maturation of host epithelial cells: in the basal layers, the viral genome is present in low copy episomal form, virus transcription and translation occur in the middle epithelial layers and end events in the virus life cycle like assembly and release occurs in the well differentiated epithelial cells present near the surface.[71,72] Unlike other oncogenic viruses, HPV takes approximately 20 years between HPV infection & the malignant transformation that marks in the progression of CC.[73]

Integration of virus into the host genome is a critical event in the malignant transformation and the persistent expression of HPV oncogenes E6/E7 in the basal and parabasal cells[74.] These oncogenes interact with the tumor suppressor genes p53 and Rb, whose function is crucial for controlling the advancement of the cell cycle in response to DNA damage.[75,76] The entire events make the cells to lose normal checks on cell cycle regulator which leads to mutations; accumulation of mutations promotes carcinogenesis[77]

CERVICAL CANCER

Cervical cancer History

A physician by name Domenico Rigoni Stern from Italy in 1842, published a series of statistics on the mortality of women who deceased from cancer in Verona.[78] Conferring to his demographic research, married women and widows are more probable to have uterine cancer than single unmarried women (e.g., nuns and virgins). This was the first case study to demonstrate a link between uterine cancer and sexual activity. Later, in 1908, Walther Schauenstein, an Austrian gynecologist, developed the theory that precancerous lesions confined to the epithelium may predict the growth of cervical cancer.[79]

This awareness was required for initiatives to prevent cervical cancer that depends on the detection and exclusion of precancerous lesions, i.e. the protecting against invasive cancer. George Papanicolaou in 1928 renowned cytological screening and this technique became a method for identifying precancerous conditions.[80] However, Herbert Taut provided Papanicolaou with vaginal smears in the same year, that was published in 1941.[81] which was the commencement of the 'Pap smear.' Direct cervical sampling was rediscovered in 1949 by J Ernest Ayre (Canadian gynecologist / cytologist) .[82]

Amongst the numerous cell varieties observed in cervical smears, some of them had enormous nuclei and vast, transparent perinuclear spaces. Ayre who thoroughly studied these cells called them as "halo cells" and proposed that they served as cervical cancer's precursors. In 1960, he recommended that "halo cells" may match up to viral infection in precancerous conditions.[83] koilocytes was resultant from the word "koilocytotic atypia" proposed by Koss and Durfee in 1956.[84] Warty atypia is the word used to describe the histological homologue of koilocytes as it has s similarities with condylomas or warts

In 1976 Meisels and Fortin of Canada suggested that Koilocytes could be related to condylomas, indicating that cancer cervix has a viral aetiology.[138] The first evidence that koilocyte nuclei contain viral components was provided by Laverty et al., in 1978, from Australia.[8]

Cervical Cancer Classification

Classification of The Papanicolaou consist of 5 classes, i.e., I to V. The Reagen system, which the WHO subsequently adopted divided abnormalities into mild,moderate & severe dysplasia, and carcinoma-in-situ (CIS).[86] Later, in the classification system by Richart

(1980), the same terminology is used as histological changes with different degrees of cervical intraepithelial neoplasia graded from CIN 1 to 3.[87]

Recently, according to Bethesda (2001) system, lesions have been classified as low- or high-grade squamous intraepithelial lesions (LGSIL or HGSIL) .[88]

Though, this classification also includes one group of lesions characterized as "atypical squamous cells of undetermined significance" (ASCUS)

Table 1: classifying systems for cervical cytology

Cytological classification used for screening		**Histological classification used for screening**	
Pap	Bethesda	CIN	WHO descriptive classifications
Class I	Normal	Normal	Normal
Class II	ASCUS	atypiaa	Atypia
Class III	LSIL	CIN1 including flat condyloma	Koilocytosis
Class III	HSIL	CIN2	Moderate dysplasia
Class III	HSIL	CIN3	Severe dysplasia
Class IV	HSIL	CIN3	Carcinoma in situ
Class V	Invasive carcinoma	Invasive carcinoma	Invasive carcinoma

Pathophysiology of cervical cancer

Cervix is the anatomical connection between the the uterus's lowest and the upper vaginal half. Cancer cervix is Clinically defined as abnormal cellular changes that originate from cervix's surface.[89]Abnormal cellular alterations in the cervix are frequently denoted to as dysplastic or carcinoma in situ (Figure 4).

In both phases, the cells change in their appearance, size, shape or rate of proliferation. However, dysplasia differs pathologically from carcinoma in situ in that dysplastic cells may regress to normal cells or progress to cancer. In in situ cancer, the biological and genetic characteristics of cells change irreversibly and abnormal cells have the ability to metastasize to other anatomical areas.[89] Based on their origin in the cervix, cervical cancer is divided into several categories. Maximum cervical cancer cases are Squamous Cell Carcinoma that arose from ectocervix or on the side facing the vaginal canal. Adenocarcinomas are cervical cancers that develop in the endocervix or part of the cervix facing the uterus, and those that affect both the ectocervix and endocervix are usually identified as adenosquamous carcinomas or mixed carcinomas.[89]

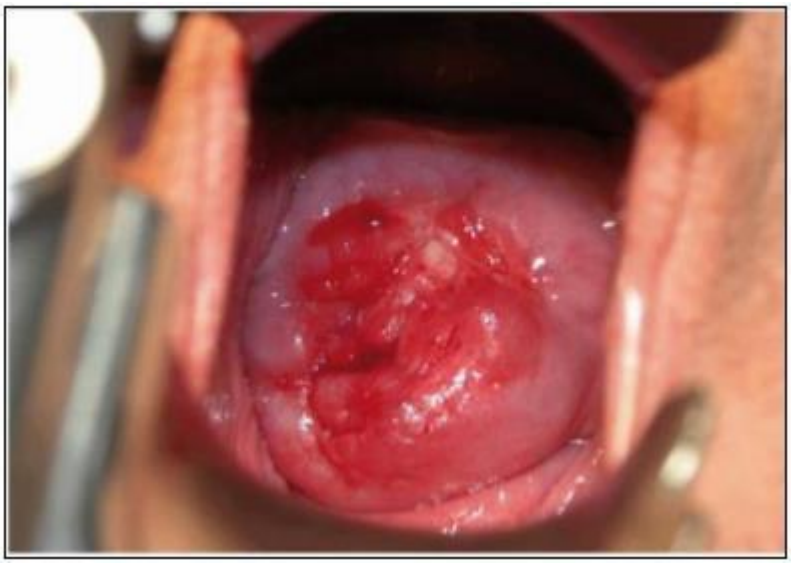


Fig. 4 : Cervical cancer's appearance upon inspection[90]

The most frequent histological type of cancer is invasive squamous cell carcinoma, preceded by Cervical Intraepithelial Neoplasia (CIN), a preinvasive stage of the disease related to HPV integration and infection processes. CIN begins to progress from CIN1 to CIN3 covering the deeper epithelial layers up to the entire thickness of epithelium [91](Fig.5).

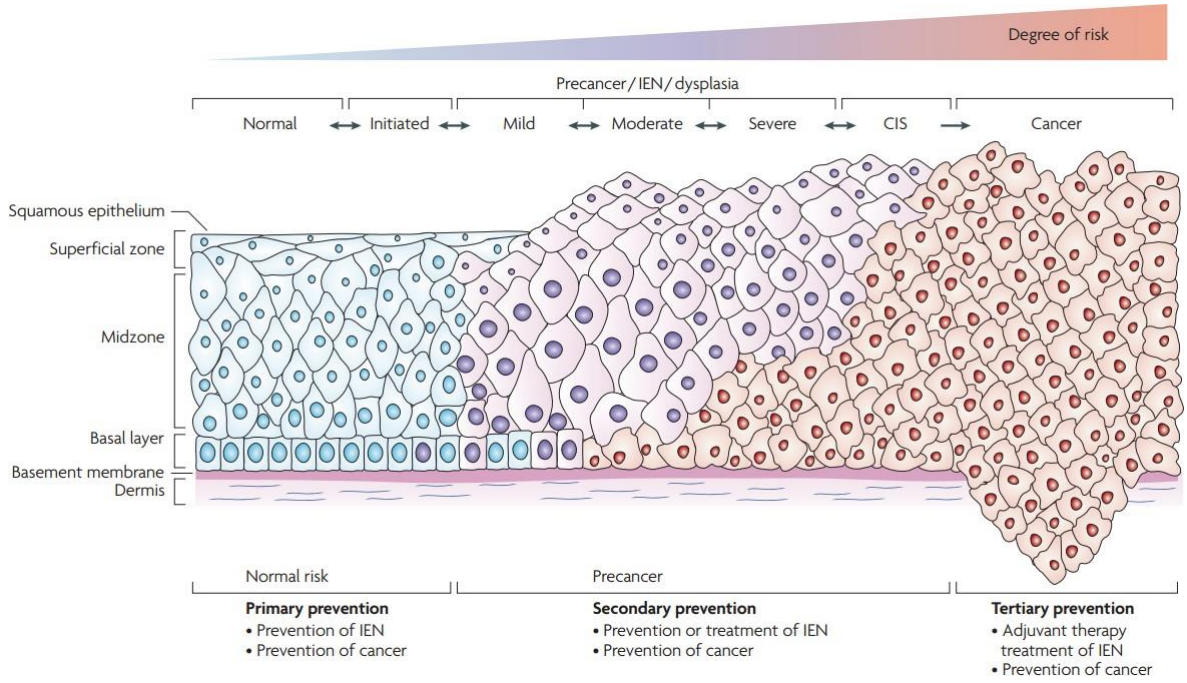


Fig 5: Transformation of normal cervical cancer cells into cancerous cells (left to right)[91]

Histology of cervix uteri

Cervix uteri

Cervix consists of fibrous elastic connective tissue and consists a relatively little smooth muscle. The elastic component of the cervical stroma is necessary for the stretchability of the cervix during the childbirth. The cervical canal is lined up by a deeply folded mucosa with a superficial epithelium comprises of columnar mucosal cells. Between the mucosa, there are branching tubular glands that have identical secretory epithelium lining them.[92]

The glands extend obliquely up and out of the canal. They secrete clear and alkaline mucus which is relatively viscous in nature except in the middle of the menstrual cycle, once it becomes more abundant and less viscous to promote sperm passage. A gland opening may become blocked at the vaginal end of the cervix, which then fills with mucus to form Naboth's follicle (or cyst).

No mucosa sheds during the menstruation, so unlike the body of the uterus it is not separated into a functional and basal layer and lacks spiral arteries. the ecto cervix's surface is covered with non-keratinizing stratified squamous epithelium, which contain glycogen.[93]

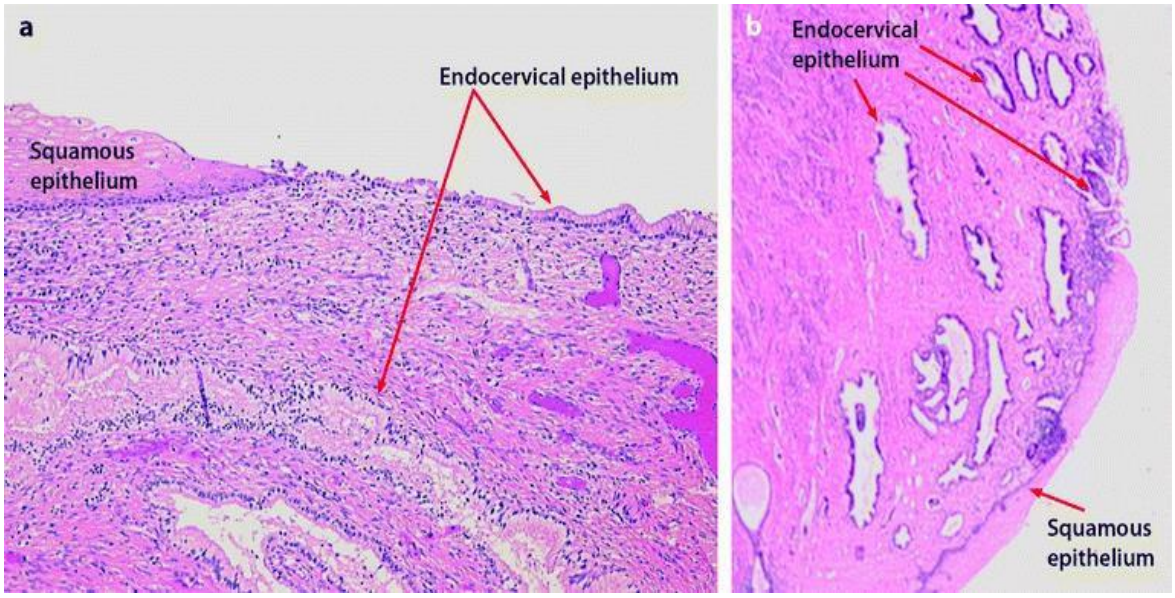


Fig 6 : Transformation zone of the uterine cervix[93]

There exists a squamous-colonary junction at the external os, which is where the columnar secretory epithelium of the endocervical canal meets the stratified squamous epithelium.[94]During puberty, the cervical os opens due to increased estrogen levels, leading to exposure of the endocervical columnar epithelium onto ectocervix. On the ectocervix's surface is an area of columnar cells called ectropion (red/raw appearance),

which is later on exposed to the vagina's acidic nature and eventually transforms into stratified squamous epithelium by a method of squamous metaplasia.

This area is therefore recognized as the transform transformation zone' (Figure 1.4). Additional hyper estrogenic conditions, such as pregnancy and oral contraceptive usage, can also lead to ectropion. This area is the common area of CIN that can progress to malignancy.[93]

Pathology of cervical cancer

Squamous Cell Carcinoma (SCC) and adenocarcinoma (ACC) are two main cancer types of cervix. SCC is the most predominant cancer and accounts for about three-quarters of all cases. With age, it emerges from a transformation zone at the squamous columnar junction cells, which migrates from the exocervix to the distal endocervical canal.[95]The second type of cancer cervix is ACC, which develop from mucus-producing cellsof end cervix (18% of all cervical cancers)

Other cervical cancers are adenosquamous - 4% and other cancers - 5% or malignancies 1.5%.[95]

Common pathological types

Squamous Cell Carcinoma:

SCC is the frequent variant of ICC. Histologically, SCC variants include keratinizing large cell, nonkeratinizing large cell, and small cell types. Large cell keratinizing tumors consist of tumor cells forming irregular infiltrating nests with laminated keratin pearls in the center[96]

Adenocarcinoma:

In recent years, the cervical adenocarcinomas cases reported in females in their 20s and 30s has been increasing. Although the overall number of adenocarcinoma cases was relatively stable. This cancer is more frequent in younger females especially as cases of invasive SCC decreases[96]

Adeno-squamous carcinoma:

Mixture of malignant glandular and squamous components are known as Adenosquamous carcinomas. Patients diagnosed with ACC are reported with worse prognosis, unlike those with pure adenocarcinoma or squamous carcinoma. [96]

Sarcoma:

The most frequent sarcoma of cervix is embryonal rhabdomyosarcoma which occur in both children and young adults. [96]

Malignant melanoma:

Melanosis can occasionally be found in the cervix. Consequently, malignant melanoma might develop here. The extent of invasion into the cervical stroma determines the prognosis and histopathologically, it mimics melanoma elsewhere.[158]

The limitations of cervical cytology:

Cytology was introduced in 1950s as the primary screening method as a part of annual preventive examination, although it has never been evaluated for efficacy in randomized trials.[97,98]

Cytology screening has become a well-established part of standard preventive care in most industrial facilities.[99,100] In fact, more than half of invasive cervical cancers in the United States continue to occur in females who have never or not often been screened.[101]

Even though a single Pap smear has a clinical sensitivity of only 60–70%, cytological screening is successful when repeated Pap smear tests are performed often, which causes significant health system costs. Numerous initiatives have been made at improving the accuracy and cost-effectiveness of cytology-based screening protocols.[102]

The introduction to liquid-based cytology reduced the proportion of inadequate preparations of slides and enabled reflex testing of other molecular markers. Because cytological techniques are subjective and dependent on the pathologist skills, the results may be false negative. 10-15% of the women mildly abnormal (low-grade squamous intraepithelial lesions, LSIL) and with equivalent (atypical squamous cells of indeterminate importance, ASCUS) have baseline cervical intraepithelial neoplasia (CIN3). [103]

Acquisition and Transmission of HPV infection

Being well-known that HPV infection is a sexually transmitted infection that is most prevalent worldwide. Thus, both men and women are engaged in the epidemiological series of infection. HPV infection alone is almost always asymptomatic, so infected individuals act as asymptomatic carriers and are transmitted to their sexual partner.[104] A longitudinal study from China in the rural population examined 874 couples, stating that 10 to 16% of sexually active couples had the same HPV types and the HR-HPV concordance was higher (16%) than for non-oncogenic types (10%). The study reported

that the male-to-female transmission rate was higher (12.13 / 1,000 person-months) than the female-to-male transmission rate (2.37 / 1,000 person-months) for HPV types.[105]

Another meta-analysis reported higher HPV (25.5%) between partners than the Chinese study. The study also showed that the male sexual partners as a reservoir for HPV infection, playing an significant part in the HPV transmission cycle. [106]

Although several studies claim HPV vertical transmission from mother/ father to offspring. The virus is thought to be transmitted from the mother to fetus during pregnancy or childbirth.[107,108]

The possibility of oocyte infection or sperm infection at the time of fertilization has also been acknowledged.[109]It is hypothesized that the fetus may obtain HPV infection from HPV-infected mothers; most likely through micro cracks in the placenta. [110] This was supported by the discovery of HPV-DNA in placental samples from the normal pregnant women.[109,111] Regardless of the route of transmission, HPV-DNA tests are negative within 6 months- 2 years.[112]

HPV with infection in sexually naïve adults occurs mainly during horizontal spread of HPV through various routes, including autoinoculation, hetero inoculation, or by means of fomits.[109,110,113,114] Possible horizontal transmission modes include close contact, fomites, kissing, and digital contact. Adults with cutaneous or genital warts can transmit HPV to children by direct contact when cleaning the anogenital area. [109,115]

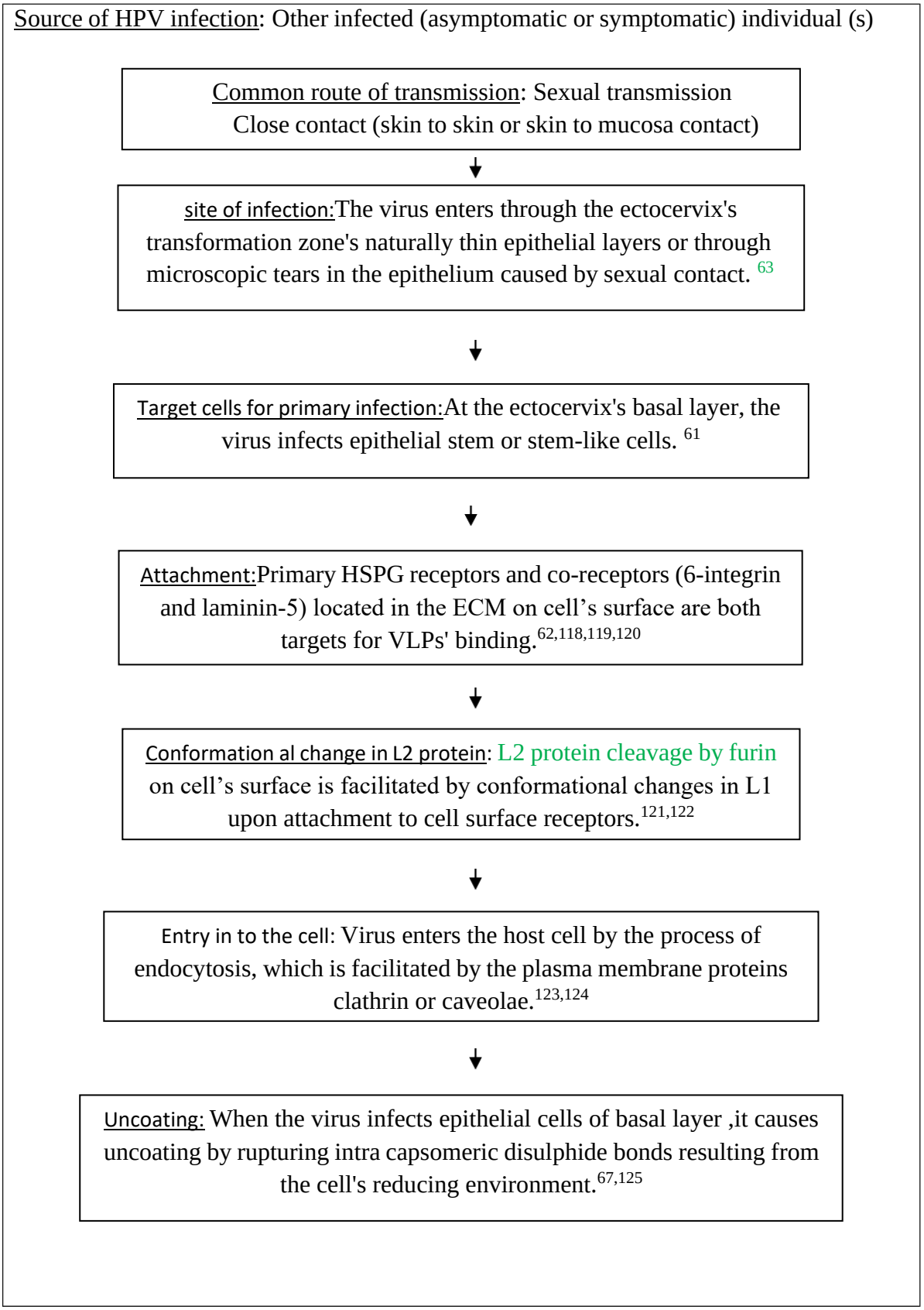
Improperly cleaned medical instruments, such as ultrasound probes and contaminated fomites, can also transmit HPV infection from an infected individual to other uninfected individuals. [116]

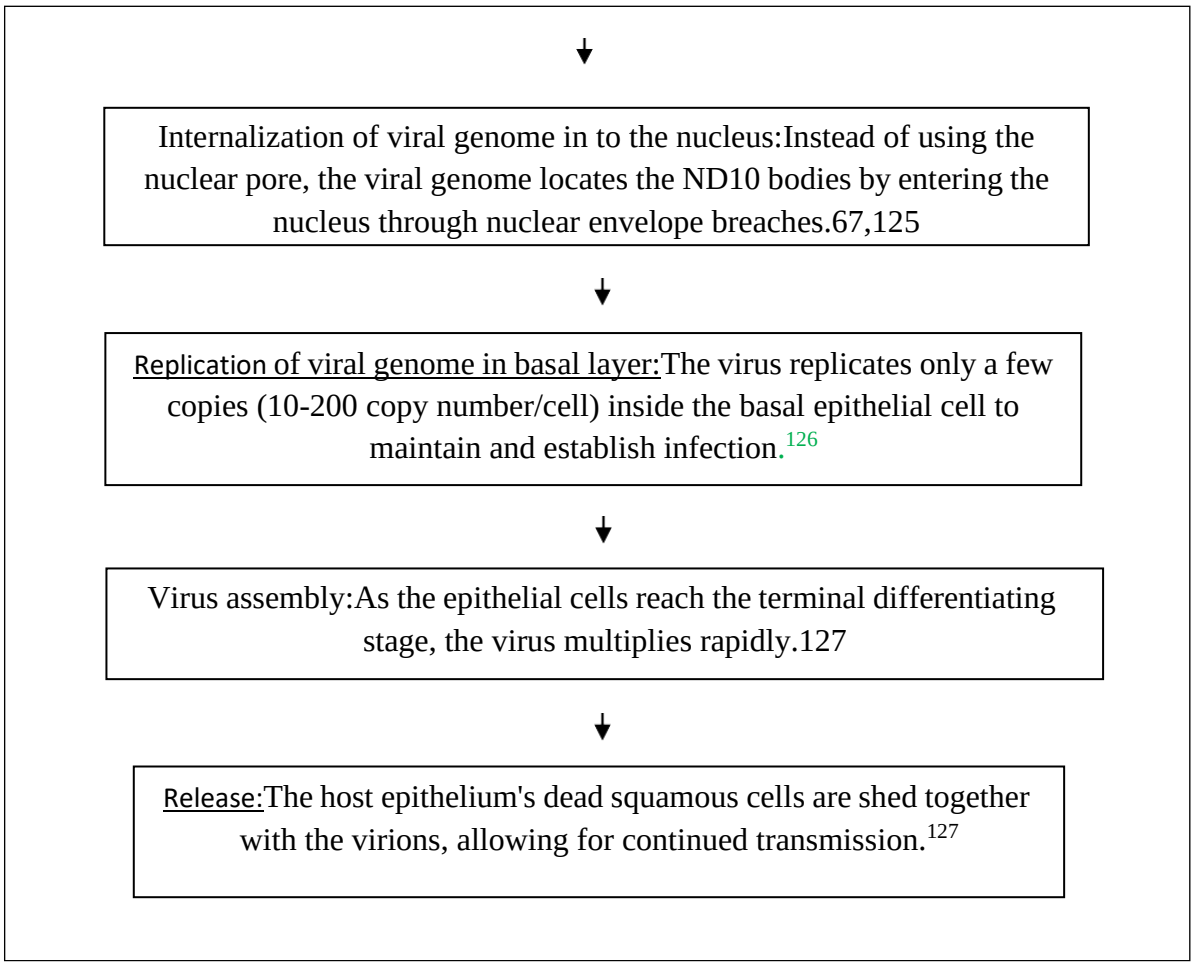
Molecular mechanisms of development of cervical cancer:

It is already well acknowledged that some oncogenic PVs produce chronic infection in infected epithelial cells and infuse the host cell's genome with theirs. Once they break the stratified squamous epithelial layers and reach the basal layer of the cervical epithelium, the virus enters the cell by endocytosis. In established infection, basal epithelial cells contain a low number of viral genomes in episomal form and transcribe low levels of viral RNA to keep the viral genome inside the cell. [72]

The development of CC begins with the acquirement of HPV infection; >50 % of infections disappear within a year and 90 percent of infections disappear by the second year's end. In a minority of infections, ASC-US or LSIL is marked at this point. Numerous risk factors play a major role in clearance rate. The chances of developing HSIL gradually increase with persistent infection. This procedure goes on for years. and the lesions grow laterally around the perimeter of the transformation zone, which remains a key time to screen for cancer and secondary prevention. It can grow into invasive cancer if not identified and treated at this stage. [117]

Fig 7: Flowchart depicting the progression from a simple, asymptomatic HPV infection to invasive cervical cancer:





Integration of HPV genome

Unlike retroviruses, HPV does not encode any special viral proteins for integration into the genome. Damage to host DNA (double-stranded DNA breaks) induced by oxidative stress[128] or by a mechanism induced by HPV oncogenes facilitates the integration of viral DNA at different sites on chromosomes (3q28, 17q21, 13q22.1, 8q24.21 and 4q13.3).

Under normal circumstances, either damaged DNA will be repaired by DNA damage response (DDR) and cell division will continue, or if not repaired, the cell will undergo apoptosis.It is a crucial step in which viral oncoproteins deceive the cell so as to bypass few important checkpoints of the cell cycle.[129,130]

The virus uses a DDR host device to support virus amplification. In order for the viral genome to integrate, it must come in close proximity with host genome, which is anchored by E2-BRD4-mediated tethering.

This fusion process occurs either by homologous or non-homologous recombination which is regulated by ATM / ATR and DNA-PK.[131] pathways. Early protein of HPV16 i.e E6 forms a complex with cellular ubiquitin protein in ligase which then degrades p53 [132,133] by linking covalently to ubiquitin. This is evidenced by two to three-fold lower p53 levels in cells of Cervical cancer compared to uninfected cells.[134]

Under normal circumstances, p53 expression stops G1 phase cells, which is critical for repairing damaged DNA or apoptosis if the damage is irreparable. The E6 protein of HPV can reduce p53 expression, thereby bypassing the host's normal defense mechanism. [135] Another vital role of the E6 protein is to activate telomerase in infected cells either by upregulating hTERT or by activating the alternative lengthening of telomeres recombination pathway (ALT). [136]

Telomere length maintenance is crucial for host cell immortalization, malignant transformation, and replication of virus. [137]

The E7 oncoprotein encoded by HR-HPV is a small molecule of approximately 100 amino acids; has functional and sequence similarities to AdE1A and SV40 Tag proteins. [138]

The E7 binds to and deregulates members of the regulatory genes and proteins of the tumor suppressor family, such as pRB, p107 and p130.[139,140]

The transcription factor E2F is important for the cell's progression from G0 to S-phase, HPV-16 E7, binds to the hypophosphorylated form of pRB through the G0 / G1 stage of the cell cycle & releases E2F, which endorses early cell entry into S-phase. [141,142]

Virus-specific genome integration disrupts not only cellular regulatory mechanisms, but also some viral genes (E1, E2, E5 and L2). The viral early protein E2 is a repressor for the viral oncogenes E6 /E7; E2 disruption results in increased regulation of E6/E7 proteins, leading to deregulation of cell cycle, resulting in the expansion of mutations series over time contributing to malignant cell transformation (Table 2.1, Figures 2.5 and 2.6) .[143]

Some host factors also aid in the development of cancer, in addition to viral causes. Viral infection stimulates the host's immune response, causing inflammation at the place of infection, resulting in oxidative damage (cellular ROS and NOS), cell proliferation, invasion, and metastasis. [144,145]

Innate immunity players like dendritic cells, macrophages, and natural killer cells identify double standard DNA (HPV genome) by TLR 9 and reduce the episomal HPV genome, leading to overexpression of E6/E7 viral oncoproteins. [146]

Overexpression of early viral E6 / E7 proteins reduces TLR9, IFN response, and expression OF MHC I, which promotes persistent HR -HPV infection. It is well known that downregulation of MHC I expression fails to activate CD8-cytotoxic T cells, that results in a slowed T cell response and continuous immune escape. [147,148]

As seen in other cancers, miRNAs play a major role in the progress of CC. They are related to numerous cellular regulatory pathways and have power to control cell proliferation & apoptosis. Upregulation of certain mi-RNAs (miR-886-5p, miR-10a,

miR-141, miR-21, miR-135b, miR-148a, miR-214 and miR-106b) and downregulation of other mi-RNAs (let 7c, miR-124, miR-126, miR-143 and miR-145) deregulates the normal cell cycle defense mechanisms, thus contributing to development of CC. [149] For instance, E6 degrades p53, resulting in downregulation of miR-23b, miR-34a and miR-218, while upregulating the expression of miR-15a/ miR-16-1 microRNAs, thus promoting the survival and invasion of host cells and inhibiting cell proliferation. [150,151]

Clinical features and staging of cervical cancers

Precancerous lesions, i.e., CIN, are asymptomatic and are usually detected by screening or pelvic examination. Invasive disease could also be asymptomatic. The utmost typical signs is abnormal vaginal or postcoital bleeding with a rise in vaginal discharge.[152]

Advanced disease stages' signs and symptoms include pelvic pain (resultant of a tumor extending into the pelvic wall), hematuria resultant of pressure on the bladder or constipation.

Cervical cytology is the common diagnostic screening test for cervical cancer. If the cytology shows abnormal results or any other indications, in that case more invasive examinations, like colposcopy, endocervical curettage, and biopsy, should be performed to confirm the diagnosis.[153]

Cervical Cancer Staging

The FIGO[154] staging system is the present standard and is appropriate to all histological types of cervical cancer.

Table 2: FIGO cancer staging system

Stage	Clinical correlation
Stage I	Carcinoma is strictly restricted to the cervix; penetration of uterine corpus should not be taken into account
IA	invasive malignancy that can only be detected under a microscope. The invasion is confined to to the calculated stromal invasion that is no wider than 7 metres and no deeper than 5 millimetres.
IA1	Stromal invasion that is measured to be no deeper than 3 mm and no wider than 7 mm in diameter.
IA2	Measured invasion of stroma > 3 mm but no > 5 mm depth & no wider than 7 mm in diameter
IB	Stage IA lesions are preclinical or clinical lesions that only affect the cervix. Stage IB malignancies are all gross lesions, even those with superficial invasion.
IB1	Clinical lesions not > 4 cm
IB2	Clinical lesions > 4 cm
Stage II	Cancer that has transmits through the cervix but not into the pelvic wall. The vagina is affected by the cancer, although not to the lower third.
IIA	Absence of a clear parametrial involvement. complicity of the vagina that extends up to the upper two thirds.
IIA1	clinically apparent lesions 4.0 cm
IIA2	clinically apparent lesions >4.0 cm
IIB	evident involvement of parametrium, but not into the sidewall of the

	pelvis.
Stage III	cancer distributed to the bottom of the vagina and/or the pelvic sidewall. A kidney that is not functioning, or cass hydronephrosis
IIIA	Although the lower portion of the vagina is affected, the pelvic sidewall is not expanded.
IIIB	Hydronephrosis, extension into the pelvic walls, or a non-functional kidney.
Stage IV	Cancer that has extended past the true pelvis or that has clinically affected the bladder and/or rectum mucosa
IVA	Invasion of nearby pelvic organs by the tumour
IVB	dissemination to distant organs

.

Associated Human papillomavirus cancers

Even though cancer cervix is the common type of HPV-related cancer, there is an increasing data that HPV is an significant etiological agent in several less prevalent but equally important types of cancer. Studies suggest that 90% of anal, 50% of vulvar, 65% of vaginal and 35% of penile cancer cases are related to oncogenic type HPV infections.[155,156]

Additionally, data recommend that around 60% of people, who have develop oropharyngeal cancer are possessing at least 1oncogenic subtype of HPV.

Interestingly a there is a geographic association between the incidence of rectal, vulva, vaginal, penile, and Head and neck cancers.[157,158]

Respiratory Papillomatosis

An uncommon condition known as recurring respiratory papillomatosis is mostly brought on by low-risk HPV strains, particularly types 6 or 11. Despite being ubiquitous worldwide, the illness is more common in some nations and areas than others.[159]

Recurrent respiratory papillomatosis with juvenile onset has been documented in both young and adult forms, and both boys and girls appear to be affected almost equally. Recurring respiratory papillomatosis with juvenile onset is thought to be the instigated by HPV being passed from mother to baby after delivery. In contrast, this preferentially affects males more than women, with a ratio of around 3:2, compared to adult-onset recurrent respiratory papillomatosis.[160]

Human papillomavirus infection in immunocompromised patients

Research indicates that the likelihood of HPV infection and HPV-related disease is high in the people who are immunosuppressed or HIV positive. Occurrence and persistence of HPV infection, both connected to elevated HIV RNA levels and CD4 counts <200/mm3.[161]

HIV-positive females with low CD4 cell counts and oncogenic HPV were considered to have a higher chance of developing a squamous intraepithelial lesion compared to females negative for HIV or with high CD4 cell counts.[162] There is a dearth of information on how highly active antiretroviral medication affects HPV infection and related diseases. Therefore, further research is needed in this area in the future.[163]

Oral infection

Benign oral lesions are related to a number of HPV types, including subtypes 1, 2, 4, 6, 7, 11, and 13. Benign verrucal-papillary lesions related to oral HPV are clinically divided into verruca vulgaris, condyloma acuminatum, multiple and single papillomas, and focal epithelial hyperplasia. HPV 2 and 4 are related to verruca vulgaris whereas HPV 6 /11 related to condyloma acuminatum and oral squamous papilloma's. Studies have identified HPV capsid in 10% of oral & 22% of hyperkeratotic papillomas.[160]

Lesions in the oral cavity brought on by HPV are very common in people with genital condyloma. Up to 50% of individuals having widespread genital condyloma also have oral condyloma acuminatum.[160]

Risk factors related to Human papillomavirus infection

An estimated 75% of sexually active individuals have contracted at least one HPV genotypes at some point in their lives (often in their early 20s). Most HPV infections and minor lesions that are linked with them nearly generally go away on their own, usually within a year or two. However, certain HPV (high risk) infections last for a very long time, causing aberrant cytology and the emergence of precancerous lesions. They might develop into invasive CC if they are not identified and treated at this point. Higher CC incidence rates in middle-aged women, which affects the productive ages of a woman's life. [164-168] Though not all women who were infected with high-risk HPV genotypes develop precancerous lesions, some cofactors, such as having smoking, multiple sexual partners, receiving long-term corticosteroid therapy, having poor personal hygiene, and having poor nutritional status, increases risk of CC.[169] The same risk factors are seen in male patients who have genital samples positive for HPV DNA.[170]

Marriage/sexual debut at an early age

It's well known. known worldwide that ageing has been proven to consistent and strong risk-factor for HPV infections. Almost all men and women acquire HPV infection at some point throughout the productive period, most often under 25 years of age.[171,172]

Some studies stated that premature sexual debut poses as risk for cervical HPV infections. [173,174] Although the underlying mechanism is unclear, squamous epithelial cell replication and differentiation in the cervical transformation zone followed by menarche facilitates HPV replication.[175,176]In addition, basal epithelial cells in transformation zone increase over time from menarche, the opportunity for the virus to become infected.[176] Although the age of sexual debut may not directly affect the development of CC, it can only be an indicator of other risky behavior.[177]

Circumcision

Several studies have reported to determine the result of circumcision, and Men who have circumcised were reported to have lower rates of penile and prostate cancer. The CDC (Center for Disease Control and Prevention) claims that the foreskin is prone to rupture during sexual intercourse, which could allow viruses to easily enter the body. A study on HPV in men reported that the time to resolution of any HPV infection was suggestively longer in circumcised men compared to uncircumcised men.[178] Though, some study specifies that circumcision was not related with an overall reduction in genital HPV detection in men.[179]

Parity:

Several studies report a relationship between the frequency of live births and the premalignant lesions development .[170] women who have > 3 pregnancy were 1.9 times more likely at risk than women with lower parity. [180,181]

Another meta-analysis on 10 case control studies performed by the IARC showed a significant difference (OR 1.81) in females who had a minimum of one or two pregnancies as compared to women who never gave birth. [182]

Other studies did not reported any association between parity numbers and the incidence of CC. [183,184] Although the above studies reported significant differences, no linear relationship was observed. There were two possible biological mechanisms to explain the connotation of parity with the HPV infection

First, high levels of progesterone & estrogen in pregnancy appears to modify the squamous-columnar junction and transformation zone on the exocervix, resulting in persistent HPV infection, a known contributing factor for malignant transformation of infected cells.[185-187] Second, physiological Immunosuppression during pregnancy may pose a risk of persistent HPV infection or delay in clearance, thereby promoting cell transformation. [188]

Smoking

Numerous research has revealed a connection between cigarette smoking and the prevalence, incidence and persistence of HPV. Smoker women are twice as likely to acquire cervical cancer in comparision with non-smokers. Smoking, however, was only recently recognized as a risk factor for detection of HPV in males and is now believed to

be linked to both anal and penile cancer in addition to the persistence of the virus. Smoking has immunosuppressive effects on the cervix by reducing the Langerhans cells, and it may also work in conjunction with other factors to increase the risk of HPV infection.[189] Researchers claim that the chemicals and metabolites in smoke harm the DNA of cervical cells, increasing cell proliferation, and perhaps promoting the growth of cervical cancer. Smoking is known to reduce the immune system's ability to fight against oropharyngeal and genital HPV infections, those that cause warts.[179]

sexual partners and risk of acquisition of new HPV infections

HPV in the genitalia is one among of the utmost prevalent STDs worldwide. Risk of HPV exposure and incidence rate increases with numerous of sexual partners, as is widely known. [190-193].Numerous serological investigations that showed a substantial correlation between the occurrence of serum HPV antibodies and having numerous sexual partners provided evidence in favor of this. [194-197] According to research done on teenagers and young women, the risk of contracting HPV rises by over ten times with each new sex partner.[198]Only a few studies found that healthy women from the community cohort had the lowest HPV prevalence, followed by women visiting STD clinics and female prostitutes of all age categories. One widely accepted theory is that divorced and separated women tend to return to dating and new sexual partners, increasing their risk of HPV infection.[199] Overall studies reported more than one sexual partners is a risk for chronic HPV infections and high prevalence rates. [199-201]the prevalence of HPV was found to be significantly higher among the divorcee, polygamous women.[202].

Sexual behaviors of male partners

Many epidemiologic studies have reported that, since HPV genital infection is a sexually transmitted illness, male sexual partners are crucial. [173,174,203-207] This was confirmed by

the discovery of HPV DNA in swabs taken from penile and scrotal regions, such as the glans penis, prepuce, distal urethra, and shaft of the penis, among other places. [208] Data from case control trials done by the IARC group further reinforced the idea that men have a part in HPV transmission; the study revealed a substantial correlation between rates of CC incidence and penile and cervical HPV. [209] Therefore, genital HPV incidence, prevalence, and infection are greatly influenced by both the sexual behavior of women and the sexual behavior of the males who have been their partners throughout their lives.[210]

Use of condoms, spermicides and vaginal lubricants

An increasing number of studies have steadily proved that using a condom decreases the incidence of genital warts and CIN by 60–70%. [211] Regular condom usage enhances the regression rate of CIN and male genital lesions in males with HPV infection along with lowering HPV incidence. [212,213]. Another cross-sectional study reported that females who never used condoms had a higher incidence of genital warts than females who used condoms frequently.[214] However, a US-based study found no noteworthy difference between these 2 groups of women.[215] This recommends that use of condom may not provide complete safety because the HPV can spread through other nonsexual routes (contact with body parts).[216] Overall, there is conflicting evidence regarding the use of condoms (CIN or ICC).

The importance of vaginal spermicides and lubricants in the prevention of HPV infection has not drew a lot of interest from in vitro research. By altering the natural architecture of the human genital epithelium, the spermicide nanoxynol-9 (N-9) has been mentioned to enhance vulnerability to HPV genital infections. [217,218,219] Even in the existence of N-9, a different ingredient known as carrageenan, which is included in various vaginal

lubricants, demonstrated anti-HPV activity and prevented HPV infections. These results presented that topical HPV microbicide Carrageenan might be utilised as an adjuvant therapeutic agent in addition to conventional treatments and vaccinations.[218,219]More women in the test arm (lubricant gels containing carrageenan) were protected from HPV genital infections in comparison to placebo arm, according to a recent placebo-controlled clinical (phase 2B) experiment on carrageenan-base d lubricant gels. This raises the likelihood that carrageenan could be used as an anti-HPV medication to treat skin-related HPV infections.[220]

Hormones and anti-estrogens:

The LCR of high-risk mucosal HPV has been found to contain hormone recognition elements. [221] Studies conducted in vitro showed that the E6 protein was overexpressed when there is exogenous hormone stimulation. [222,223] Enhanced 16-hydroxylation of estradiol activity, which recognised to boost cell proliferation and might be a risk factor for the development of malignancies, was seen in HPV 16 immortalized cervical and foreskin cells. [224] The presence of increased progesterone and estrogen receptors in stromal cells in the precancerous cervical lesions is indication that hormones have a role in the process of neoplastic disease. [225] Overall, the evidence indicates that using synthetic birth control pills and other medications containing progesterone or estrogen may increase the chance of developing CC. [226]

Consumption of quid, paan, gutka

Chewing betel nut is a long-standing tradition in several Asian nations, including India. Depending on the region, "quid" may contain areca nut, betel leaf, tobacco, slaked lime, and other components. It is a combination of substances that is inserted and maintained in the mouth and frequently swallowed. The terms "paan" and "betel quid" are

interchangeable on the Indian subcontinent and other nearby nations, but "gutka" comprises only the ingredients for paan masala and tobacco. [227-229]Alkaloids, proteins, sugars, lipids, polyphenols, crude fibre, and minerals can all be found in areca nuts. [230] The primary component of betel leaves, betel oil, includes phenolic chemicals including phenol, chevicol, hydroxychevicol, and eugenol as well as vitamin C, trace minerals, carotenes, and other antioxidants.[231]

Current research firmly supports their connection to cancer, but their relation with the growth of CC is still controversial. However few studies report its association with CC. Raj Kumar et al. reported that all women who took betel quid were positive for CC, all of whom were non-smokers (95% CI: 1.20-13.33) .[232] Another cross-sectional study showed that greater prevalence of cervical dysplasia in women who were in the habit of chewing the betel pound.[233]

Nutrition

Some food ingredients and nutrients shorten the duration of HPV infection and developing cancer. Carotenoids, vitamins C and E are involved in oxidative reactions and protect cells from oxidative damage. Folic acid,vitamin B6 & B12 and cysteine (serum homocysteine) involved in single carbon transfer reactions and protect cells from DNA methylation damage. Other food metabolites, like retinoic acid and its isomers, show hormone-like activity that plays a part in shielding cells from damage triggered by viruses. Some fruits and vegetables that contain carotenoids (antioxidants), vitamins C and E, can offer resistance to the persistence of HPV infections and inhibit the malignant transformation of HPV-infected cells by quenching reactive oxygen species. These supplements also strengthen the cellular & humoral immunity of the host.[234]

Another necessary nutrient in order to produce purines, S-adenosylmethionine, and thymidylate is folic acid. Thymidylate and purine nucleotides are necessary for the synthesis and repair of DNA. S-adenosylmethionine, is the main source of methyl groups in a variability of methylation reactions.[235] Low folate levels in tissues lead to an rise in fragile DNA sites, a decrease in DNA repair, and decreased DNA methylation, all of which are crucial steps in HPV DNA integration.[236]

Retinoic acid hinders cell proliferation and DNA replication, which is essential for terminal differentiation of cervical epithelial cells.[237] It has been revealed that retinoic acid increase healing of CIN2[238] and down lesions regulate HPV mRNA expression by regulating AP-1α TGFβ activity [239,240]

Genetic predisposition

According to the Swedish Cancer Registry, fewer than 30% of cervical tumours are thought to be caused by genes (heritability estimates). [241] For all CC cell types, a case control analysis found a family trend. Risk factors for Adenocarcinoma, adenosquamous carcinoma, and squamous cell carcinoma were 2.49, 9.93, and 3.11 times higher in females with family history of CC, respectively. [242] Other research reported the risk to be 2.20–2.87 times higher. [243,244]

Role of infections other than HPV

It is thought that co-infections with other STDs can have an impact on how development of CC either directly, as when HPV replication and transcription are altered, or indirectly, such when inflammation results in epithelial damage. One of the few viruses that is most commonly investigated as a possible co-factor for CC is HSV. Early in vitro tests conducted in the 1970s showed that HSV had cancer-causing potential. Galloway et al.

[245,246] offered the "hit and run" theory for HSV-2-induced malignancies, in which transformed tissues lacked HSV-2 DNA. [247] In comparison to controls, patients of SCC had a greater seroprevalence of HSV-2, according to a multicenter investigation by the IARC group[248]

Cervical neoplasia is positively associated with CMV, HHV-6, and HHV-7, three other members of the herpesvirus family. Chlamydia trachomatis is a sexually transmitted illness that can become chronic, and hypertrophic ectopy is believed to be linked to squamous metaplasia. [249,250] [251] The majority of C. trachomatis genital infections are asymptomatic and, if ignored can last for months or years. [252]

Cervical lesions or CC in a man's sex partner are significantly correlated with C. trachomatis antibodies in males, according to studies. [253,254] However, number of studies that revealed that these infectious agents had little to no influence on the expansion of cervical neoplasia. [255-259] Adeno-associated viruses (AAV), contrasted with aforementioned infectious viruses, have been found to lower the possibility of cervical neoplasia by inhibiting the HPV replication and cellular transformation in vitro.[260,261]

Diagnosis

Traditional cell cultures are useless for diagnosing HPV since HPV cannot be generated artificially, and serological testing is ineffective because it cannot differentiate between current and prior infections.[262] Therefore, the detection of viral DNA may be used to accurately diagnose HPV infection. [263,264]. Additionally, genotyping should be included in addition to the HPV DNA detection. [265,266] Important methods to identify HPV infection include colposcopy and acetic acid testing, biopsy, DNA testing, and Pap smear.[267]

Colposcopy and acetic acid test

The Colposcope, a low power microscope, is used during colposcopy, which is an outpatient treatment performed by professional doctors. This treatment is performed to inspect the vagina, cervix and vulva and to gather biopsy material following the administration of acetic acid. More anomalies of these measures correlate with more severe lesions.[268]

In acetic acid test, 3-5% acetic acid is administered for 5–10 minutes to suspected lesions to make them more visible by making them white. The acetic acid test is not advised for routine screening. It can be used to recognize lesions for surgical therapy, choose lesions for target biopsies, and spot HPV-related lesions in the genitalia that are still in the subclinical stage.[269]

Biopsy

Koilocytes, mature squamous cells with a distinct perinuclear zone, are the most distinctive feature of genital warts. Biopsy plays a key role in colposcopy because the treatment depends on the sternness of the biopsy sample, and treatment is only advised when the results show any signs of precancer or cancer.[268] Koilocytes' nuclei may be enlarged, and double nuclei and hyperchromatic chromatin are frequently found.[268]

DNA TECHNIQUES

Direct probe hybridization techniques, such as Southern blot & dot blot, were initially used for detectionof HPV. They were labor- and time-intensive, had a limited sensitivity, and need a lot of DN A from clinical samples. Following are the recognized standard procedures:

Polymerase chain reaction

The HPV sequence is exponentially amplified during this phase of selective target amplification, which results in the production of billion copies from a single HPV DNA molecule after 30 cycles.[268]Primers that target the viral capsid L1 gene (a consensus primer set includes PGMY09/11, GP5+/6+, and SPF10) and those that identify several HPV subtypes in a single amplification are utilized for the detection of HPV.[269-271]As the name suggests, type specific PCR amplifies a single genotype of HPV by focusing on a DNA sequence specific to that type. Several PCR cycles may be required for type specific PCR in order to recognize the precise sequence present in the sample. [271] The following discussion covers several target amplifications uses for HPV genotyping and detection.

Real time PCR

The very sensitive target amplification method real-time PCR is available to detect HPV DNA. The accurate quantification of viruses contained in a sample is made possible by the combination of fluorescent probes and PCR primers. Real-time PCR has the capability to estimate HPV viral load specifically because it uses the nuclear genome to control the sample's cellular composition. [269]

Multiplex HPV genotyping kit

The Multiplex genotyping kit (Multimetrix, Heidelberg, Germany), a PCR-based fluorescent bead assay that can identify 24 HPV strains with low and high risk, is another innovative genotyping test. The multiplex HPV genotyping kit bead mix is combined with the PCR products. This mixture of beads comprises 26 beads with 24 HPV probes, 1 -globin probe, and 1 control probe attached. The PCR products are hybridized, tagged with R-phycoerythrin marked streptavidin, and then read on a luminex analyzer.

Different HPV kinds may be distinguished by the individual beads. Although the multiplex HPV genotyping kit is now only available for research usage, it has demonstrated great sensitivity in applications in large-scale epidemiological investigations and has the possibility to be utilizsed in regular HPV diagnosis in the future.[234]

Hybrid capture HPV DNA test 2 (HC2)

Additional to the Pap test, the hybrid capture HPV DNA test 2 is now approved for use by Food and Drug Administration.[273]. The sensitivity and specificity of the FDA-approved HC2 are practically on par with PCR-based detection techniques, since it can detect as low as 1 pg of HPV DNA/mL. The best features of this test are its ease and excellent repeatability of results. Low-risk and high-risk HPV genotype groupings can be found, but the exact HPV type cannot be determined.[268]

Reverse line blot and linear assay

A very early, widely used prototype techniques was the reverse line blot test from Roche Molecular Systems (Alameda, CA). The line blot test makes use of PCR using L1 consensus primers and PGMY 09/11 primers. Multiple HPV kinds' probes are glued on a membrane strip, which is then hybridized with the PCR result before being visually detected. The line blot assay is a research use only test, and the original assay is now commercialized as the linear assay HPV genotyping test.[272] The results are read with the unaided eye, based on a visible band in specific areas of the hybridization strip. The assay detects 27 different HPV types, and the extended edition adds 11 low-risk types, which include 61, 62, 64, 67, 69 to 72, 81, 82, and.[274]

PCR and restriction fragment length polymorphism (PCR-RFLP)

After amplification, restriction enzymes perhaps utilised to examine a PCR product's sequence makeup. Restrictions endonucleases can break down PCR results into a variety of fragments that can then be separated by gel electrophoresis to produce a specific banding pattern. This approach is simple, but time-consuming.[269]

Direct sequence analysis of PCR products

There are 2 ways to infer the genotype using the HPV sequence. First, a homology search on the sequence might be utilized to query a sequence database. Numerous archives are openly available online and may be found at http://www.ncbi.nlm.nih.gov. Fast homology searches of a sequence inside a continually updated sequence database are possible with BLAST software [277].The second option is phylogenetic analyses. The new sequence may be combined with a known collection of HPV sequences that represent various HPV genotypes in a multisequence alignment. A genotype can be determined based on the sequence alignment and a phylogenetic tree that illustrates the evolutionary connections between the discovered sequence and reference sequences. It must be highlighted that while genotyping of clinical samples is carried out by examination of only a small but representative portion of the viral genome, the official categorization of genotypes is solely based on sequence analysis of the viral genome. [269]

Pap smear test

In the majority of developing nations, including India, the pap smear test is the most often utilised screening procedure. This test was first explained by Papanicolaou and Traut in

1928 .[270]Not only premalignant and malignant changes be detected, but also viral infections like HPV infections and herpes can also be identified. If the PAP result is positive, additional confirmatory tests such as colposcopy, cervical biopsies, and DNA tests such as PCR must be performed.[268]

Clinical utility of molecular Human papillomavirus diagnosis

A major shift in HPV infection diagnosis has occurred in recent years., which has followed the development of highly sensitive DNA detection tests. However, it requires careful laboratory validation of test results and interpretation should be performed with the utmost care.[269]

Well-characterized international quality control panels are needed to compare different diagnostic tools. In addition, an assessment of the implications of HPV DNA detection for patient treatment is required. Additionally, prior research specifies that having many HPV genotypes may indicate exposure to the virus repeatedly and be associated to a complex risk of the disease progressing.[278,279] Particular molecular instruments will be mandatory due to extensive HPV genetic heterogeneity and possible clinical significance of specific subtypes. New low- or high-density DNA probe assays (DNA chips) may provide useful technology for such research [231]

Prevention of Human papillomavirus infection

There are two approaches to disease prevention, ie primary and secondary, which are discussed below.

Primary Prevention

The disease can primarily be prevented by eliminating the risk before it is detected. In the case of cervical cancer, examples of primary preventive vaccination approaches are to prevent high-risk HPV infections or male circumcision to reduce the possibility of chronic HPV infection and transmission. Unlike primary prevention, secondary prevention methods do not seek to completely prevent the risk of disease, but to screen for early pathological changes before clinical signs appear.[280]

Secondary Prevention

Secondly, early detection of HPV, through screening and treatment of precancerous lesions, can prevent cervical cancer. Screening is considered the most affordable and sustainable approach to cervical cancer's prevention. In addition, primary approaches to the prevention of cervical cancer is not beneficial for women who are already infected with HPV and / or at risk of developing cervical cancer.[280]

Prevention of cervical cancer

Vaccines

In the United States, there are two licenced HPV vaccinations.: a quadrivalent vaccine (Gardasil, Merck and Co., Inc.) & a bivalent vaccine (Cervarix, GlaxoSmithKline). Both are recombinant vaccines because they consist of virus like particles (VLPs) prepared from recombinant L1 capsid protein of HPV target types.[281]

Cervarix is focussed against two types HPV 16 /18, while Gardasil is directed against four types of HPV, two oncogenic types HPV 16 /18 and two non-oncogenic types HPV 6

and 11. Both the vaccines are prophylactic, not therapeutic, because they are ineffective in the progression of HPV-related disease.134 Gardasil has been accepted by the FDA as an aid in women (2006) and men (2009) aged 9-26 years. While Cervarix was licensed by the FDA in 2009 for use in women aged 10-25.[282,283]

Clinical studies in> 18,000 women aged 15–25 years for Cervarix and> 20,000 women aged 16–26 years for Gardasil have shown high levels of efficacy of both vaccines in the inhibition of cervical precancerous lesions (CIN 1, 2 and 3) caused by target types. HPV in women inexperienced with vaccine-type infection at vaccination [284,285]

Gardasil has also been known to be highly effective against genital warts linked to HPV 6/11, vaginal related HPV 16/18 and vulvar precancerous lesions, and HPV 16/18-related anal precancers in men.[284]

Studies on immunogenicity and security were conducted on women of ages 9-15 years with quadrivalent vaccine [286]and women aged 10-14 years with bivalent vaccine [287] to bridge antibody titers in women in efficacy studies.

Following vaccination with both vaccines, more than 99% of study participants developed antibodies and the titers were identified to be higher in young girls than in older women who participated in efficacy trials.

20 g of HPV 6 L1, 40 g of HPV 11 L1, 40 g of HPV 16 L1, and 20 g of HPV 18 L1 protein is all present in each dosage of Gardasil (0.5 mL). The VLPs are adsorbed on 225 g of an adjuvant made of amorphous aluminium hydroxy phosphate (alum). While Cervarix includes 20 g of HPV 16 L1 protein and 20 g of HPV 18 L1 protein in each 0.5 mL dosage. The VLPs are adsorbed on 50 g of 3-O-desacyl-4' monophosphoryl lipid an adjuvant and 500 g of aluminium hydroxide. [288]

Barriers to cervical cancer control programs

Though the significance and efficiency of cervical cancer prevention in the course of screening has been demonstrated, the socio-demographic populations of females who are less probable to participate in the screening have been quantified, the underlying reasons to make clear why most women do not utilize available screening services have not been described well.

One of the major obstacles in detecting HPV-mediated diseases and cervical cancers particularly in the developing countries are cost and technical requirements.[289]

There is an urgent requirement to build up less expensive and fast HPV tests that are easy to use.[290]

The second reason could be the acceptability of HPV screening, as cervical cytology requires the introduction of a vaginal speculum for examination, which is a major barrier to screening. Therefore, a non-cytological screening method may solve the problem of insufficient screening in developing countries, or the self-collection method may also increase screening practice in many resource-poor areas where there is a limited number of physicians trained in performing speculum examinations.[290]

Lack of data and awareness about HPV infection and cervical cancer in women is the third and main reason in India.[291-297] on the other hand, factors such as socio-economic restrictions and a lack of nationalized guidelines and policies also add to barriers for cancer screening programs.[298]

Objectives

OBJECTIVES

1. To determine the Human Papilloma Virus (HPV) genotypes in women attending R.L. Jalappa hospital in Kolar region
2. To identify socio-demographic risk factors associated with HPV infection
3. To determine the association of High Risk (HR) HPV genotypes in development of premalignant and malignant cervical cytology in women

Materials and Methods

MATERIALS AND METHODS

Materials

Table 3: list of the supplies that were purchased for our study.

Name of the product	Name of the company
Cytobrushes	Digene HC2 DNA Collection Device (Cat. No. 5126-1220)
DNA extraction kit	QIAamp DNA Mini kit (50) (Cat. No. 51304)
Primers	Sigma Aldrich Private India Limited, Bangalore
PCR thermal cycler	Applied Biosystems, USA
UV trans illuminator	Major Science, USA

Methodology

Study type: Cross-sectional Descriptive Study

Study setting: This was a prospective hospital-based study which was done at the Departments of Microbiology (sample processing) and Obstetrics and Gynecology (sample collection) of Sri Devaraj Urs Medical College, Kolar during the period of 2018 to 2021.

Ethical clearance:

Ethical clearance is obtained from the Institutional Ethical Committee of Sri Devaraj Urs Medical College, Kolar (SDUMC/KLR/IEC/14/2019-20).

Study population:

Women who are suspected to have HPV infection and attended Obstetrics and gynecology's outpatient department (OPD) with symptoms like abnormal bleeding, unusual vaginal discharge with/without foul smell, pelvic pain, discomfort while urinating and pain during sex.

Study eligibility criteria

Inclusion criteria: Married women showing symptoms like: Abnormal Vaginal Discharge, Pain during coitus and Clinical suspicion of cervical cancer was included in our study.

Exclusion criteria:

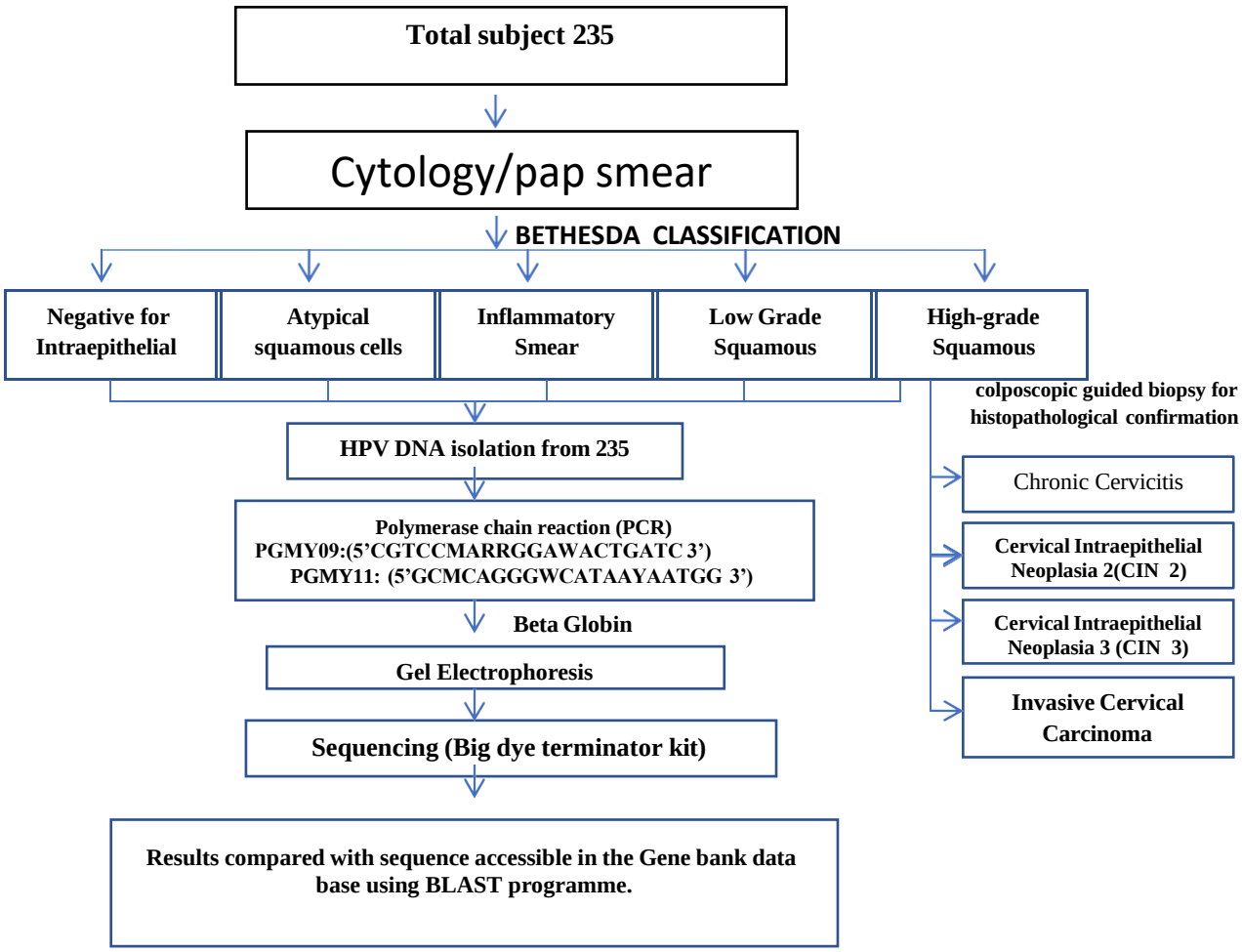
Unmarried women, pregnant cases and Patients undergoing treatment for other infections/diseases were excluded.

Sample size calculation:

Previous study showing the occurrence of single HPV genotype to be 44.5%.[373.]

With 15% relative precision and confidence level of 95% the smallest sample size required was estimated to be 213 (calculated using n Master 2.0). Accounting for poor quality samples 10% of the additional samples was selected. So the final sample size was 213+22 = 235 samples

Fig 8 : Work plan of the study



Sample Collection

Informed consent and Patient information form was provided to all the participants before collecting the sample. The study was described to all the participants.

Papsmear and Cytobrush sample collection

a. Procedure for Cytobrush sample collection:

A cytobrush sample was taken from a patient who was suspected of having HPV infection during a single pelvic exam by inserting the cytobrush into the cervical region and

rotating at 90^0C. cytobrush head was removed, placed in a vial which contains Phosphate buffered saline, and kept at 4°C for molecular analysis.

b. Procedure for Pap smear sample collection: A pap smear was performed on same patient after cytobrush sample collection. To view the cervix, a speculum was utilized. while the patient was asked to lie down in the lithotomic position. The smaller side of the Ayres spatula was placed in front and inside the external os. By rotating the spatula 360 degrees, the squamocolumnar junction was scraped. The smear was created by evenly spreading the scraping onto a glass slide, which was fixed with fixatives right away to prevent air drying and sent right away to the pathology lab for cytological analysis.[277]

Tissue biopsy sample collection

From the patients with confirmed diagnosis of HSIL or suspected of having cervical malignancy, colposcopy guided tissue biopsies were collected, for histopathological-examination and confirmation. The biopsies taken for histopathological examination were sent to the pathology laboratory of the Sri Devaraj Urs Medical College& Hospital.

Pathological analysis of tissue biopsies and Pap smears (for cytobrush samples)

Pathological analysis of tissue biopsies and Pap smear were done in accordance with the standard protocols.[299] The results of histopathology (tissue biopsies) and cytology (Pap smears) were attained from the medical records of the Sri Devaraj Urs Medical College& Hospital.

Pap smear preparation

The patient was asked to lie down in lithotomic position and with the support of a speculum, the cervix was examined. Ayres spatula was inserted with the smaller side in front, inside the external os. The squamocolumnar junction was scraped by rotating the

spatula to 360°. The smear was then prepared by evenly spreading the scraping onto a glass slide, that was immediately fixed using fixatives to avoid air drying and immediately sent to the pathology laboratory for cytological examination.[300]

Papanicolaou (Pap) staining for cytology

Principle: For cytological specimens, the Papanicolaou technique is the staining method that is most frequently used. Both basic and acidic dyes are used in papanicolaou stain. The basic components of the cell are stained by acidic dye, and the acidic components are stained by basic dye. Orange G (OG), Harris's alum-haematoxylin, and an EA 36 or EA 50 triple-dye mixture are the required solutions.

Procedure:

1. After being removed from the fixative, smears were washed in descending alcohol concentrations (80, 70, 50%), (8-10 sec each).
2. Harris alum-haematoxylin stained (4 min).
3. Washed beneath tap water for 1-2 min.
4. 0.5% HCL differentiation was done until only the nuclei were stained.
5. Washed in tap water for 3-5 min.
6. Transferred to 70% alcohol, then received two adjustments at 90% alcohol (few sec each).
7. Stained in OG for 2 min.
8. Rinsed in three changes of 95% alcohol.
9. Stained in EA solution for 2-4 min.
10. Rinsed in three changes of alcohol (95%). Completely dehydrated in absolute alcohol and cleared in xylene.
11. Slides were then inspected under the oil immersion microscope (x100)

Interpretation of results: Under the oil immersion microscope the following structures should be appreciated from the Pap smears of cervical samples

Table 4 : Interpretation of Papanicolaou staining

No.	Structure	Color
1	Cytoplasm cyanophilic (basophilic)	Blue, green
2	Cytoplasm eosinophilic (acidophilic)	Pink
3	Cytoplasm (keratinised)	Pink, Orange
4	Nuclei	Blue, dark violet, black
5	Microorganisms	Grey blue
6	Erythrocytes Orange	Red

Hematoxylin & Eosin (H &E) staining for tissue biopsies

Principle: Hematoxylin containing alum acts as a mordant and dyes the nucleus a pale blue color. This turns red in the presence of acid because differentiation requires treating the tissue with an acid solution. The bluing process transforms the initially soluble red colour within the nucleus into an insoluble blue tint. Eosin, a counterstaining agent that gives the cytoplasm a pink hue, was used.

Procedure:

1. Sections were deparaffinized: slides were flamed on burner and placed in xylene. Treatment was repeated.
2. Tissue sections were hydrated by passing them over decreasing alcohol concentration (100%, 90%, 80%, and 70%) and water.
3. Hematoxylin Stained (3-5 min).
4. Washed beneath running water till sections become ‖blue‖ (5 min or less).
5. Differentiated in 1% acid alcohol that is 1% HCl in 70% alcohol is done for 5 min.
6. Dipped in an alkaline solution, then washed in flowing tap water until the portions were blue once more.
7. Stained in 1% Eosin Y (10 min).
8. Washed in tap water (1-5 min).
9. Dehydrated in increasing alcohol concentration (70%, 80%, 90%, and 100%) and cleared in xylene.
10. Mounted in mounting media and sections were seen under oil immersion microscope (x100).

Interpretation of results: Under the oil immersion field of microscope, the following structures should be appreciated from tissue biopsy samples

Table 5: Interpretation of H and E staining

No.	Structure	Color
1	Nuclei	Blue, Black
2	Cytoplasm	Pink
3	Fibrin	Deep Pink
4	Red blood cells	Orange Red
5	Muscle Fibres	Deep red

Preparation of samples prior to DNA extraction

Samples were brought to the room temperature before the isolation of DNA. By using Qiagen DNA extraction kits, DNA was isolated from cytobrush samples. (For cytobrush samples, catalog number – 51304)

HPV DNA extraction

DNA was detected from the samples (n = 235) according to the kit literature provided with the QIAamp DNA mini kits.

The procedures followed for DNA extraction is as follows:

i. Proteinase K (20 µL) was poured into a microcentrifuge tube (1.5 mL).

ii. Sample (200 µL) added to the tube.

iii. Buffer AL (200 µL) was added, mixed by pulse vortexing for 15 sec and incubated @ 56°C for 10 min. Then briefly centrifuged.

iv.Ethanol (96-100%; 200 µL) was added, mixed by using pulse vortex for 15 sec and briefly centrifuged.

v. Mixture was then moved to QIAamp mini column, cap closed , centrifuged at 8000 rpm for one min. QIAamp mini column was then positioned in a new collection tube (2 mL) and the filtrate was discarded.

vi. buffer AW1 (500 µL) was added to QIAamp mini column. Centrifuged at 8000 rpm for 1 min. QIAamp mini column was placed in a clear collection tube (2 mL) and collection tube containing filtrate was discarded. viii. Buffer AW2 (500 µL) was added to the mini column and centrifuged at 14,000 rpm for 3 min.

vii. Mini column was placed in a clear 1.5 mL microcentrifuge tube and the filtrate was discarded. Finally, Buffer AE (200 µL) was added. Incubated at room temperature for 5 min , centrifuged at 8000 rpm for 1 min. x. Extracted DNA was collected & stored at -20°C until further processing.

Polymerase chain reaction (PCR)

DNA was amplified by primer sets MY09 [F5'-CGTCCMARRGGAWACTGATC-3] and MY11 [R5'-GCMCAGGGWCATAAYAATGG-3'] targeting L1 gene with a band size of 450 base pair[301,302,303]

Stepwise preparation of PCR reaction mixture

1. Required number of PCR tubes (0.2 mL) were labelled and kept back on ice.

2. After thawing, PCR reagents were gently vortexed and briefly centrifuged.

3. A thin-walled PCR tube was placed on ice and the following components were added for each 25 µL reaction.

4. The amount of water necessary for the desired volume was first calculated and added to the tube. The premix contained following components in a closing volume of 25 µL / aliquot.

i. Ampliqon red master mix (2X): 12.5 µL
ii. HPV MY09 (Forward primer): 1 µL
iii. HPV MY11 (Reverse primer): 1 µL
iv. Template DNA: 3 µL (10 pg-1 µg)
v. Water : Added to make final volume to 25 µL

5. Samples were gently vortexed.

6. Tubes were then kept in the thermal cycler (Applied Biosystems, USA) and DNA amplified using standard PCR conditions.

Table 6: Polymerase chain reaction conditions

Primer	Initial Denaturation	Final Denaturation	Annealing	Extension	Final Extension
		35 cycles			
MY09/ MY11	95 °C , 5 min	95 ° C, 30sec	55 ° C, 1 min	72 ° C, 30 s	72 ° C, 5 min

Gel electrophoresis and interpretation of results under UV transilluminator

PCR products were detached on 2% agarose for detection of specific bands. The amplified products were loaded on to the gel and allowed to run for 2 hours at 60V. The gel was stained by Ethidium bromide (0.5 µg/mL) for visualization of the bands.

Reagents required Agarose Ethidium bromide Amplified product 50X TAE buffer

Tris base: 12.1 g Glacial acetic acid: 2.850 mL 0.5M EDTA : 5 mL Distilled water : Made up to 50 mL. Store at -20°C Preparation of agarose gel (2%)

1. Agarose powder (2 g) was mixed in 1X TAE Buffer (100 mL) (for 2% agarose) and boiled with gentle stirring till a clear homogenous solution was formed.
2. Cooled for some time and added ethidium bromide (2 µL; concentration 0.5 µg/mL).
3. Poured into gel mold and placed the comb. Allowed to set for 20 min.
4. Comb was removed carefully. Kept the gel in electrophoresis unit containing 1X TAE buffer (200 mL), such that, it is completely submerged in the buffer.
5. The amplified product (20 µL) was loaded carefully into the wells; molecular weight marker (DNA ladder) was loaded in the last well.
6. Electrode was fixed, power supply was turned on and current (60 V) was adjusted.
7. gel was left to run for 2 hr.
8. Photographs of gel were taken under UV light transilluminator and the bands were recorded using the Gel documentation system (Major Science, USA). Note: Inert red dye in the PCR master mix acts as loading dye which will not allow the samples to float over the gel. The gel was then visualized under UV transilluminator

Hpv 16 used as control and Each gene had specific band size based (450 bp) on the primer sequences selected. The DNA ladder was also added in one well of each gel to

obtain the bands of known sizes which was used in locating the band positions of test samples

DNA Sequencing

After finishing the purification, genomic DNA was sequenced by Sangers Didcon Chain Termination technique. The primers for sequencing are MY09/MY11. DNA sequencing was done in the department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka.

The sequences were specified using an ABI Prism 310 Genetic Analyser (The sequences were obtained in FASTA format.

Sequencing PCR

The following are the PCR conditions and the composition of the sequencing mix:

10µl Sequencing Reaction , Big Dye Terminator Reaction Mix: 4µl Template (100ng/ul): 1µl Primer (10pmol/λ): 2µl Milli Q Water: 3µl PCR Conditions: (25 cycles) Initial Denaturation: 96°C,5 min; Denaturation: 96°C for 30 sec Hybridization: 50 °C for 30 sec Elongation: 60 °C for 1.30 min

Instrument Details of Sequencing Machine:

ABI 3130 Genetic Analyzer

Chemistry Cycle sequencing kit: Big Dye Terminator version 3.1‖

Polymer & Capillary Array: POP_7 pol Capillary Array.

Analysis protocol: BDTv3-KB-Denovo_v 5.2

Data Analysis: Seq Scape_ v 5.2

Software Reaction Plate: Applied Biosystem Micro Amp

Optical 96-Well Reaction plate

Statistical Analysis

The data obtained from the study observations was recorded on a predesigned proforma and managed using Microsoft Excel All quantitative measures are prepared through mean and standard deviation. Categorical data are represented by frequency and percentages. The association between socio-demographic risk factors and HPV is done by odds ratio and confidence intervals. P value <0.05 is considered significant.

Results

RESULTS

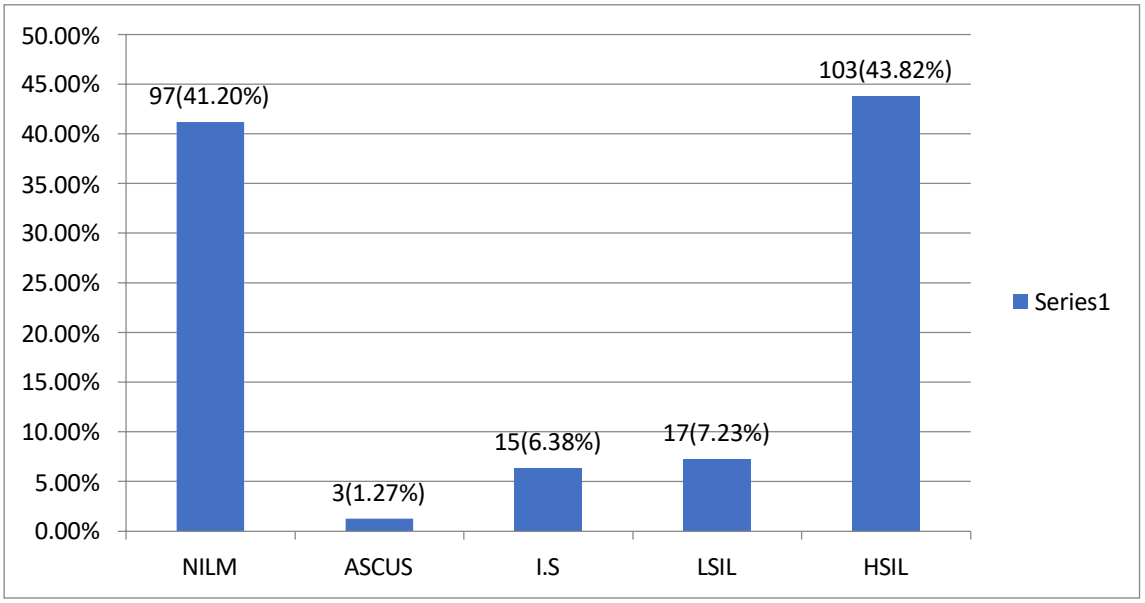
DISTRIBUTION OF CERVICAL ABNORMALITIES BASED ON CYTOLOGY(n=235)

The study included 235 women with cervical abnormalities and suspected HPV infection. The specimens consisted of cytobrush samples (n=235). Pap smear was collected from the same patient after cytobrush sample and reports were taken from the pathology department. Examination of the pap smear are as follows: Negative for intraepithelial lesions/malignancy (NILM) (41.2%), Atypical squamous cells of undetermined significance (ASCUS) (1.2%), Inflammatory Smear (6.3%), Low grade squamous intraepithelial lesions (LSIL) (7.2%), and High grade squamous intraepithelial lesions (HSIL) 103(43.8%)

Table7: Distribution of cervical abnormalities based on cytology(n=235)

Sl. No	Pathological Findings (N=235)	Frequency (%)
1	NILM	97(41.20%)
2	ASCUS	3(1.27%)
3	Inflammatory Smear	15(6.38%)
4	LSIL	17(7.23%)
5	HSIL	103(43.82)

Fig 9: Distribution of cervical abnormalities based on cytology(n=235)



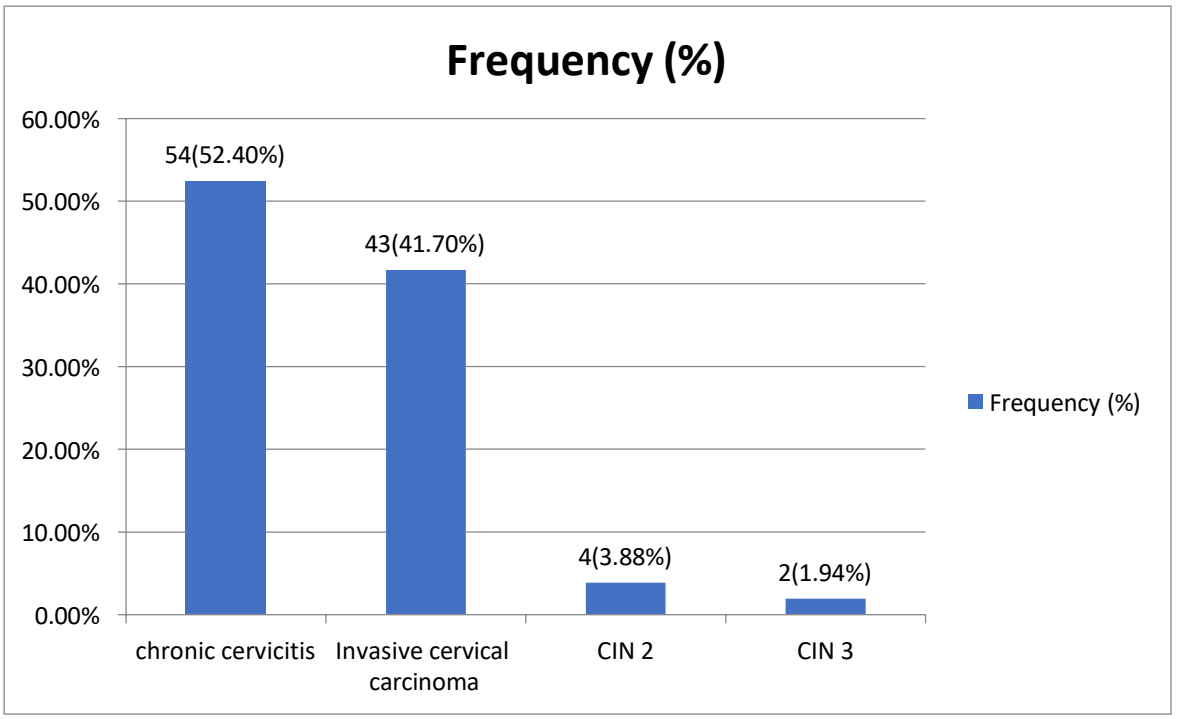
Distribution of cervical abnormalities based on histopathological confirmation of High-grade lesions(n=103):

Patients with HSIL underwent colposcopy-guided biopsy (n=103) and Histopathological tests were performed on the samples for confirmation. Histopathology reports showed that 54/103 (52.4%) patients had chronic cervicitis, 4/103(3.88%) had Cervical intraepithelial neoplasia 2 (CIN 2), 2/103 (1.94%) had Cervical Intraepithelial Neoplasia 3 and 43/103(41.7%) had Invasive cervical carcinoma (ICC).

Table 8: Distribution of cervical abnormalities based on histopathological confirmation of High-grade lesions(n=103)

Sl. No	Pathological Findings (N=103)	Frequency (%)
1	Chronic Cervicitis	54(52.40%)
2	ICC	43(41.70%)
3	CIN 2	4(3.88%)
4	CIN 3	2(1.94%)

Fig 10 : Distribution of cervical abnormalities based on histopathological confirmation of High-grade lesions(n=103)



Percentage of HPV positive women in total samples screened (n=235)

All the collected cytobrush samples (n = 235) were subjected to DNA extraction.78 of the 235 samples tested positive for HPV infection, whereas 157 tested negatives. As a result, 33.19% of all screened women in our study had HPV infection.

Fig 11: Percentage of HPV positive women in total samples screened (n=235)

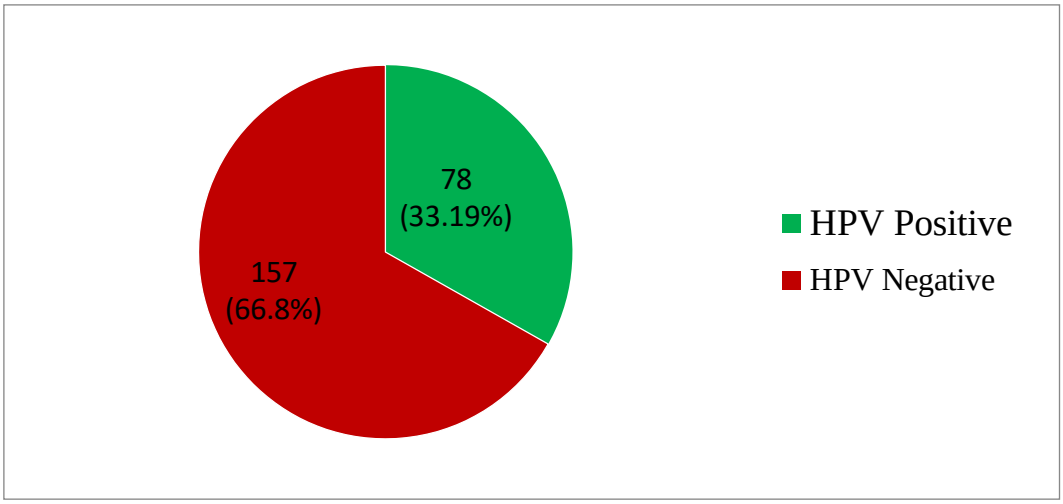
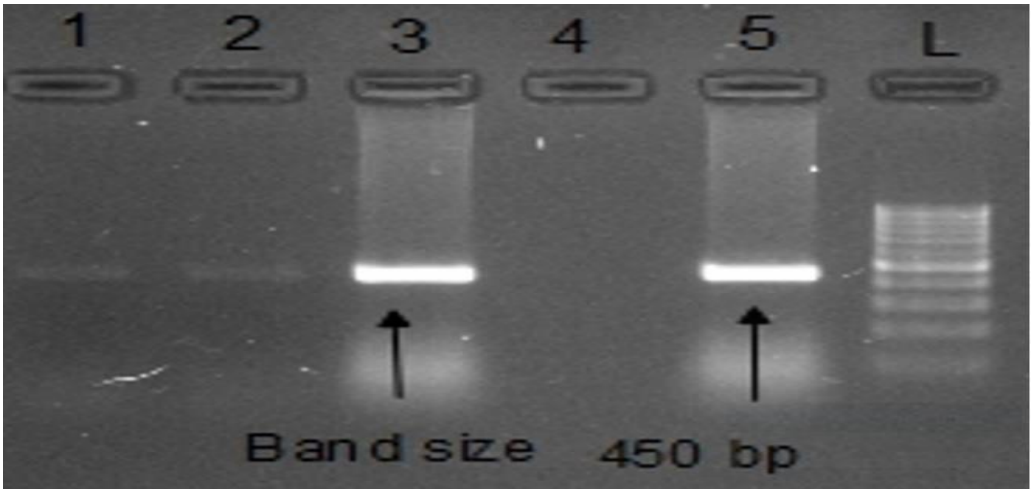


Fig: 12 Agarose gel for amplification of the HPV DNA



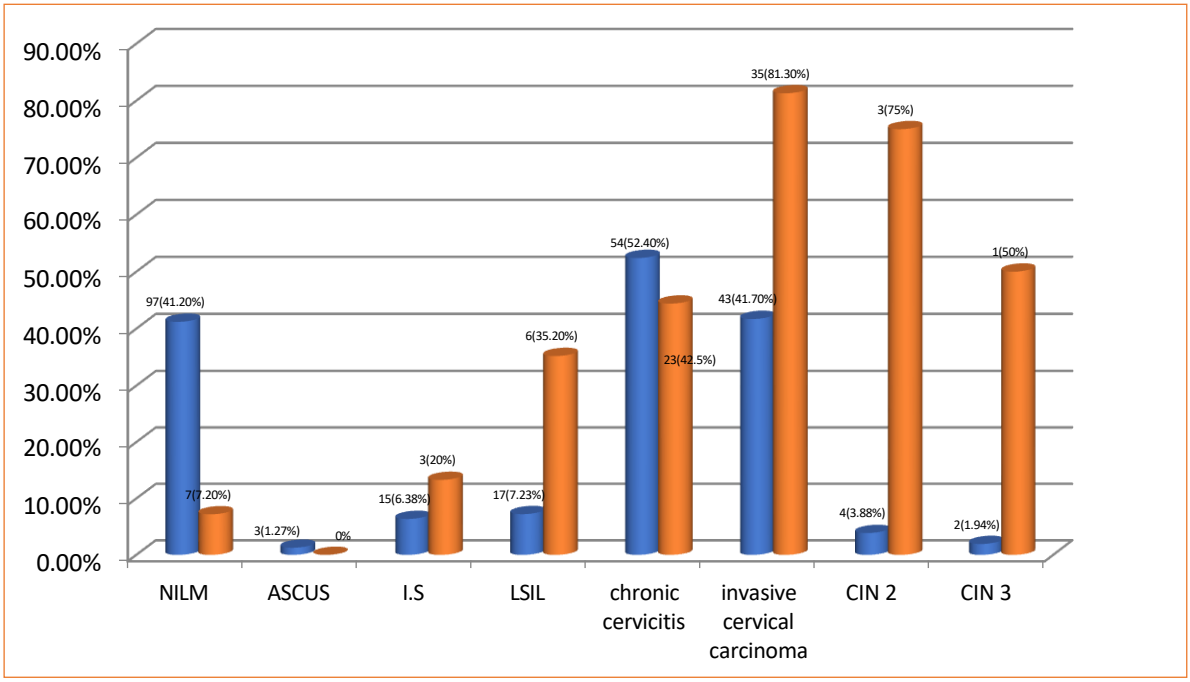
Agarose gel of the product of the Human papillomavirus gene amplified by conventional PCR 100 base pair (bp) DNA ladder (NEX-GEN, GENETICS) is on the right (L), and 1,2,4 Negative for HPV DNA, 3,5 positive HPV DNA

Distribution of total participants (n=235) and the HPV DNA positive samples(n=78) based on their pathologies:

Table 9: Frequency of HPV DNA positives in different cytology's

Sl. No	Pathological Findings (n=235)	Frequency n (%)	HPV DNA Positive n (%)
1	NILM	97(41.20%)	7(7.2. %)
2	ASCUS	3(1.27%)	0(0%)
3	Inflammatory Smear	15(6.38%)	3(20%)
4	LSIL	17(7.23%)	6(35.20%)
5	Chronic Cervicitis	54(52.40%)	23(42.5%)
6	ICC	43(41.70%)	35(81.30%)
7	Cervical Intraepithelial Neoplasia 2	4(3.88%)	3(75%)
8	Cervical Intraepithelial Neoplasia 3	2(1.94%)	1(50%)
	Total	235(100%)	78(33.19%)

Fig 13: Frequency of HPV DNA positives in different cytology's



DNA sequencing and interpretation of results using BioEdit software

DNA sequencing and interpretation of results using Bio Edit software has been done to validate the PCR results. All 78 HPV DNA positive samples, were selected for DNA sequencing. PCR amplicons were then sequenced to assure the alleles. A best selected portion of the nucleotide sequences on the chromatogram was selected and analyzed using Bio Edit software. Finally, the sequences were then examined by means of the software NCBI BLAST. Further, nucleotide sequences were evaluated by compared for the similarity or uniqueness with the other sequences, which are already available in the NCBI Gene Bank

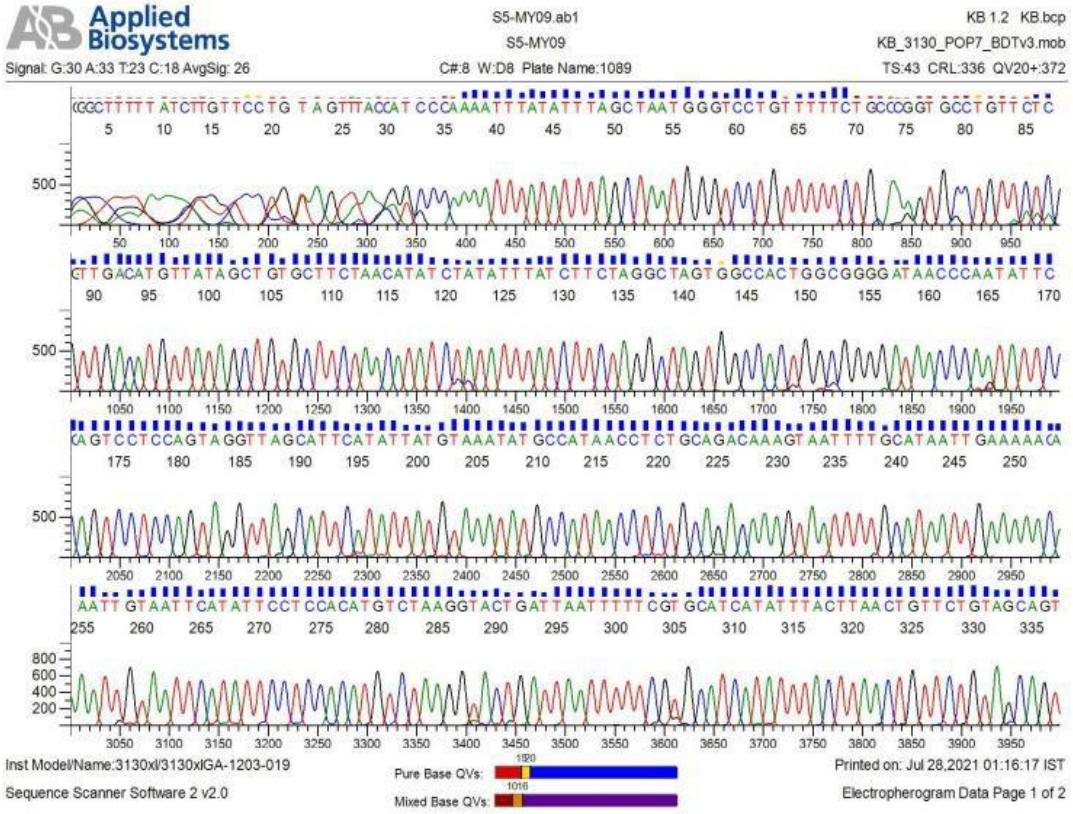


Fig 14: Chromatogram obtained after sequencing of the HPV positive cytobrush sample

NCBI BLAST

DNA sequence comparison from our study with known alleles in the NCBI's GeneBank, showed that, among few alleles novel mutations were observed.

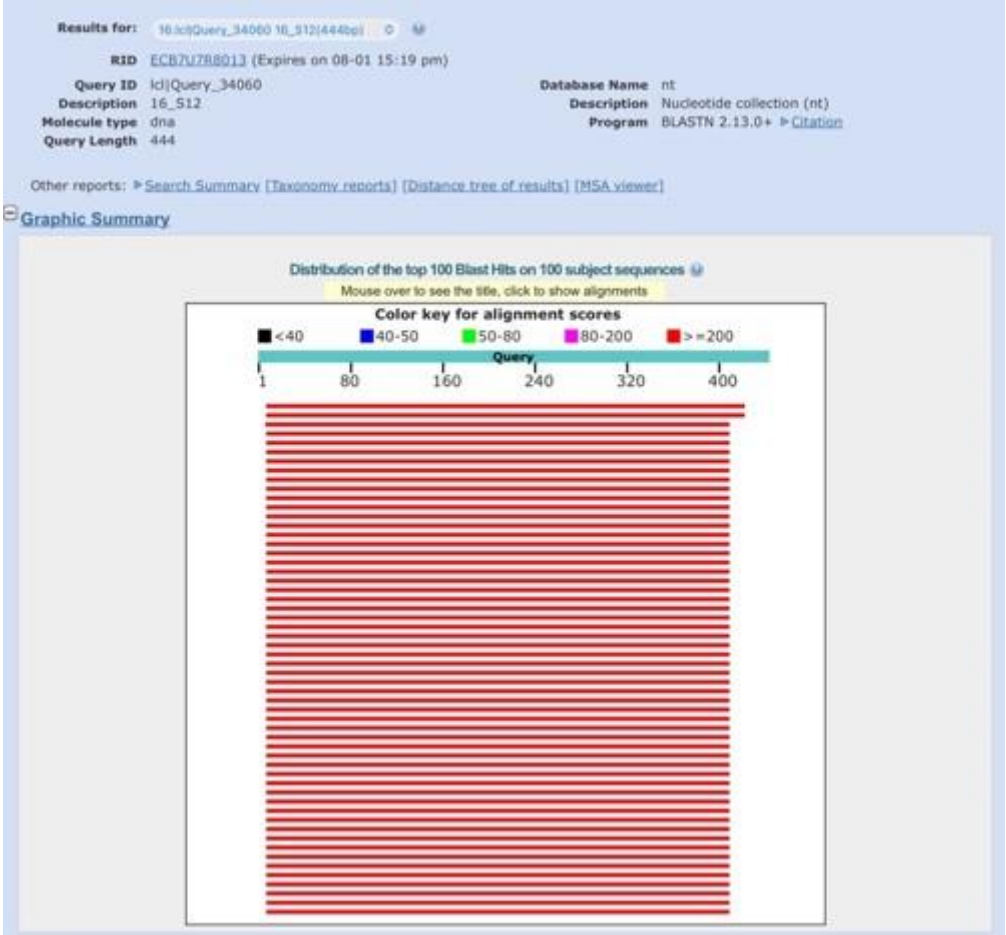


Fig 15: NCBI BLAST result for sequencing

Out of 78 HPV DNA, 42 were HPV genotyping positive on Sanger sequencing. Among LSIL group 3/42 (7.1%) were infected with HPV 16. Out of 42 samples only 1 (2.3%)sample showed HPV 16 genotype among CIN 2 and Only 1(2.3%) sample out of 42 among CIN 3 were positive for HPV DNA and showed HPV 16 genotype.Distributionof genotypes in ICC cases revealed that HPV 16, 20/42 (47.6%) and 18, 7/42 (16.6%) were the most predominant genotypes, preceded by HPV 56, 4/42 (9.5%), which is consistent with studies from India and around the world. In our study, the prevalence of HPV 16 was identified to be high 31/42 (73.8%), followed by HPV 18, 7/42 (16.6%), and HPV 56, 4/42 (9.5%).

HPV Genotypes distribution Among different cytology's

Out of 78 HPV DNA positive samples, 42 were positive for HPV genotyping on Sanger sequencing and gave good sequencing results. Among LSIL group 3/42 (7.1%) were infected with HPV 16. Infection with HPV 16 genotype was detected in 1 out of 42 CIN 2 samples, i.e., 2.3% and Only 1/42(2.3%) sample among CIN 3 showed positivity for HPV DNA with HPV 16 genotype. Analysis of genotype distribution in cases of ICC revealed that HPV 16,20/42 (47.6%) and 18, 7/42(16.6%) were the most prevalent genotypes, followed by HPV 56, 4/42 (9.5%) which is very close to research reported from India and around the world.

Table 10: HPV genotype distribution in different cytology's

Genotypes (n=42)	**LSIL (n=3)**	**Chronic Cervicitis (n=6)**	**CIN 2(n=1)**	**CIN 3 (n=1)**	**ICC (n=31)**
HPV 16	3 (7.1%)	6(14.2%)	1(2.3%)	1(2.3%)	20(47.6%)
HPV 18	0(0%)	0(0%)	0(0%)	0(0%)	7(16.6%)
HPV 56	0(0%)	0(0%)	0(0%)	0(0%)	4(9.5%)

Distribution Of HPV Genotypes(n=42)

In our study amongst the different cytology's identified, HPV 16 was predominant Genotype 31/42(73.8%) followed by HPV 18, 7/42(16.6%) and HPV 56, 4/42(9.5%). All study participants exhibited a single genotype and no multiple genotypes were observed.

HPV 56 was identified to be the novel genotype in this Kolar region which belongs to a non-vaccine targeted group.

Table 11: HPV genotype distribution

Genotypes	HPV genotype (Prevalence %)
HPV 16	31(73.8%)
HPV18	7(16.6%)
HPV56	4(9.5%)

Age wise distribution of study participants based on presence of HPV DNA

Table 12: Age wise distribution of study participants

Age group (In years)	Study participant's n (%)	HPV DNA +ve (%)	HPV DNA –ve (%)
21-30	24(10.2%)	4(16.6%)	20(83.3%)
31-40	71(30.2%)	18(25.3%)	53(74.6%)
41-50	89(37.8%)	41(46.0%)	48(53.9%)
51-60	37(15.72%)	12(32.4%)	25(67.5%)
>60	14(5.9%)	3(21.4%)	11(78.5%)
Total	235(100%)	78(100%)	157(100%)

235 women were screened for our study and divided into 5 age groups, as follows:24(10.2%) women from age group 20-30 years, 71(30.2%) women from age group 31-40 years, 89(37.8%) women from age group 41-50 years, 37(15.72%) women from age group 51-60 years and 14(5.9%) within the age range of 60 or older. A

maximum of 89 (37.2%) women were in the age range of 41 to 50 as shown in the table 13.

The participant's mean age was 42 years ± 10 years. Participants' HPV DNA positivity rates according to age groups: 21-30, 31-40, 41-50, 51-60 and >60 years was 4(16.6%),18(25.3%),41(46.0%),12(32.4%),3(21.4%),78(100%) respectively. However, highest occurrence of HPV DNA positivity was in age group, 41-50 years (41/89, 46.06%), followed by age group 51- 60 years (12/37, 32.4%).

Sociodemographic and personal characters as risk factors for HPV infection

It was observed that the highest incidence of infection with HPV was seen in the age group > 40 years 56(40%) in comparison with age group <40 years ,22 (23%) HPV DNA which is statistically significant (p=0.0078*)

The samples of women in our study were divided based on parity into 2 groups, parity <3 and parity >3. When parity was considered, it was found that the incidence of HPV infection increased as the number of children increased. There were 193 participants in our current study with >3 children, of them 56 showed HPV infection, whereas 22 patients tested positive with <3 parity (n=42). Thus, compared to women with one or two children, those who had three or more children were significantly linked with HPV infection.

We also looked at the subjects' methods of contraception and their connections to the HPV infection. Maximum women (231/235) didn't use any method of contraception. Oral

contraceptives were used by Only four out of 235 women, while the same number also utilised barrier contraception.

HPV infection was at its highest rate in women who dont use oral contraception (33.7%), and at its lowest rate in those who used. However, in our study use of contraception is not seen significantly associated with HPV infection. A questionnaire was asked to the study subjects on whether they use cloth/ pads during menstruation and also how frequently do they change their sanitary napkins. Based on this data 154 study participants presented with poor menstrual hygiene and HPV infection found in these patients was 46(29.87%) and 81 subjects presented with good menstrual hygiene and HPV infection was 32(39.5 %). In our study menstrual hygiene is not associated significantly with HPV infection. Among the 235 study subjects 65 were from urban and 170 belonged to rural areas. The highest number of HPV positivity was seen in rural resident subjects i.e., 56/175 (32%). Place of residence shows no significance with HPV infection. Among 175 pre-menopausal women, 56 HPV positives were detected and among 60 post-menopausal women 22 were having HPV infection. Our study, however, found no connection between HPV and menopause.

In our study, only 12 participants were going for regular screening and 223 patients never had any HPV screening before. No participants in our study ever took any HPV vaccination before**.** Majority of the participants, 33.5% (62/185), belonged to the below poverty line status. pertaining to the habits like smoking, alcohol and tobacco, 37.5% (62/165) of the study participants consume tobacco and 22.8% were non tobacco users. There were no smokers or drinkers among our study participants.

Overall Age group >40 years, parity>3, age at marriage<20 years and social habits like chewing tobacco was identified to be strongly linked with HPV infection.

Table 13: Sociodemographic and risk factors related to HPV

Factors	HPV +VE	HPV –VE	OR (95%CI)	*P*-VALUE
Age				
>40=140	56(40%)	84(60%)	2.2121(1.2331 to 3.9685)	**0.0078***
<40=95	22(23.1%)	73(76.8%)		
Parity				
<3=42	22(52.3%)	20(47.6%)	2.6714(1.3523 to 5.2775)	**0.0047***
>3=193	56(29%)	136(70.4%)		
Age at marriage				
<20years=88	38(43.1%)	50(56.8%)	2.0330(1.1652 to 3.5472)	**0.0125***
>20years=147	40(27.2%)	107(72.7%)		
Oral contraceptives				
Yes=4	0(0%)	4(100%)	0.2173(0.0116 to 4.0870)	0.3079
No=231	78(33.7%)	153(66.2%)		
Menstrual hygiene				
Poor=154	46(29.8%)	108(70.1%)	0.6522(0.3712 to 1.1458)	0.137
Good=81	32(39.5%)	49(60.4%)		
Residence				
Rural=170	55(32.3%)	115(67.6%)	0.8733(0.4786 to 1.5937)	0.659
Urban=65	23(35.3%)	42(64.6%)		
Menopause				

Pre=175	56(32%)	119(68%)	0.8128(0.4400 to 1.5015)	0.5081
Post=60	22(36.6%)	38(63.3%)		
Regular screening				
Yes =12	5(41.6%)	7(58.3%)	1.4677(0.4504 to 4.7825)	0.5243
no=223	73(32.7%)	150(68.6%)		
HPV immunisation				
yes= 0	0(0%)	0(0%)	2.0064 (0.0394 to 102.0710)	0.7283
No=235	78(33.1%)	157(66.8%)		
Economic condition				
Low=185	62(33.5%)	123(66.4%)	1.0711(0.5492 to 2.0892)	0.8402
High=50	16(32%)	34(68%)		
Social habits(tobacco)				
Yes=165	62(37.5%)	103(62.4%)	2.0316(1.0705 to 3.855)	**0.03***
no=70	16(22.8%)	54(77.1%)		
Smoking/Alcohol				
Yes=0	0(0%)	0(0%)	2.0064 (0.0394 to 102.0710)	0.7283
No=235	78(33.1%)	157(66.8%)		

Assessment of awareness and knowledge about cancer cervix and HPV/HPV vaccine among study participant

The entire the participants (100%) were unsure of how they could get cervical cancer, who can possibly get this cancer, what are the risk factors linked to this cancer, etc. However, 5% (12/235) of patients unknowingly underwent Pap smear testing for cervix examination. Whereas, 94.8% (223/235) patients were not aware of Pap smear screening for cervical cancer, and therefore never had undergone for Pap testing. This study shows that, none of the study participants, 100% (235/235), has neither heard of HPV, nor is aware of its effects on health of the infected individual. Therefore, none of the participants were vaccinated, and had no knowledge about HPV vaccine and its schedule.

Association of High Risk (HR) HPV genotypes in development of premalignant and malignant cervical cytology in the women

A significant increase in severity of disease was observed showing HPV as an important risk factor in disease progression ($p=0.03^*$). An increase in frequency of HPV increases while disease is progressing from premalignant to malignant.

Table 14: Association of High-Risk HR-HPV genotypes in development of premalignant and malignant cervical cytology in women

Clinicopathological Parameters	Total Sample	HPV Positive	Genotype positive	HPV 16	HPV 18	HPV 56	HPV Negative	P VALUE
LSIL	17	6(35.2%)	3	3	0	0	11(64.7%)	**0.03***
CIN 2	4	3(75%)	1	1	0	0	1(25%)	
CIN 3	2	1(50%)	1	1	0	0	1(50%)	
Invasive Cervical Carcinoma	43	35(81.3%)	31	20	7	4	8(18.6%)	

Discussion

DISCUSSION

Cervical cancer is common in India[304] but there is a deficit of national statistics on HPV infection and its genotype distribution, which would be supportive for a more extensive vaccination campaign. To the extent of our knowledge our study is the very first to report the HPV infection prevalence and distribution of genotypes in the women with cervical infections in the Kolar district. Samples were obtained from R.L. Jalappa hospital. Patients from nearby villages in Kolar districts visit for a consultation. Thus, our study offers a detailed estimation of the incidence HPV and its genotype distribution in symptomatic females of Kolar district. However, in this study any bacteriological, mycological or HIV infections were excluded which that would predispose participants to HPV infection.

Sociodemographic and risk factors assessment:

The participants' mean age was 45 years, with 37.8% (37/235) within the age range of 41-50 years, followed by 31-40 years, 30.2% (71/235), which was steady with former studies done in the Asia Pacific region.[305] In our study, the majority of HPV DNA positive samples 41/78(46.0%) were from patients in the age spectrum of 41 to 50 years, indicating that the persistence of the virus is an important factor in the progress of HPV infection. This was in accordance with the results of a few studies that showed HPV infection increases with age.[306-312] The process of ageing causes waning of immune system and the likelihood of contracting infection rises. It was observed in our study that the age of the women affected the occurrence of HPV infection significantly (p value = 0.0078*). Contrarily, a small number of other research revealed,HPV infection decreased with advancing age. [313-316]. However, Yildirin et al., and Akcali et al., in Turkey reported there is no connection between women's age and HPV prevalence.[317,318]

According to Kymberlee Montgomery et al, Women over 40 are not necessarily at a higher risk for HPV, but many of them have tested positive and are now dealing with the impact of HPV, It is recognized as a risk factor for cervical cancer.[319] None of the participants in our study had received the HPV vaccine. Less people were aware of HPV vaccinations and immunization schedules. Despite the reality that vaccinations are available for the two main high-risk oncogenic types, HPV 16 and HPV18, the lack of awareness and the high cost of vaccination remain the two main obstacles to immunizing women in underdeveloped nations.

Age at marriage correlates with the beginning of a woman's sexual life. Females in our Indian society do not openly divulge their age at their first intercourse due to social stigma, Therefore, in our study, the age of marriage criterion was used rather than the age at first sexual encounter. Our study identified a significant association between HPV infection & a woman's age at marriage (p value = 0.0125*), and additionally, statistical results demonstrated that younger women who made their sexual debut before age 20 had a higher probability of contracting the virus than other groups. Therefore, in our study, the highest incidence of HPV was seen in women who married below 20 years of age. But women who married at age 20 and beyond have lower rates of HPV infection. Our study is in harmony with ley et al and kataja et al.[320,321] However, our findings oppose earlier research that found no significant link between HPV and marriage age. [307,322]

In our study, participants with parity >3 are more (193/235) when compared to participants with parity <3(42/235). Out of 78 HPV positive DNA, 56(71.4) positives were seen in women having >3 parity whereas 22(28.2%) positives were seen in women with <3 parity. Our study's results are statistically significant (p=0.0047), which is steady with the findings of Srivastava et al's study,[323] who stated that women having greater parity are 3-5 times higher at risk of acquiring cervical cancer. Few studies revealed

comparable outcomes and noteworthy correlations among females who begun their sexual lives earlier.[309-311,322,324,3265] The hazard of squamous cell cervical cancer in females positive for HPV increases with greater parity. This proves that early marriages increase women's sexual life and increase their likelihood of having more children, which raises the chance of cervical cancer in these women due to an immature cervix and increased wear and tear brought on by longer sexual life and more pregnancies. However, Aggarwal et al., Duttagupta et al., and Lazcano et al. did not report a clear association between HPV and age at marriage, which is in dissimilarity to our study.[307,308,326]

Smoking causes the body's immune system to be suppressed. Together, smoking and HPV infection change the levels of certain bodily substances including cytokines, which promotes the growth of tumors and some cancers. We didn't find any smoker women in our study. Hence no significant correlation is seen associated with HPV. Our study is in accordance with other authors, reported an inverse or a complete lack of association between smoking and HPV persistence.[327,328] Our study is contradicting with Kataja et al., who reported current smoking as an individual risk factor causing HPV infection.[321] Smoking was related to a 2-fold increased risk of HPV infection, according to Rohan et al. and Shields et al. Adittionally , Confortini et al., also reported increase in risk of HPV infection with the history of cigarette smoking.[329-331]

Our study advises that use oral contraceptives (Oc's) will provide definitive protection against HPV infection, while women in other categories were found to be at noticeably higher risk. No oral contraceptives were utilized by most of the study participants, 98.2% (231/235), and among them 33.7% (78/231) tested positive with HPV DNA Only 4 out of 235 individuals take oral contraceptives, and none of them were recognized to have HPV. However, our study's results weren't statistically significant, which is consistent with findings by Kataja et al. and Garg et al.[321,332] that reported no significant correlation

between the kind of contraceptive technique used and risk for HPV infection. However, our study was in contrast with Ley et al. who reported that use of Oc's was highly and independently correlated with higher occurrence of HPV infection.[320] Shields et al., also reported that the use of oral contraceptive was a reliable indicator of HPV seropositivity. [330] According to Goodman et al., prolonged usage of oral contraceptives reduces the likelihood of cervical HPV acquisition.[333] Varghese also reported decreased occurrence of HPV infection in patients using barrier method of contraception. [334]

Participants in our study come from urban and rural locations. The incidence of HPV infection was 55/170 (32.3%) in rural women and 23/65 (35.3%) in urban women. This finding is concurrent with a study by Alhamany et al. and they also reported higher frequency of infection with HPV among urban females than in rural females.[335] Our study's findings conflict with those of Aggarwal et al. and Gupta et al., who claimed that women from rural people are far more probable to be diseased with HPV than females from urban populations.[308,309] The findings of our study however, were not statistically significant.

Generally, HPV positivity is high in women who practice poor menstrual hygiene Where they use cloth all through their periods than those who use sanitary napkins. Use of subpar fabric or poor cleanliness while washing and reusing the similar cloth during periods may be to blame for this association. But in contrast according to our study, women who practiced good menstrual hygiene had higher HPV positivity rates 32(39.5%) which was consistent with Abulizi et al., who also stated that females who use sanitary napkins or toilet paper during their periods are more probable to get HPV than those who use regular cloth.[336] This could be attributed to the low-quality products.

In several Asian nations, especially India, it is a long-standing custom to chew betel nut. Areca nuts, betel leaves, tobacco, slaked lime, and other ingredients may be seen in

"quid" based on the location. It is a mixture of ingredients that is commonly chewed and

also inserted and kept in the mouth. Kolar district residents consume a lot of tobacco. Participants in our study who chew tobacco make up 70.2% (165/235), and 62/165 (37.5%) of them were HPV DNA positive. This data firmly confirms the link between cigarette use and HPV infection and demonstrate statistically significant findings (p=0.03*). This is in agreement with another cross-sectional study that reported cervical dysplasia was more prevalent in females who had a practise of chewing the betel pound. [337] Additionally, Raj Kumar et al. reported that all females who smoked betel nut tested positive for CC.[338]

Early detection is key to treating and preventing cervical cancer. Over 5 times more cases are reported in developing nations than in wealthy ones. The absence of effective screening programs is a major factor behind the much higher prevalence of cancer cervix in underdeveloped countries.

In India, the Pap test remains the gold standard procedure for cervical cancer screening. However, India has very few formal screening programs to choose from. Invasive cervical carcinoma is frequent, especially in rural India, even though Pap tests are readily available. Similar findings were seen in our study, which conveyed that even though screening facilities are accessible, 94.8% (223/235) of participants had not ever undergone any cervical cancer screening. Contrarily, none of them (235/25) had ever heard of HPV infection or vaccination, as a result they had never undergone an HPV test or vaccine.

Moreover, since this was a cross-sectional descriptive research and subjects were not monitored/followed up as the disease progressed. The age that was reported was the age upon diagnosis, not the actual age at which the cancer first appeared. The results were therefore ambiguous and required confirmation in large-scale population-based longitudinal investigations, such as integration with regular screening programs.

HPV genotype distribution in women with cervical abnormalities:

The incidence of HPV infection in our study was 33.1%. Similar outcomes were also reported by Joshi et al who also reported 33% HPV prevalence.[339] Our study is closely related to Aggarwal et al and Peedicayil et al who also reported HPV prevalence of 36.8% and 38.7 % respectively.[308,340] Our study's prevalence was more than that of previous studies, which ranged from 8.8% to 27.7%.[307,310-312,325,341-343]

Incidence of HPV amid the Invasive Cervical Cancer (ICC) cases was 81.30% (35/42) in our study. It is usually accepted that HPV effectively causes 100% of cervical carcinoma. Henceforth, the variance in results could be partially explained by variances in the quality of the sample sensitivity of the HPV detection techniques used, and other infections causing ICC [344]

Sequencing has been regarded as the gold standard for HPV genotyping, despite its limitations, as it allows for the recognition of all virus types without the risk of false classifications due to cross-reactions between similar types that can occur in tests based on hybridization. [345,346] Sanger sequencing has been the gold standard up until now, but it lacks the resolution produced by next-generation sequencing (NGS) methods.[347] To be able to achieve high sensitivity and efficiency, molecular detection of HPV DNA in clinical specimens using a single set of consensus general primers sacrifices specificity in favor of the recognition of all potentially relevant genotypes, including variations that are less common. The target DNA can be confirmed by DNA sequencing the inter-primer area of the PCR amplicon.[348,349] All 78 samples tested positive for HPV DNA in our study were thus subjected to sequencing in order to validate the PCR results. 42 of them had successful sequencing findings. Comparing known alleles in the NCBI's Gene Bank allowed the identification of three HPV genotypes: HPV 16, HPV 18, and HPV 56.

In our study, genotypes distribution in ICC cases reported that HPV 16(47.6%) and 18(16.6%) were the most prevalentt genotypes which matches with studies conducted in India and around the world. HPV 16/18 detected among 5% of women at any given time in the general population according to few Indian studies and and 82.7% of them were invasive cervical carcinomas showing the presence of HPV 16 or 18[351]

In our study, 20 (47.6%) of ICC cases had HPV 16 infection, 7 (16.6%) had HPV 18, and 4 (9.5%) had HPV 56 infection. In Kolkata, HPV 16 and HPV 18 infection rates are 59–74% and 2–13.9% respectively of all ICC cases.[352,353] South Indian reports revealed that 58–69% and 5–19.4%, of all ICC cases are caused by HPV 16 &18, respectively.[353,354] Similar research from Delhi found that HPV 16 &18 were responsible for 73.6 and 14.2% of cancer cervix cases, respectively.[355] Similar data from other study demonstrate that HPV type 16 (72–73.6%) is the most predominant genotype in cervical cancer, followed by HPV 18 (5–11.9%).[353,356] In Pakistan, ICC cases involving HPV 16 &18 range from 45.1 to 95% and 2 to 43.1%, respectively.[357-360] In contrast, HPV 16 and HPV 52 are more common in the China population than HPV 18. [361] The most predominant genotypes in ICC cases is similarly comparable with the genotypes reported elsewhere. [362,363]

The percentage of normal women with HPV DNA was 7.2% (7/97). This is steady with the reviewed literature. According to the International Agency for Research on Cancer's pooled meta-analysis, Asian women with no cervical cancer had an overall HPV prevalence of 9.6%. Additionally, it is comparable to the HPV prevalence we observed in research conducted in north India. [364,365]

HPV positive was discovered to be (6/17)35.20% in low-grade illness, and (3/42)7.1% linked to high-risk type HPV 16. Very varying rates of HPV-positive cases—between 29% and 100%—have been recorded in the category of LSIL.[366]

In CIN 2 & CIN 3, we found (3/4)75% and (1/2)50% cases to be HPV positive and linked to high-risk HPV 16 only.

In our study 15/235 samples i.e 6.38% of samples showed inflammatory smears on pap test whereas 3/15 i.e 20% of them showed HPV DNA positivity. None of them were positive for genotyping on sanger sequencing. According to a study done by Matha Manjari et al, 50% of the total samples showed inflammatory smears whereas 8% of them were HPV DNA positive[374]. In another study done in Odisha , out of 162/ 595 samples showed inflammatory smears, of which 88 samples showed HPV DNA positivity with HPV 16 (89.7%) as predominant genotype followed by HPV 18 (28.4%)[373]

The 6 commonest HPV types seen in group/study were as follows: Low grade squamous intraepithelial lesions: HPV 16; chronic cervicitis: HPV 16; CIN 2: HPV 16; CIN 3: HPV 16 and in Invasive cervical carcinoma: HPV 16, HPV 18, and HPV 56

In all histological groups, HPV-16 was by far the most prevalent single type. Other south Indian research have also revealed outcomes that are similar. [367,368] HPV-52 was shown to be the most prevalent infection overall in one study from a rural area in south India (29.4%), followed by HPV-16 (17.6%).[369]

Females who were healthy and had low-grade diseases likely to have low-risk genotypes. Even though HPV-6 is frequently reported in literature, our study didn't find any instances of low-risk HPV-6 or HPV-11.

HPV 56 was identified to be the 3rd most predominant genotype in our study. Out of 42 genotyping positives by sequencing 4 cases were reported with HPV 56 i.e 9.5% in ICC.

In our study, we assessed the relationship between High-Risk HPV genotypes and the emergence of premalignant & malignant cervical cytology in women. A significant rise in severity of disease was observed showing HPV as an important risk factor in disease progression (p value 0.03*). This is steady with a study done by shrivatsav et al.[370]

HPV vaccination of choice for this population:

Fortunately, since 2006, two HPV vaccinations have been made available: CervarixTM (GlaxoSmithKline Biologicals, Rixensart, Belgium) and Gardasil® (Merck & Co., Inc., Kenilworth, NJ, USA) for the primary prevention of HPV 16/18 and HPV6/11/16/18 associated cancer cases, respectively.[371,372] A nonavalent vaccination that recently came

out and protects against the genotypes HPV6/11/16/18/31/33/45/52/58 is another one to the list.

There is no HPV vaccine available in India's national immunization programs. Therefore, bestowing to our study findings we would recommend a cervarix (Bivalent) vaccine for the population in Kolar as HPV 16 was the predominant genotype which is followed by HPV 18. we also detected another genotype – HPV 56, a non-vaccine targeted genotype seen in only 4 study participants. Hence larger population-based studies are needed for the confirmation of the newer genotypes.

Conclusion & Summary

CONCLUSION & SUMMARY

In the Kolar region, the current study provides the first information on the genotype distribution of women with and without cervical cancer cases. This information will be useful in helping to create an effective approach to disease surveillance. Additionally, this study provides the region with baseline information for upcoming research.

This study identified a novel genotype (HPV 56) which were not included in any HPV vaccinations which would be helpful in devising new vaccine strategies in future.

Our findings and many epidemiological research indicate that adult females should undergo cervical screening to lower the incidence of cervical cancer linked to HPV.

Despite the availability of HPV vaccinations in the market, only the most common kinds of HPV can be prevented from infecting a person. We must still rely on early diagnosis of infection, through a variety of screening techniques, as cervical cancer may be brought on by other HPV genotypes as well.To identify patients who are at higher risk for developing precancerous lesions, molecular screening for HPV infection should also be used.

There is utterly no knowledge of HPV infection or HPV vaccination in our study. Therefore, these results may have significant repercussions for cervical cancer prevention strategies.

Limitations of the study:

This a cross-sectional study and no longitudinal data are available is the study's biggest drawback. As a result, no significant causal conclusions can be made because the patient's simple HPV infection does not assure that they will acquire cervical cancer in the future. Many times, the immune reaction will cause the body to naturally eliminate the HPV when the aberrant cervical cell returns to its normal state.

RECOMMENDATION

During our study, it was discovered that no single woman was aware of either cervical cancer screening (an important secondary prevention strategy) or the availability of HPV vaccines.

The observation held true even among the study's educated female participants. Furthermore, there are several myths and misconceptions about vaccination in the population, particularly in rural areas; this is partly because of failure of the PATH-initiated free HPV vaccination program in India in 2009.[370]

To be able to raise awareness among pre-adolescent girls, their parents, and guardians, it is essential to provide health education to all stake holders. Given the aforementioned scenario, the Indian government has two tasks to overcome first, HPV vaccination should be included to the regular national immunization schedule. Secondly, getting the general public to accept HPV vaccination.[371]

Having said that, developing laboratory capacity in HPV testing techniques and technologies is extremely essential as routine cervical cancer screening or clinical diagnosis and follow-up.

HPV genotyping is currently limited to research studies at reference labs. Through technology transfer, HPV testing must be implemented at every district hospital or tertiary care facility. In light of the launch of HPV vaccine, HPV testing is strongly recommended the need to assess how the immunization may affect diseases linked to HPV. Thus, it is advised to increase laboratory capacity for HPV testing.

Future studies should focus on the assessment of the HPV vaccines and the inclusion of predominant types at the regional level, and the long-term effects on the effect of HPV disease in real-world settings. India has a diverse population that is primarily made up of poor socioeconomic classes and individuals who experience widespread nutritional inadequacies; these factors may alter the degree of protection. Therefore, to track the effects of HPV vaccination, longitudinal cohort and surveillance studies that are connected to a network of HPV testing labs are required. Effective screening centers to be established in rural/remote areas.

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Annexure-I

LIST OF PUBLICATION

SL. No.	Paper Title	Authors	Journal	Indexation
1.	Cervical Screening and Assessment of Risk Factors For Human Papilloma Virus Infections Among Women Attending a Tertiary Care Hospital.	**Prathyusha.M[1]** Sheela S.R[2]	International Journal of Health Sciences (IJHS)2022 6(S1), 7041–7049.	Scopus

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Cervical screening and assessment of risk factors for Human papilloma virus infections among women attending a tertiary care hospital

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Abstract---Aim: To assess the presence of HPV among women who were visiting gynaecological out patient department for any cervix related reason. Material and Methods: Cervical scrapings were collected using Cytobrush. Pap smear results were collected from the pathology department and Histopathological confirmation for high grade lesions was also collected. PCR has been performed to look for the presence/absence of HPV DNA in all the samples by using MY09/11 primers. Results: Risk factors responsible for acquiring HPV infection were examined. Out of 60 Cytobrushes samples 32 samples had squamous intra epithelial lesions, out of which 16 were again Histopathologically confirmed as malignant cases. Chronic cervicitis were seen in 16 samples. Whereas, 8 were inflammatory smears and 20 were Negative for Intraepithelial Lesions. Only 16 HPV positives were confirmed by PCR. Conclusion: This study showed the incidence of HPV is very low in contrast to other studies in India.No awareness of HPV infections has been found in our region either. However, these findings could have important connection in the prevention of cervical cancer.

Keywords---HPV, HPV DNA, Kolar, Socio - Demographic, Cervical Carcinoma.

Introduction

Cervical carcinoma is one of the major health issues for women living in the region of India & every year almost 120,000 women suffer from this disease. Available data states India records 15.2% deaths of cervical carcinoma among the

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POSTER PRESENTATION

Title	Authors	Conference/ Webinar	Date
Cervical Screening and Assessment of Risk Factors for Human Papilloma Virus Infections Among Women Attending a Tertiary Care Hospital	Prathyusha.M[1] Sheela S. R[2]	International Webinar on microbiology	August 02-03;2021

ORAL PRESENTATION

Title	Authors	Conference/ Webinar	Date
Molecular genetic study to detect prevalence of High risk Human papilloma virus genotypes(16 and 18) in asymptomatic healthy subjects in southeastern karnataka state.	Prathyusha.M[1] Sheela S. R[2]	Global Webinar on Molecular and Genome Evolution	August 27-28,2021

Annexure-II

PATIENT INFORMATION SHEET

TITLE OF THE STUDY: Distribution of Human Papilloma Virus genotypes in women with or without cervical cancer in Kolar district.

Name of the Principal Investigator: **Prathyusha M**

Purpose of the study:

Human papillomavirus (HPV) is the main cause of Cervical cancer. The association between certain high-risk genotypes of HPV and cervical cancer is well established. Some genotypes of HPV are referred to as "low-risk" because they rarely cause lesions that develop into cancer. Both high-risk and low-risk types of HPV can cause the growth of abnormal cells, but only the high-risk types of HPV lead to cancer. Although HPV is essential in transformation of cervical epithelial cells, it is not sufficient, and a variety of cofactors and molecular events influence whether cervical cancer will develop. Early detection and treatment of precancerous lesions can prevent progression to cervical cancer. This study is an attempt to find the incidence of HPV DNA in premalignant & malignant conditions of the cervix.

Information on Procedures:

Each participant will be administered a standardized questionnaire on socio economic status , sexual behavior, reproductive history , contraceptive practices , history of STD, demographic profile , etc. cytobrush sample, Cervical tissue sample will be collected from you.

Storage of samples:

Genetic material prepared from your cervical biopsy sample may be stored for future research projects. In such an event, permission from the Ethics Committee will be obtained prior to us.

Duration:

The research takes place over 3 years in total

RISKS and benefits:

Participation in this study is purely voluntary. There is no risk involved in this study expect for the mild discomfort while taking biopsy sample. You will be given appropriate treatment for your disease as per the hospital norms but you will not be given any compensation for participation in the study. Confidentiality: All information that you provide will be considered confidential and no mention of your name or any other identifying information will appear on the samples or in any publication in connection with this study

Right to refuse or withdraw:

You do not have to take part in this research if you do not wish to do so. You may also stop participating in the research at any time you choose. It is your choice & all of your rights will still be respected for any further clarification or information you are free to contact the investigator.

M.Prathyusha

Mobile no: 9908550658

ANNEXURE - II

WRITTEN CONSENT FORM

Title of the study: "Distribution of Human Papilloma Virus Genotypes in women with or without cervical cancer in Kolar district."

Declaration by the participant:

- I understand that I remain free to withdraw from this study at any time.
- I have read or had read to me & understood the purpose of this study & the confidentiality of the information that will be collected & disclosed during the study.
- I have had the opportunity to ask my questions regarding the various aspects of this study & my questions have been answered to my satisfaction.
- I agree to participate in this study & authorize the collection & disclosure of my personal information as outlined in this consent form.

Participant's name & signature/thumb impression **Date**

Name and Signature of the witness **Date**

Signature of the principal investigator **Date**

ANNEXURE - II

PROFORMA OF DATA COLLECTION

Name: **Lab No:**

Sample: **Department:**

Age / Sex: **Education:**

Risk Factors

- Oral Contraceptives/ Steroids:
- Regular Screening: Yes/No
- Parity (number):
- Age at marriage:
- Nutrition:
- Socioeconomic Status:
- Smoking:
- Alcohol:
- Tobacco:
- Use of Sanitary Pads/ Cloth:
- Immunization if any:

Clinical History:

Histopathological Findings:

PCR Findings:

New Knowledge Generated

NEW KNOWLEDGE GENERATED

HPV56, the third most prevalent genotype, of this region can't be protected even by the new 9v vaccine for being genetically unrelated. The risk of development of invasive cervical carcinoma associated with these genotypes needs to be estimated