Immunohistochemical Detection of High Risk Human Papilloma Virus -16 in Cervical Smears- A Hospital Based Study

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Abstract
Cancer of the uterine cervix is one of the most common cancers among women worldwide, the highest incidence being observed among developing countries. The medical screening method commonly known as the Pap smear or the Papanicolaou test used for cervical cancer screening has many disadvantages like interobserver and intraobserver variability, low predictive value and a high false negative rate. Due to these limitations, there is a need for more objective parameters for accurate diagnosis of cervical intraepithelial neoplasm. The aims of the study were to find out the prevalence of high risk HPV-16 in cervical smears by immunohistochemical method and to identify the risk factors associated with HPV-16 infection. 173 patients were screened in the study for HPV infection by using immunohistochemical marker Anti HPV-16. A total of 82 patients showed positivity with Anti HPV-16.A prevalence of 47.40% for HPV infection was obtained. The risk factors known to be associated with Human Papilloma Virus (HPV) infection analysed were age of the patient, age of marriage, parity and history of intake of Oral contraceptive pills. No statistically significant association was found between the above risk factors and HPV infection.
Keywords: *Cervical cancer, HPV infection, Immunohistochemistry, Anti HPV 16, risk factors.*

Introduction

Cancer of the uterine cervix is one of the most common cancers among women worldwide, the highest incidence being observed among developing countries. In India, an estimated 1,32,082 new cases are reported annually, whereas in Kerala, cancer of the cervix is the second most common malignancy in females constituting 24.49% of all cancers in females [1]. It is encouraging to note that the incidence of cervical cancer is declining due to screening by cervical smear examination. This medical screening method commonly known as the Pap smear or the Papanicolaou test however has many disadvantages like interobserver and intraobserver variability, low predictive value and a high false negative rate. Due to these limitations, there is a need for more objective parameters for accurate diagnosis of cervical intraepithelial neoplasm. Persistent infection with Human Papilloma Virus (HPV) is the principle cause of cervical cancer implicated in greater than 99% of cervical cancers worldwide.HPV 16 is the type most frequently found in squamous cell carcinoma of the cervix. Therefore testing for high risk HPV 16 has now been incorporated into the management of

abnormal Pap smear results. This study is an initial step in evaluating all the patients attending the Outpatient Gynaecology Dept. of a tertiary care centre by detecting presence of HPV infection. Although there are advanced methods like Polymerase Chain Reaction (PCR), In Situ Hybridisation (ISH) etc. immunohistochemistry (IHC) has been attempted because of its ease and cost effectiveness. Despite the high incidence of cervical cancer in India, large scale population based studies on the HPV prevalence are few. In view of the clinical trials for HPV vaccine taking place in India, it is of utmost importance to understand the prevalence of HPV.

Materials and Methods

The study was a descriptive hospital based cross sectional study conducted in a tertiary care centre. All the patients attending the Gynaecology outpatient department, who are subjected to cervical smear test during the period of June 2011 to November 2012 were included in the study. A total of 173 patients were analysed. Routine cervical smears were collected from patients attending Gynaecology outpatient department subsequently stained by Papanicolaou stain. An additional cervical smear using Ayre's spatula was also collected and the material was put in a Koplin's jar containing 80% Isopropyl Alcohol for cell block preparation. Details as per the proforma were collected from the patient at the time of sample collection. Samples for cell block preparation were later collected in a test tube, centrifuged at 1500 rpm for 10 min. After decanting the supernatant, equal amount of AAF solution (Acetic Acid-1 ml,95% Ethyl Alcohol-16 ml,10% Formalin-2ml) was added to the sediment and was allowed to stand overnight. The cell button thus obtained was dislodged and subjected to histopathological processing and paraffin embedded.

Of the cell blocks thus obtained, 5 micrometre sections were obtained and immunohistochemical staining by Anti HPV 16 (Polymer HRP IHC detection system, Peroxidase antiperoxidase method) was done.

Papanicolaou stained smears of all the samples were screened and were categorized as per Bethesda grading system for cervical smears. Nuclear and/or cytoplasmic positivity in the squamous cells were taken as positive result.

The results thus obtained, and the patient details were entered in Microsoft Excel. The prevalence of Human Papilloma Virus (HPV)-16 and the risk factors associated with it were thus analysed using SPSS software.

Result

During the study period from June 2011 to November 2012, a total of 270 samples were collected out of which cell blocks with adequate cellularity could be prepared in 173 samples. Complaints of patients were also recorded and analyzed during the study. Discharge, itching, increased frequency of micturation, dyspareunia were some of the complaints reported by the patients.(Table 1). In some patients, cervical smear was taken as part of routine pre op check up .The most common complaints patients presented with were itching and discharge.

Table: 1 Clinical Presentation

Symptoms	No. of Women	%
Discharge	53	30.63%
Itching	58	33.52%
Increased Frequency of Micturation	18	10.40%
Routine Pre op check up	40	23.12%
Dyspareunia	4	2.3%
	173	**47.40**

Of the total 173 patients screened HPV positivity was observed in 82 cases(47.4%) and 91 cases (52.60) cases were Anti HPV 16 negative (Ref table 2)

Table 2 Prevalence of HPV - 16

Total	HPV 16+	HPV 16-
173	82(47.4%)	91(52.6%)

The age distribution and the presence of HPV infection in each age group were as follows. (Refer Table 3)

Table 3: Age distribution of HPV-16 infection

Age	No. of Women	No. of cases with HPV-16 Positivity	%
<30	21	13	61.90
31-40	63	40	63.49
41-50	54	20	37.04
51-60	22	5	22.73
>60	13	4	30.77
Total	**173**	**82**	**47.40**

As observed in the above table and in figure 3,the total prevalence of HPV-16 infection was found to be highest in the 31-40 years age group closely followed by the <30 years age group. As age increases, it was observed that the prevalence of HPV was greatly reduced to nearly half of that in the lower age ranges.

Age at Marriage

As observed in the table, of the total no of patients selected for the study, maximum number of the subjects (108) were married between 22-24 years of age. (Table 4)

But higher prevalence of high risk HPV16 infection was observed in subjects who got married at the age of less than 18 years although the total number in this group was less statistically significant.

There was no statistically significant correlation between the age of marriage and prevalence of HPV-16 infection (Students t test,p value=0.249)

Table 4: Age at marriage and HPV-16 positivity

Age of Marriage	No. of Women	No. of cases with HPV positivity	%
<18	8	6	75.00
19-21	53	25	47.17
22-24	108	49	45.37
25-27	4	2	50.00
Total	**173**	**82**	**47.40**

Parity

Among the total sample of 173 females selected for the study, 6 women were nulliparous and 167 were multiparous. HPV positivity was seen in 33.33% of nulliparous women while it ranged from 45.65% to 60% in multiparous women according to parity. (Table 5) There was no statistically significant correlation between the parity and prevalence of HPV-16 infection (Chi square test,p value=0.89)

Table 5: Parity and HPV -16 positivity

Parity	No. of Women	No. of women with HPV positive	%
0	6	2	33.33
1	15	9	60.00
2	102	48	47.06
3	46	21	45.65
>3	4	2	50.00
Total	**173**	**82**	**47.40**

History of Intake of Oral Contraceptive pills

History of intake of oral contraceptive was also taken into consideration for the study. Oral contraceptive pill (OCP) intake was noted only in a minority of patients. Out of the total sample of 173 females, only 13 women reported of intake of oral contraceptive pill of which 7(53.85%) showed HPV positivity (Table 6)

p value was observed to be 0.926 (Chi square test) which indicated that it was not statistically significant.

Table 6: History of OC pill intake

OC Pill Usage	No. of Women	No. of women with HPV 16 positive	%
-	160	75	46.88
+	13	7	53.85
Total	**173**	**82**	**47.40**

There was no statistically significant correlation between OCP intake and HPV -16 positivity.(Chi square test,p=0.926)

Diagnosis on Pap Smear

Papanicolaou stained cervical smears were screened and classified on the basis of Bethesda system (Ref Table 7).

Table7: Diagnosis on Pap Smear and HPV -16 positivity

Diagnosis on PAP Smear	No. of Women	No. of women with HPV positive	%
Inflammatory Smear	167	77	46.11
Atypical squamous cell of unknown significance (ASCUS)	2	1	50.00
Low-grade squamous intra-epithelial lesion (LSIL)	1	1	100.00
Squamous cell carcinoma	2	2	100.00
Atypical glandular cell – NOS	1	1	100.00
Total	**173**	**82**	**47.40**

Out of 173 smears, vast majority (167) showed only inflammation of which 77 (46.11) women were found to be HPV positive. In the remaining 6 cases,2 revealed a diagnosis of atypical Squamous cell of unknown significance (ASCUS), 1 showed low grade Squamous intraepithelial lesion (LSIL), 2 showed Squamous cell carcinoma and 1 showed atypical glandular cell-NOS. Almost all of these were found to be HPV positive

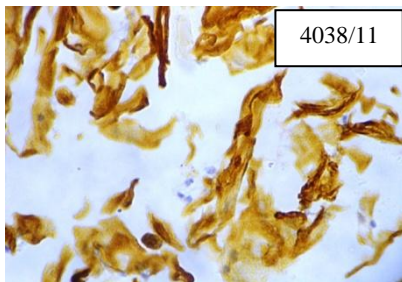**Figure 1:** Anti HPV 16(IHC) stained smear showing nuclear and diffuse cytoplasmic positivity (400X)

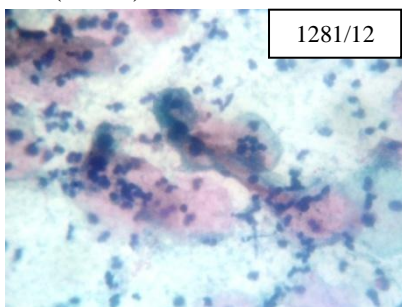**Figure 2:** Pap stained smear showing severe Inflammation (400X)

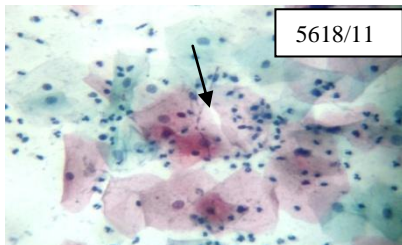**Figure 3** Pap stained smear showing pseudohyphae of Candida species (400X)
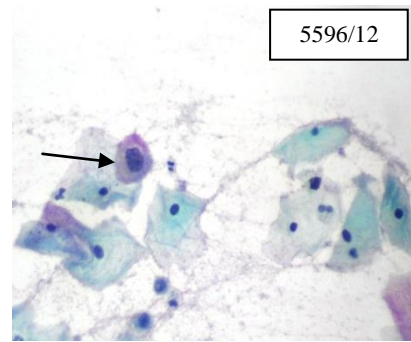

Figure 4 Pap stained smear: ASCUS (400X)
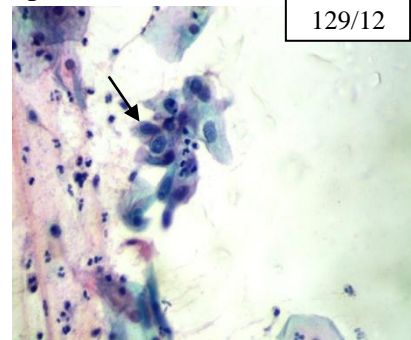

Figure 5 Pap stained smear: LSIL (400X)

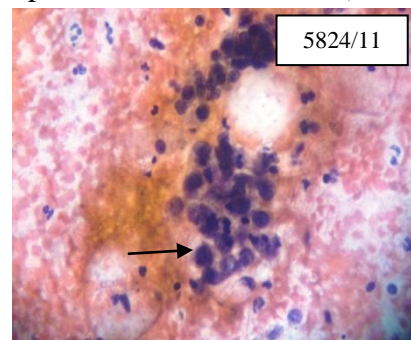**Figure 6** Pap stained smear: AGUS (400X)

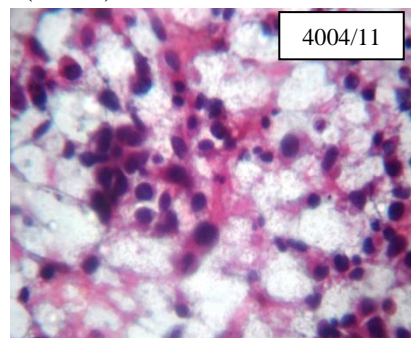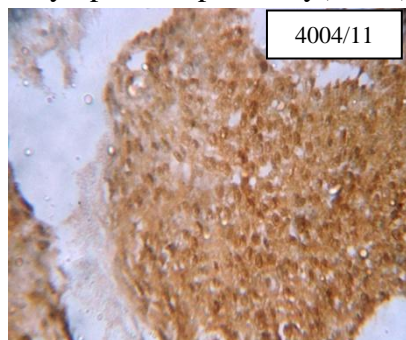**Figure 7** Pap stained smear: Squamous Cell Carcinoma (400X)

Figure 8 Anti HPV 16(IHC) stained smear: Nuclear and cytoplasmic positivity(400X)

Discussion

Clinical Presentation

Majority of the patients infected with HPV (50.45%) presented with discharge per vaginum and itching. This may be attributed to poor hygiene which has been found to be associated with a higher prevalence of HPV in women as in a study conducted by Franceschi et al[2]

Prevalence of HPV -16

Of the 173 patients screened, 82 tested positive for HPV and 91 tested negative. A prevalence of 47.4% was obtained in the total no. of cases screened. Diffuse cytoplasmic positivity was obtained in 51 cases and diffuse cytoplasmic and nuclear positivity was obtained in 30 cases.

Comparison with other studies is provided in Table 8

S.No	Study	HPV prevalence
1	Aggarwal R.Gupta et al (2006) [3]	36.8%
2	Thomas et al (2004) [4]	24.8%
3	Ekalaksananan et al(2001) [5]	21%
4	Present Study	47.4%

As observed in the above table, a wide variation in the total prevalence of HPV-16 infection is noted in the various studies conducted. This can be attributed to the number of patients screened in each study, the type of case group(high risk or low risk group),the cultural limitations and the method used in each study.

There is a relatively high prevalence of HPV 16 in the present study. Franceschi et al [2] as recognized low socioeconomic status as a risk factor for HPV 16 infection and cervical carcinoma as well. A predominantly non vegetarian diet has also been considered to be a risk factor for HPV infection.

Serum levels of beta carotene, beta cryptoxantin, lutein, alpha and gamma tocopherol which act as protective factors against HPV infection are low in people consuming a predominantly non vegetarian diet.

Low socio economic status in most of the patients and a predominantly non vegetarian diet in most of the subjects screened could be contributing factors for the high prevalence rate of HPV infection in the present study. In the study by Mrudula Soma and Suhasini Kamaraj[6], cytoplasmic and nuclear positivity was seen in cells of the basal and middle layer and nuclear positivity alone was seen in the cells of the basal layer. The difference in nuclear and cytoplasmic positivity in the present study could be possibly due to the different cell types.

Age

The most common age group affected in our study is between 31-40 years. This is comparable to study by A.Pavani et al[7]. In our study the youngest patient screened was 24 years of age and the oldest person screened was 72 years of age.

Table 9-Age groups affected

S.no	Study	Most common age group
1	A.Pavani et al[7]	30-35
2	Dunne EF et al[8]	20-24
3	Aggarwal et al[3]	<30
4	Present Study	31-40

It can be noted from the above table that according to majority of the studies, HPV infection is most prevalent in the younger age group. Most HPV infections are transient in nature, with the viral infection resolving itself within a 12 month period. This may be attributed to the lower prevalence seen in the older age group.

Age at Marriage

According to studies by Howayda S Abd El All, Amany Refaat and Khadiga Dandash[9] and Nor Hayati Othman [10], sexual activity with multiple sexual partners, sex with a promiscuous partner, history of sexually transmitted infections, sexual intercourse at a younger age group have all been implicated as risk factors in acquiring HPV infection.

All the patients included in our study were married. As HPV infection is as sexually transmitted disease, there are less chances of infection in unmarried women. Also in our scenario it is difficult to get information about premarital sexuality. Age at first intercourse was not elicited and age at marriage was recorded instead No significant correlation of HPV-16 prevalence with age at marriage was obtained. This is in concordance with study conducted by Shikha Srivastava, Sadhana Gupta and Jagat Kumar Roy. [11]

Parity

96.5% of patients included in the study were multiparous .No significant correlation was observed between parity and the presence of HPV infection .This is comparable to the findings in the study by Nubia Munoz et al [12] and Aggarwal R .Gupta et al[3]

History of Use of Oral Contraceptive Pills

Only 13 patients in our study gave a history of use of Oral Contraceptive pills. The association between HPV positivity and ever use or long term use of Oral contraceptives was again not found to be statistically significant. This could be compared with the following studies.

Table 10: Association of HPV 16 and OC pill intake

Sl.No	Study	Association between HPV positivity and OC pills
1	Smith JS et al [13]	Nil
2	Malcolm Moore [14]	Nil
3	Nubia Munoz et al[12]	Nil
4	Present Study	

However according to study by Howayda S Abd El All, Amany Refaat and Khadiga Dandash[9]. HPV infection was more pronounced in women having history of use of oral contraceptives.

Diagnosis on Pap Smear

46.11. % of the smears reported as inflammatory smear/NILM on Pap smear evaluation showed positivity with Anti HPV 16 .In a study conducted by Gary P.S Yeoh et al. [15]. 25% of smears reported as normal in cytology showed positivity for HPV. This further brings out the importance of objective methods of detection of HPV infection as subtle changes in cervical squamous cells as seen in the

Pap smear is subject to a very high levels of inter observer and intraobserver variability. In our study there were only 6 cases with abnormal cytological results on Pap Smear. In a study conducted Shaira Sahebali et al[16]. 24.1% cases reported normal on cytology, 11% cases reported as ASCUS, 30.2% reported as LSIL and 2.7% cases reported as AGUS showed positivity for HPV. Our results could not be compared with this study due to the lower no. of samples reported as abnormal in cytology.

In a resource poor country like ours, cervical cancer screening practices are inconsistent. The cytological interpretation of Pap smear becomes faulty especially if the smear is inflammatory, which is commonly encountered in women belonging to the lower socio economic groups. In such a situation, screening with a test of higher sensitivity can prove to be much more beneficial to the patients.

1) 173 patients were screened in the study for HPV infection by using immunohisto-chemical marker Anti HPV-16 A total of 82 patients showed positivity with Anti HPV-16.A prevalence of 47.40% for HPV infection was obtained.

2) The following risk factors known to be associated with Human Papilloma Virus(HPV) infection were analysed-
   - Age of the patient
   - Age of Marriage
   - Parity
   - History of intake of Oral contraceptive pills

No statistically significant association was found between the above risk factors and HPV infection

Limitations of the study

1) As the study was conducted in a hospital set up in the outpatient department, it was difficult to obtain adequate patient details for evaluation of additional risk factors.

2) Immunohistochemical method on cell blocks prepared from the cervical smears collected, a less sensitive method was used to detect the presence of HPV-16. More advanced methods like PCR and In Situ Hybridisation

etc may lead to a better and accurate determination of presence of HPV-16.

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