Journal of Cardiovascular Disease Research

ISSN:0975 -3583,0976-2833 VOL14, ISSUE 01, 2023

Original research article

Evaluation of HPV in selected cases of premalignant and malignant lesions of cervix by real time PCR

[1]Dr. Uma K, [2]Dr. Ankita Das, [3]Dr Rashmi SP

[1,2]Assistant Professor, Department of Pathology, Akash Institute of Medical Sciences and Research Centre, Devanahalli, Bangalore, Karnataka, India

[3]Associate Professor, Department of Pathology, Akash Institute of Medical Sciences and Research Centre, Devanahalli, Bangalore, Karnataka, India

Corresponding Author:
Dr. Ankita Das

Abstract

Aim: The aim of the present study was to evaluate HPV in selected cases of premalignant and malignant lesions of cervix by Real Time PCR.

Methods: The present study was a prospective study done over a period of 2 years from Jan 2019 to Jan 2021 in the Department of Pathology, Akash Institute of Medical Sciences and Research Centre, Devanahalli, Bangalore. The study material comprised of 75 Cervix punch biopsies received from the Department of OBG, Akash Hospital, Devanahalli.

Results: Analysis of the background characteristics of women in the study revealed that three fourths of the women in the study were in the age range of 31-50 years and had no education, with only 24.67% having primary education. Most of them were housewives attending only farm, married after 20yrs of age (61.35%). 523 cases, .i.e., 68.69% of patients had 1-2 children and most of them do not give history of any contraceptive use. It was observed that 56% (421) of the cases were reported as Chronic Non-Specific cervicitis followed by a significant number of cases presenting as Carcinoma Cervix (30%). On the whole CIN accounted for 10% and cervical polyps in 4% of cases respectively.

Conclusion: A combination of HPV testing and Pap testing is an option for screening women age 30 and older. Regular surveillance can increase the possibility that cancer could be found at an early stage when treatment is most likely to produce a cure. With the advent of molecular techniques, particularly PCR, it is now possible to detect very low quantities of HPV and also to subtype the commonly occurring HPV in cervical scrape smears.

Keywords: Immunohistochemistry, PCR, premalignant, malignant

Introduction

Cervical cancer is the second most common malignant tumor in women worldwide, and a leading cause of cancer deaths of females in developing countries [1]. Human papillomavirus (HPV) infection is the etiologic agent for virtually all cases of cervical squamous cell carcinoma (SCC) and a large portion of endocervical adenocarcinoma. Among >200 human papillomavirus phenotypes, 14 high-risk HPV (hrHPV) phenotypes have been reported to be closely associated with the initiation and progression of cervical cancer.

Two genotypes of hrHPV, including HPV 16 and 18, are responsible for ~75% of all cases of cervical cancer. Continuous production of the E7 protein from oncogenic genotypes of HPV is required for progression of malignancy [2]. Thus, sensitive and specific detection of E7 HPV protein expression in the clinical samples of exfoliated epithelial cells or biopsies from cervix may provide a clinical benefit for early detection of precancerous conditions. The HPV genome consists of six early open reading frames (E1, E2, E4, E5, E6, and E7), two late open reading frames (L1 and L2). Among six proteins encoded by early open reading frames, E6 and E7 are critical for the development of cervical cancer by regulating cervical epithelial cell immortalization [3]. Transient viral infection usually resolves spontaneously within 6 to 12 months without increasing the risk of cervical cancer [4]. However, in certain cases, viral DNA can integrate into the host genome to cause persistent HPV infection, resulting in an abnormal accumulation of HPV E6 and E7 proteins within host cells [5].

Viral genotyping analysis to identify the oncogenic HPV has not produced good predictive values for the development of intraepithelial CIN lesions [6]. It has been reported that 12-14% of low-grade squamous intraepithelial lesions (LSIL) progress to high-grade squamous intraepithelial lesions (HSIL) [7]; without treatment, a few of those lesions progress to invasive cancer [8]. Protein biomarkers involved in the development of cervical cancer, including SCC antigen, serum fragments of cytokeratin, carcinoma embryonic antigen, soluble CD44 and matrix metalloproteinases, have been considered as potential

ISSN:0975 -3583,0976-2833 VOL14, ISSUE 01, 2023

diagnostic markers for cervical cancer screening [9]. However, these potential protein biomarkers are encoded by the human genome; therefore, diagnostic use is inhibited by the endogenous expression of these biomarkers within noncancerous cervical cells. The oncoprotein E7 encoded by the hrHPV genome with specific expression in HPV-transformed human cervical cells may serve as an ideal biomarker for the early detection of cervical cancer [10].
The aim of the present study was to evaluate HPV in selected cases of premalignant and malignant lesions of cervix by Real Time PCR.

Materials and Methods
The present study was a prospective study done over a period of 2 years from Jan 2019 to Jan 2021 in the Department of Pathology, Akash Institute of Medical Sciences and Research Centre, Devanahalli, Bangalore. The study material comprised of 75 Cervix punch biopsies received from the Department of Gynaecology, Government General Hospital, Kurnool.
The cervical biopsies showing features mentioned in the inclusion criteria were selected for further evaluation by immunohistochemistry to detect HPV. Since typing was not possible by IHC, the positive cases were subjected to Real Time PCR for HPV confirmation and typing. A detailed clinical history pertaining to the selected cases including age, marital status, parity, symptoms at clinical presentation and other relevant details were obtained from the Department of Gynaecology, Akash Hospital, Devanahalli.

Inclusion criteria
Cervix Biopsies from patients showing CIN I, CIN II, CIN III, Carcinomas all below 40 years of age are taken and submitted for immunohistochemistry.

Exclusion criteria
Cases of chronic non-specific cervicitis, cervical polyps and all the cervical biopsies (including dysplasias, carcinomas) above 40 years of age are excluded for immunohistochemistry.

Tissue collection and processing
All the samples obtained were fixed in 10% buffered neutral formalin and submitted for processing. Tissues were processed in YORCO TISSUE PROCESSOR (Automatic) with cycle of 16 hours, after which the processed tissues were embedded into wax blocks. The wax blocks were trimmed, sections were made using the WESWOX ROTARY MICROTOME (MT- 1090A). Sections were taken on to slides and stained by routine H&E staining.

Procedure of HPV detection and typing by real time PCR
Processing of samples
1. Thick sections from the samples were transferred to sterile (2 ml) eppendroff tubes.
2. Paraffin was removed with warm xylene (60 °C) followed by washing twice with ethanol (90% v/v).
3. The samples were dried in a thermomixer for 30 minutes.
4. The dried samples were transferred into centrifuges (15 ml capacity) containing 1X phosphate buffered saline (pH 7.4).
5. The tubes were then vortexed to dissociate the cells, and centrifuged at 10,000 rpm for 10 minutes to get the cell pellet.

PCR for HPV
1. The aliquot of extracted DNA was subjected to PCR, for amplification of HPV DNA type 16 and 18, using type-specific primers. These type-specific primers amplified the complementary sequence of forward HPV DNA type 16, which was 5'-ATTAGTGAGTAT AGACATTA-3' and that of the reverse was 5'-GGCTTTTGACAGTTAATACA-3'. The forward and reverse sequences of HPV DNA type 18 were 5'-ACTATGGCGCGCTTTGAGGA-3' and 5'-GGTTTCTGGCACCGCAGGCA-3', respectively.
2. The reaction mixture was carried out in a volume of 50 µl, containing the following: KCl, 100 mM; TrisHCl, 20 mM; MgCl2, 2.0 mM; dNTP, 2.5 mM; 1.5 units of taq polymerase; 25 picomole of each primer was added to 13 µl of sterile distilled water. Then 2 µl of template DNA was added to each reaction.
3. The reaction was performed in a DNA thermocycler subjecting the mixture to 40 cycles of amplification.
4. Denaturation was done for three minutes at 94 °C for the first cycle. This was followed by one minute each of denaturation at 94 °C, annealing at 54 °C, and extension at 72 °C, for 40 cycles. The last cycle was extended for one minute at 72 °C.
5. The PCR product was electrophoresed in 1.5% agarose gel. The gel was stained with ethidium bromide, to visualize the amplified PCR product, and the bands were visualized with the help of a

ISSN:0975 -3583,0976-2833 VOL14, ISSUE 01, 2023

UV transilluminator and results recorded.

Results

Table 1: Clinical details of patients

Age in years	Cervical biopsies (No. of cases) (n=758)	% age
< 30 yrs	78	10.30%
31 -40 yrs	244	32.19%
41 – 50 yrs	239	31.53%
Above 50 yrs	197	25.98%
Education		
No education	571	75.33%
Primary education	187	24.67%
Occupation		
House wife	584	77.04%
Other works	174	22.96%
Age at marriage		
< 17 yrs	26	3.43%
18 -20 yrs	267	35.22%
>20 yrs	465	61.35%
	Parity	
Nil	45	5.94%
1-2	523	69%
3+	190	25.06%
Contraceptive Use		
Yes	85	11.21%
No	673	88.79%
Clinical History		
White discharge	633	83.51%
Bleeding P/V	125	16.49%

Analysis of the background characteristics of women in the study revealed that three fourths of the women in the study were in the age range of 31-50 years and had no education, with only 24.67% having primary education. Most of them were housewives attending only farm, married after 20yrs of age (61.35%). 523 cases, .i.e., 68.69% of patients had 1-2 children and most of them do not give history of any contraceptive use.

Table 2: Distribution of cervical lesions on the basis of histopathological diagnosis

Cervical lesions	No. of cases (n=758)	% age
CNC	421	55.54%
Cervical polyps	32	4.22%
CIN I, Koilocytic change	59	7.78%
CIN II,CIN III	19	2.51%
Carcinoma Cervix	227	29.95%

It was observed that 56% (421) of the cases were reported as Chronic Non-Specific cervicitis followed by a significant number of cases presenting as Carcinoma Cervix (30%). On the whole CIN accounted for 10% and cervical polyps in 4% of cases respectively.

ISSN:0975 -3583,0976-2833 VOL14, ISSUE 01, 2023

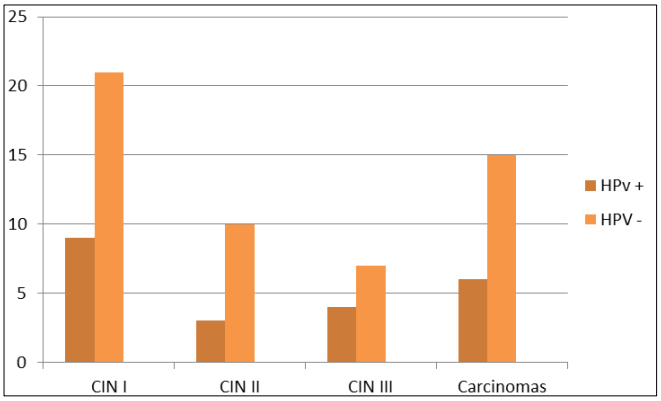

Fig 1: Immunohistochemistry for HPV

Immunohistochemistry for HPV showed a positivity of 29.33%on the whole. There was 30% positivity in CIN I, 23.08% positivity in CIN II, 36.36% positivity in CIN III and 28.57% positivity in carcinoma cervix cases. P value is 0.9144597.

Table 3: HPV positivity on IHC correlation with age

Age	CIN I	CIN II	CIN III	Ca cervix	Total (n=22)
20-30 yrs	2	0	1	-	03 (13. 64%)
31-40 yrs	7	3	3	6	19 (86.36%)

Present study shows HPV incidence of 13.64% in the age group of 20-30 years, where as in the age group of 31- 40 yrs the incidence is 86.36%.

Table 4: HPV type detected by real time PCR

HPV Type	No. of cases (n=22)	%age
HPV 16	14	63.64
HPV 18	06	27.28
HPV 16&18	01	4.54
Not detected	01	4.54

Real time PCR was done on 22 cases which showed positivity on IHC to detect the HPV genotype. It is observed that HPV 16 was the commonest genotype detected in 63.64% of the cases, followed by HPV!8 in 27.28%. A combination of HPV 16 and 18 was found in a single case accounting for 4.54%. In one case no HPV genotype could be detected.

Discussion

Cervical cancer is a leading cause of cancer-associated mortality in females worldwide and the second most common cancer in females in developing countries. Fortunately, cervical cancer is preventable and curable at the early stages due to the relatively slow progression of cervical carcinoma [1] The morbidity and mortality of cervical cancer has been remarkably reduced by the early screening tests, including Pap-smear and liquid-based cytology [7]. Low sensitivity and false positive results from the abnormal morphology of ASC-US are major limitations of cytology tests used in primary screening. According a meta-analysis of Pap-smear tests and LBC, the sensitivity and accuracy of cervical cytology exhibited wide variation in different regions and institutions [11].

The clinical details of the cases in the present study are compared with that of Quamrin Nahar et al. [12] It was observed that the majority of cervical lesions presented in the age group of 31 -50 yrs (63.72%) which compared well with the study of Quamrin Nahar et al., [12] (65.4%). Regarding the education level of the study cases, the present study did not co-relate, and this can be attributed to the rural background of our cases. Most of the cases, i.e., 77.04% of our cases were housewives similar to that of Quamrin Nahar et al. [12] In the present study it is noted that 61.35% of the cases are married after 20yrs, 69% of the cases had 1-2 children and 88.79% never used any contraceptives. In Quamrin Nahar et al., [12] study it is observed that 22.4% of the cases were married at 18-20 yrs, had 1-2 children in 42.6%, never used contraceptives in 86.7% of the cases. All these factors are dependent on the education and socio- cultural factors prevailing in the concerned study area. In the present study 78 cases of cervical intraepithelial lesions account for 10.29% of all cervical biopsies. Thus the incidence of cervical intraepithelial lesions is higher in the present study when compared to the other studies. The incidence is least in the study of Solapurker [13] which is 2.54%. Thus the present study was accordance to the study of Balkachew Nigatu et al [14].

In the present study of 75 cases submitted for immunohistochemistry, 22 cases showed positivity for

ISSN:0975 -3583,0976-2833 VOL14, ISSUE 01, 2023

HPV accounting for 29.33% positivity. Highest incidence of HPV positivity is seen in the study of Ghosh et al. [15] which is 97.50%. Highest incidence of HPV positivity in low grade squamous intraepithelial lesions is seen in the study of Mrudula Soma [16] which is 100% positivity whereas lowest incidence is seen in the present study (30% positivity). Highest incidence of HPV positivity is noted in High grade squamous intraepithelial lesions in the study of Bramhacharimayum 83 which is 75% positivity whereas lowest incidence is seen in the present study which is 29.16% positivity. Highest incidence of HPV positivity is seen in the study of Rasheed [17] which was 55%, whereas lowest incidence is seen in the present study which is 28.57% positivity. Real time PCR was done to find the HPV genotype which cannot be assessed by IHC. In all the studies HPV 16 was the commonest genotype, followed by HPV 18. Both HPV 16&18 infections were detected in few cases. In a small fraction of cases none of the types were detected on PCR. The present study also shows that HPV 16 is the most common infection detected followed by HPV.

Conclusion

Cancer of the cervix has been the most important cancer in women especially in India over the past two decades. The chance of an individual developing cancer depends on both genetic and non-genetic factors. A combination of HPV testing and Pap testing is an option for screening women age 30 and older. Regular surveillance can increase the possibility that cancer could be found at an early stage when treatment is most likely to produce a cure. With the advent of molecular techniques, particularly PCR, it is now possible to detect very low quantities of HPV and also to subtype the commonly occurring HPV in cervical scrape smears. There is ample data on prevalence of HPV in women with cervical cancer. However data on HPV prevalence in women with clinically normal cervix from India is sparse. Hence the necessity to take up this study was considered.

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ISSN:0975 -3583,0976-2833 VOL14, ISSUE 01, 2023

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