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Early cervical cytological changes in human papilloma virus (HPV) positive cases

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Abstract
Introduction and objective: Many strains of Papillomavirus are well known to be associated with cervical neoplasia, and thus it is expected that some precancerous cervical cytological changes may be present in Papillomavirus-positive cases where florid precancerous dysplastic changes may be completely absent.
Methodology: In this pilot study, we had selected five Papilloma virus DNA positive cases by Real time PCR; where there was no classical cytological precancerous changes were observed and compared their morphological parameters with five Papilloma virus DNA negative cases, to find out some minor morphometric parameters that may be significantly different in positive cases.
Results: Our findings indicated that some cytological parameters are constantly present in all HPV DNA positive cases. Those including irregular nuclear membranes, anisochromasia, vesiculation, and significantly fewer degenerative cells. Although not so significant, some other parameters may also be associated with positive cases: Increased cellular thickness, mildly decreased nuclear: cytoplasmic ratio, and increased cell population.
Conclusion: Thus, these initial cytological changes may be of some value in identification of the abnormal activities of the Papillomavirus in early cervical infections.

Keywords: Human Papilloma Virus (HPV), High-risk genotypes, PAP smear, Real-time PCR, Cervical Cancer screening

Introduction
The Human Papilloma Virus (HPV) is a group of viruses that are members of the Papillomaviridae family, which comprises 52–55 nm small, epitheliotropic, unenveloped, double-stranded DNA viruses that infect the mucosa and cutaneous epithelia in humans and trigger cell proliferation [1]. HPVs infect the genital tract and are strongly linked to cervical cancer in women. The expression of viral oncogenic proteins E6 and E7 targets several negative regulators of the cell cycle, such as p105Rb and p53, that hinder the steady establishment of viral episomes and the transition of cells into S-phase [7]. The E6 protein suppresses the p53-mediated effects that regulate cell proliferation and apoptosis, thereby promoting cell expansion. E7 binds to pRb and acts as a functional inhibitor of pRb and related proteins, such as EF2, which results in the expression of transcriptionally growth-related proteins [2].
Cervical cancer is the second-most common malignancy among women worldwide. According to Globocan 2020, there were 604,100 new cases of cervical cancer detected globally in 2020, and 341,831 deaths were attributed to this malignancy. In 2020, cervical cancer was responsible for 9.4% of all cancers and 18.3% (123,907) of new cases in India [3]. The connection between HPV and cervical invasive cancers is crucial, and the number of such cases is soaring. Until now, more than 100 distinct types of HPV have been identified, 40 of which may involve urogenital tract lesions. The virus subtypes can be broadly classified into those that infect stratified squamous epithelium and those that infect mucosal epithelium. The mucosal types can be further divided into low-risk and high-risk varieties. The low-risk varieties have been linked to the development of genital warts. The most frequently identified low-risk variants include 6, 11, 40, 42, 43, 44, 54, 61, 72, and 81 [11].
The high-risk categories are primarily associated with the occurrence of intraepithelial neoplasia, and they include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 69, and 82.

These subtypes are considered carcinogenic; they are present in 99% of cervical cancers, with types 16 and 18 present in 70% of them [2]. Long-term HPV infection is the cause of the majority of precancerous lesions and cervical cancers. They usually originate at the junction of the ectocervix and endocervix. In the pathway leading to cervical neoplasia in women, persistent infection with oncogenic types of HPV followed by the incorporation of HPV DNA into the cellular genome is a crucial precursor. The emergence of cancers of the uterine cervix, which have been established as high-risk HPV inoculations, is imperative [14]. It is estimated that 99.7% of cervical cancers are caused by high-risk HPV infections [13].

An analysis by the Indian Cancer Society shows that there are no signs or symptoms until an advanced stage of infection for women with precancers and early lesions. Still, some of them are expected to show certain common manifestations, which include irregular inter-menstrual bleeding (between cycles of menstruation), abnormal vaginal bleeding after coitus, odorous, bloody vaginal discharge, pelvic examination bleeding and pain, loss of appetite and weight, swollen legs, etc. [4]. The condition of HPV infection is usually transient, and most individuals successfully eradicate the virus from their bodies using their effective immune systems after 5.1-15.4 months. Furthermore, HPV persistence and, consequently, cervical cancer (CC) is influenced by other variables such as age, high parity, smoking, long-term use of contraceptives, sexual conduct, and co-infection with other sexually transmitted infectious agents [6].

Screening for cervical cancer aims to identify curable precancers, identified histopathologically as CIN3 and AIS, to minimize cancer mortality and morbidity, along with its complications. In India, the percentage of women who have ever undergone cervical cancer screening is 1.9% (2.2% urban and 1.7% rural) [9]. The supercilious sensitivity of HPV testing for the detection of precancers provide greater assurance against cancer when testing is negative, which is why screening strategies are shifting from cytology tests to oncogenic HPV DNA testing. Despite the increasing use of HPV testing, remarkable aspects of its use remain unresolved, including the ideal management of positive results [5]. HPV DNA testing helps to identify the correct genotype of HPV, followed by a cytological Papanicolaou test (known as the PAP test or PAP smear) or co-testing. The screening process mainly focuses on populations of women belonging to the sexually active group, encompassing the age range of 21–65 years.

According to the Guidelines for the Prevention and Treatment of Opportunistic Infections in Adults and Adolescents with HPV, possible PAP test results include the following:

- Normal (negative for intraepithelial lesion or malignancy).
- LSIL (low-grade squamous intraepithelial lesion) or CIN1 (cervical intraepithelial neoplasia grade 1).
- HSIL (high-grade squamous intraepithelial lesion) or CIN2, 3 (cervical intraepithelial neoplasia grade 2, 3).
- ASC-US (atypical squamous cells of uncertain significance).
- ASC-H (atypical squamous cells that cannot rule out a high-grade lesion).
- AGC (atypical glandular cells).

The most cost-effective, straightforward method for detecting early cervical cancer is a visual inspection with acetic acid (VIA), which mainly highlights the white lesions on the mouth of the cervix, if any, and thus differs from the usual conditions of cervical cytological changes. However, for the general population of women, the primary screening test suggested is HPV DNA detection with regular testing every 5 years, co-testing, or a cytology-based PAP smear with regular testing every 3 years. Furthermore, women with abnormal cytology reports are advised to undergo a Colposcopy test to examine the cervix thoroughly too efficiently detect advanced or high-grade CIN 2/3 and dysplasia [8].

The primary prevention stage of HPV vaccines for girls aged 9-13 years before the onset of sexual activity can reduce the risk of HPV infection, or CC. There are many other practices, like following safer sexual practices, living a healthy lifestyle, and maintaining proper personal and menstrual hygiene that can be a way to avoid any reproductive tract infections (RTIs). Secondary prevention stages such as CC screening, HPV genotyping, and treatment of pre-cancerous lesions are common for people with high-risk behaviours. Tertiary preventive measures include women being diagnosed with proper and advanced cancer treatments that are cost-effective as well as curative for early-stage cancer detection and treatment. Follow-up screening should be done at regular intervals according to the doctor's recommendations [10].

In this small population-based pilot study, we conducted a survey among the females presented to Gynaecology OPD at Peerless Hospitex Hospital and Research Centre Limited, Kolkata, West Bengal, India, to assess the prevalence of HPV infection associated with cervical cancer and cervical cytological abnormalities. All participants in the survey underwent a basic primary cervical cytology screening using a cervical PAP smear and HPV DNA testing, and any cytological alterations were further diagnosed by performing a colposcopy after a year. Our study aimed to compare the HPV-infected and non-HPV-infected cervical changes and the HPV genotype prevalence, employing cervical brushing fluid collected from women living in suburban areas of the north, south, and east of Kolkata [12].

Materials and Methods

A. Study population

Our study was conducted over last one year at Peerless Hospitex Hospital and Research Centre Limited, Kolkata, India. The individuals who were interested in participating in the survey were selected, and their written consent was taken. Through various interviews and counselling processes, it was revealed that a few of them were associated with high-risk behaviours like consumption of alcohol and smoking, multiple partners, and prolonged use of contraceptives. 20–60 years age group individuals were included for the study. Some of the individuals showed symptoms like irregular inter-menstrual bleeding, abnormal bloody vaginal discharge, and post-coital bleeding. They were educated about the risks of cervical cancer, and awareness of cervical screening test was spread amongst them. Rejection criteria included women with a history of hysterectomy, previous history of invasive cancers, HIV or any other active infections during the past 5 years, or unable to bear follow-up screening. They all underwent a thorough pelvic examination, and their cervical samples were collected for PAP testing and HPV DNA genotyping by gynaecologists [12]. Details of the procedure are given in

Fig.1. In this pilot study, we have selected five confirmed PCR positive HPV cases and with no classical cytological pre-cancerous change and compared their morphological details with five confirmed PCR negative HPV cases, and thereby try to find out some minor morphometric cytological differences between them.

B. Sample collection and preparation

The gynaecological oncologists performed a pelvic examination, and with the help of a cytobrush, tissue samples were collected from the junction of the cervix and uterus. The cytobrush was transferred into transportation medium vials and stored in HPV sample collection containers. Liquid-based cytology, which includes cervical smears or PAP smears, was made on slides following the procedures of Papanicolaou's stain [12]. The pap stain principle is to differentiate between basophilic and acidophilic cell components and to acquire a comprehensive chromatin pattern. The cytological slides were observed under a high-power microscope to detect any abnormalities or alterations in the cervix, and the cytology results were analysed following standard protocols by pathologists at Peerless Hospitex Hospital according to the Bethesda 2001 system [12].

C. Diagnostic procedures

The cytological specimens were categorized according to the Bethesda 2001 System into atypical squamous cells of undetermined significance (ASC-US), low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), atypical glandular cells (AGC), and cancer. All women with PCR positive for HPV high risk genotype(s) test results or abnormal cytology reports were advised to undergo colposcopy, endocervical curettage (ECC), and cervical biopsy. For individuals without an abnormal report, a random biopsy was done. The ECC and biopsy reports were interpreted by pathologists at Peerless Hospitex Hospital [12].

D. HPV DNA genotyping

The test of HPV genotyping was carried out using the TRUPCR® HPV HIGH-RISK GENOTYPING KIT (Real Time PCR-Based Detection and Genotyping of 14 High-Risk HPV Genotypes, 3B Blackbio Biotech India Ltd., India) according to the manufacturing company's protocols. This kit was intended for the detection and genotyping of 14 HPV high- and intermediate-risk types (16/18/31/33/35/39/45/51/52/56/58/59/66/68). It uses a TaqMan Probe-Based Real-Time PCR Assay based on E6 or E7 oncoproteins, which mediate cervical cancer development. It consists of an endogenous internal control DNA, which allowed for the elimination of untrustworthy results. Furthermore, it is based on the idea of oligonucleotide hydrolysis, which enables greater specificity and sensitivity of the E6 or E7 region by primer and probes specific for the detection and genotyping of 14 High-Risk HPV Genotypes. Extracted DNA from cervical cells in liquid media or a swab was used as a sample for this test kit.

E. Statistical investigation

Age, cytological findings, and the circulation and frequencies of different HPV strains were all determined using descriptive statistics.

Results

A. Participation rates

In the hospital screening programme, 150 women (37.5%) participated among 400 women aged between 20–60 years. 50 of these women were excluded, leaving 100 women to participate in this study. The most common grounds for exclusion were living outside our survey location, denial to participate, or failure to appear for appointments. Among those 100 women, we selected a team of 10 women for this research. In this pilot study of ten cases, all women were between 25–57 years of age. General findings of all these subjects are given in Table 2. Some risk behaviours like smoking, alcoholism, use of contraceptives and high parity were present in HPV DNA positive cases.

B. Minor cervical cytological abnormalities and HPV infection

Routine cytological report details of all these patients were within normal limits. When we screened minor morphometric details of the cytological smears of all the patients, we have noted some parameters which were constantly present in all HPV PCR positive cases (Fig.2-Fig.6). Those parameters were irregular nuclear membranes, anisochromasia, vesiculation, and fewer degenerative cells in the smears. We also observed that some morphological changes, although not constantly present but seen in some smears of positive cases. They were increased cellular thickness, mildly decreased nuclear: cytoplasmic ratio, and increased cell population. Details of which are given in Table 1.

Discussion

A population-based study is done to examine the cytology of the cervical cavity results, HPV genotyping PCR, and histopathological diagnosis for all aberrant categories. When compared to hospital-based research, this study has less selection bias. The relationship between screening results and histological diagnosis reveals that screening tests are effective [12]. There has been no prior population-centred investigations study using liquid-based cytology, HPV High-risk Genotyping PCR (using TRUPCR®) and histological diagnosis were done to our information.

The cytological observations mentioned in Table 1 are due to alterations of cell cycles at molecular levels which includes gene regulation and protein expression. The HPV replication cycle is thought to begin with virus entrance into cells found in the germinativum stratum (basal layer) of the epidermis. HPV infection of the superficial layer is likely to necessitate modest epidermal damage or microtrauma. 6-Integrin has been postulated as an epidermal cell receptor for HPV-6, although it is not required for HPV-11 or HPV-33 adherence [15, 16, 17, 18]. Like many other viruses, HPV-16 and HPV-33 connect to host cells via heparan sulphate on the cell surface [16, 18]. The virus replicates itself as a low-copy-number episome in the basal layers by exploiting the host genome replication machinery to synthesise its DNA around once each cell cycle [18, 19, 20]. By binding and deactivating tumour inhibitor proteins, cell cyclins, and cyclin-dependent kinases, the E6 and E7 gene products disrupt the host cell growth cycle [18, 21]. During a successful HPV infection, the E6 and E7 gene products disrupt cell growth-regulatory mechanisms and change the molecular milieu to enable viral replication in a terminally divided cell that has exited the cell cycle [18, 21]. In positive cases of HPV, the E7 proteins which have low binding affinity for pRB gene binds with cellular proteins like cyclin E which stimulates excessive cellular DNA synthesis and cell proliferation. Following that, the E5 gene transcript

increases mitogen-activated protein kinase activity, boosting cell responses to differentiation and growth stimuli. As a result, the host cell continues to proliferate and differentiates slowly. Thus, the overall cellularity of HPV PCR positive cases is revealed to be much higher than the PCR negative cases of HPV. The count of degenerated cells is sharply unnoticed in positive cases due to abnormal cell proliferation and absence of apoptosis as compared to negatives where the number of degenerated cells is spotted quite often [18].

It has been known that the nucleus assembles viral particles, and full virions are discharged as the cornified layers of the epithelium shed. The E4 gene transcript is involved in papilloma virus particle development and discharge. It is unlikely that the procedure is cytolytic. During the genome replication process, viral DNA spreads across the epithelium, but complete virions are only present in the higher layers of the tissue. Hence if the viral content inside the cells is very high, it is likely that it influences the epithelial cell thickness to thin or thick [18].

The nuclear membrane irregularities of HPV infected epithelial cells of cervix are prominently observed in all HPV PCR positive cases taken into account. Koilocytosis which includes dysplastic nucleus, abnormal nuclear enlargement, coarse chromatin, along with nuclear membrane changes can be an indication of early pre-cancerous stages or low-grade squamous intraepithelial lesions (LSIL) [22].

In this study, we observed genotypes 45, 66, 51, and 56 other than the common genotype 16. The high-risk HPV-45 was first described in 1987 [23] and it is a member of the same phylogenetic species as HPV18 [24, 25]. HPV type 45, has been implicated as the third most common genotype in invasive cervical cancer [26], varies between 3-5% of cervical cancers worldwide [27] and more commonly it is associated with adenocarcinoma [28, 29]. HPV genetic variants include A and B lineages and five sublineages [30]. Among them, the B2 sublineage is associated with a higher risk of cervical cancer [31]. HPV-66, a α-papillomavirus is rarely found [32]. However, it is associated with cervical intra-epithelial neoplasia 1 [33, 34]. In one study HPV-51 was found in 10.8% unvaccinated cases [35]. However, it is not common in cervical cancers and this finding was unexpected [36]. HPV-56 was first described in 1989 and it accounts for less than 1% cervical cancer cases globally [37] although in Asia it is found more where it may be as high as 6% [38].

These initial morphometric changes may be of some value in interpreting the abnormal activities of the Human papilloma virus in early cervical infections.

Table 1: Minor differences present in cervical smears prepared from HPV PCR positive and negative cases in patients without classical pre-cancerous changes.

HPV high risk genotype(s) detected by real time PCR	Cell thickness	Cellularity	Deg. cells	Irregular nuclear membrane	Aniso-chromasia	Vesiculation	N: C ratio
45	Thin	1+	-	2+	2+	2+	8
16	Thick	3+	-	2+	2+	2+	8
66	Thick	2+	-	2+	2+	2+	6
51	Thick	4+	-	2+	2+	2+	6
56	Thin	3+	-	2+	2+	2+	8
HPV -	Thin	2+	1:2	-	-	-	8
HPV -	Thick	2+	1:5	-	-	-	8
HPV -	Thin	1+	1:10	-	-	-	8
HPV -	Thin	2+	1:10	-	-	-	7
HPV -	Thick	2+	1:5	-	-	-	7

*Deg. Cells: Degenerated cells; N: C ratio: Nuclear: Cytoplasmic ratio.

Table 2: General features of the HPV DNA positive and HPV DNA negative cases

Name	Age	Clinical history	H/O risk behaviour	HPV genotype detected	Ancillary Findings
RBR	40	Irregular inter-menstrual bleeding	Smoking and alcoholism	HPV- 45	Superficial and intermediate squamous cells with scattered endocervical cells & plenty of Polymorphs.
RM	30	Irregular inter-menstrual bleeding	Nil	HPV- 16	Superficial & Intermediate squamous cells, Inflammation and repair ++, Lacto bacillary flora.
AD	38	Abnormal vaginal bleeding	Nil	HPV- 66	Superficial & Intermediate squamous cells, Inflammation and repair ++, Lacto bacillary flora.
KM	25	Abnormal vaginal bleeding	Use of contraceptives	HPV- 51	Superficial & Intermediate squamous cells, Inflammation and repair +, a Lacto bacillary flora.
RK	32	Bloody vaginal discharge	High parity	HPV- 56	Superficial and Intermediate squamous cells with few metaplastic cells and background Neutrophils
SJ	47	Vaginal discharge and bleeding	Nil	HPV DNA Ngative	Superficial & Intermediate squamous cells, Inflammation and repair ++++, Lacto bacillary flora.
MS	37	Post-coital bleeding for 2 years	Nil	HPV DNA Ngative	Superficial & Intermediate squamous cells, Inflammation and repair +++, Lacto bacillary flora.
SP	42	Vaginal discharge and bleeding	Nil	HPV DNA Ngative	Superficial & Intermediate Squamous cells, Inflammation and repair ++++, Lacto bacillary flora.
RG	52	Vaginal discharge and bleeding.	Nil	HPV DNA Ngative	Superficial & Intermediate squamous cells, Inflammation and repair +++, Lacto bacillary flora.
AR	57	Vaginal discharge and bleeding.	Nil	HPV DNA Ngative	Superficial & Intermediate squamous cells, Inflammation and repair +, Cocco-bacillary flora.

Specimen: Cervical Smear-LBC (Cervical brushing fluid); Specimen adequacy: Adequate, Transformation zone cells present; in general categorization all were negative for intraepithelial lesion or malignancy. There was no history of HPV vaccination. All superficial and intermediate squamous cells were benign in nature.

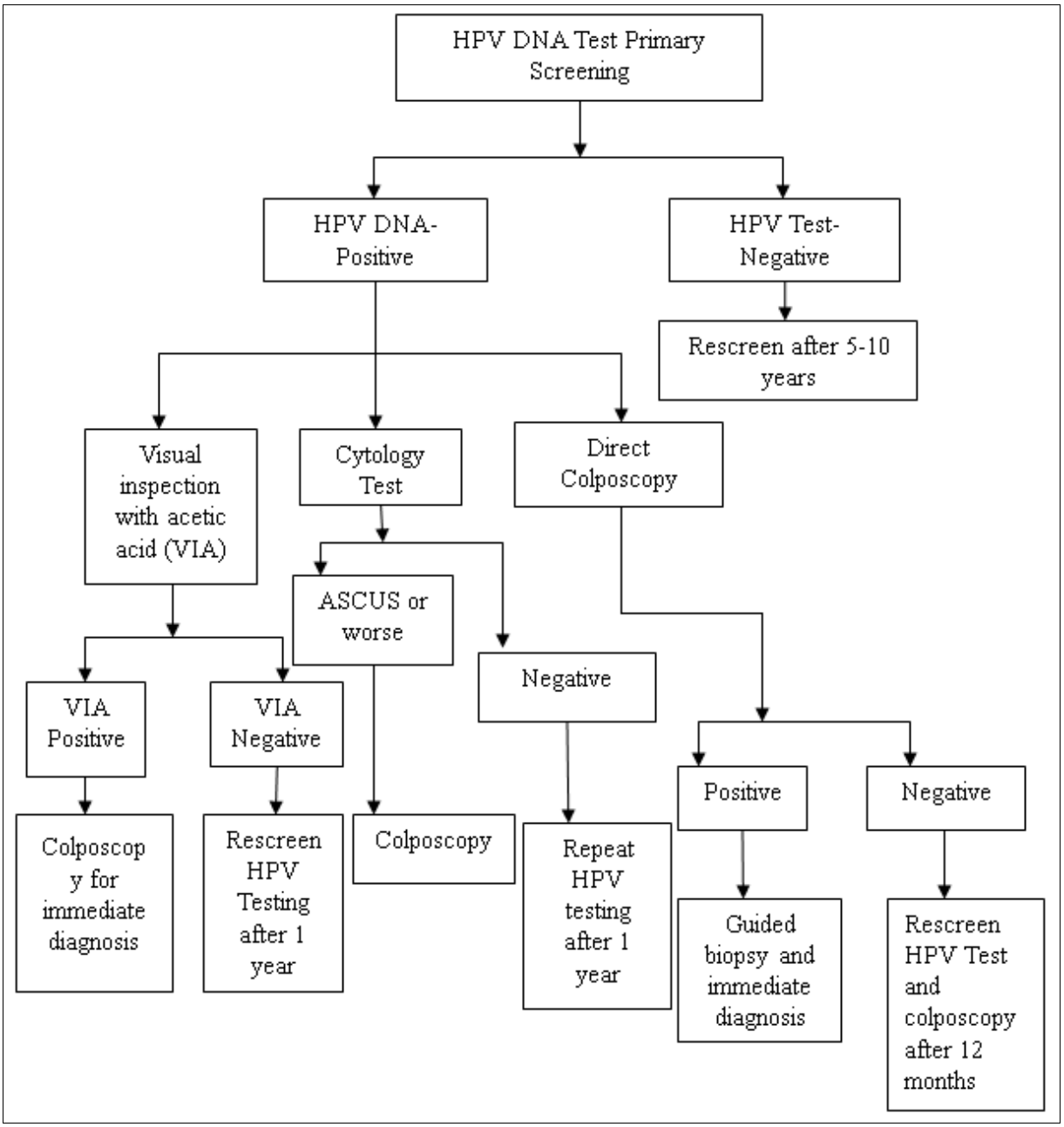

Fig 1: Cervical cancer screening by HPV DNA Test and Cytology Test

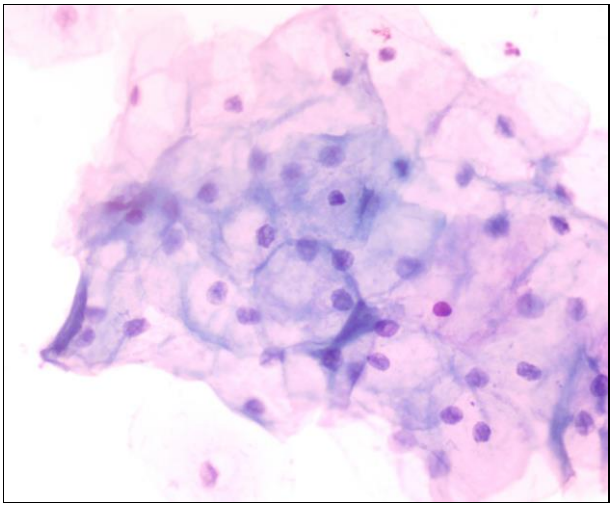

Fig 2: Irregular nuclear membrane in many cells

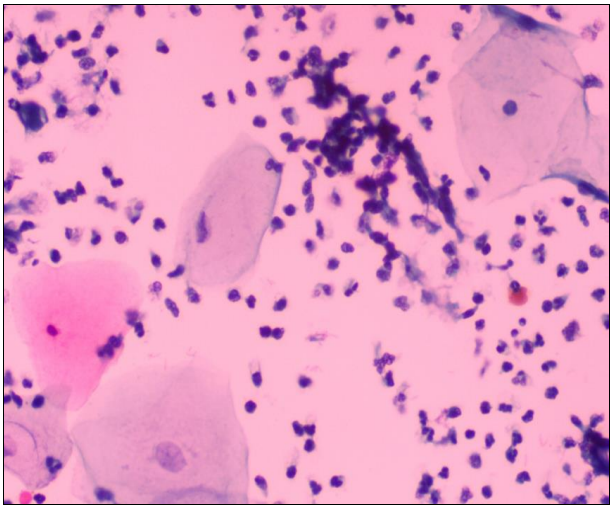

Fig 3: Showing distinct vesiculation in some areas

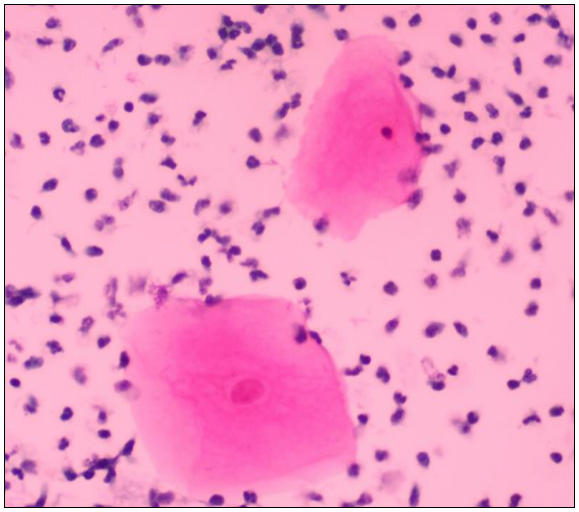

Fig 4: Increased nuclear size in some cells.

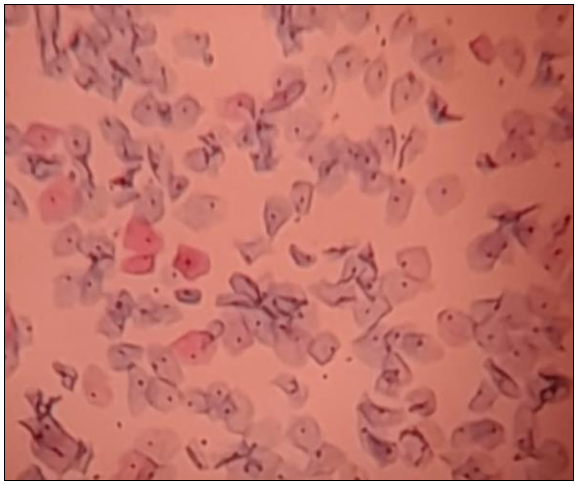

Fig 5: Increased cellularity and cell thickness with few degenerated cells in HPV positive cases

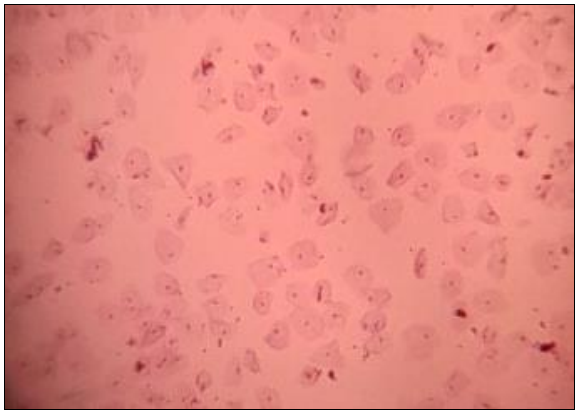

Fig 6: Less cellularity and cell thickness with many degenerated cells in HPV negative cases

Consent

All the participants willingly gave their written consent information for the study as per Institutional Ethical Committee guidelines.

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Potential Conflict of Interest

There was no conflict of interest of any Author.

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