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DETECTION AND QUANTITATION OF HIGH RISK HUMAN PAPILLOMAVIRUS, HRHPV 16 AND 18 IN TISSUE OF INDIAN WOMEN WITH CERVICAL CANCER: A CASE CONTROL STUDY

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ABSTRACT

Background: Cervical cancer is the second most common cancer in women worldwide. High-risk infection with HPV type 16 or type 18 is the most important risk factor associated with the development of cervical cancer. **Objective**: The present study was carried out with an aim to investigate the presence and type of HPV infection in cervical cancer cases as well as controls and to determine the association of HPV positivity with various patient and lesion characteristics. **Material & Methods**: A total of 96 histopathological confirmed cases of cervical cancer (CC), and 100 control samples with matched aged groups were enrolled in the study. Specimens were obtained from the site of lesion. All the samples were subjected to histopathological analysis and detection of hr-HPV-16 and 18 was done by using real-time polymerase chain reaction (RT-PCR). **Results**: Majority of cases from upper middle social class (91.67%) and (8.33%) from lower middle class while 82% and 12% in controls respectively. Most of the cases were Stage II (57.30%), and Stage I (21.87%), while Stage III (15.62%), and Stage IV (5.21%) respectively. Size of the tumors were 2–4 cm^2 (59.38%), while <2cm (19.79%) and >4cm (17.71%). Majority had Keratinizing SCC (85.42%). Most of the cases were HPV 16 positive (65.63%) followed by HPV 18 positive (18.75%) respectively. **Conclusions**: The present study highlighted the fact that presence of HPV 16, 18 associated with development and progression of cervical cancer.

KEYWORDS: Human papillomavirus (HPV), cervical cancer (CC), real-time polymerase chain reaction (RT-PCR).

INTRODUCTION

Cancer is a growing health problem in developing countries. Worldwide, Cervical cancer (CC) is the 4th most common cancer among women and seventh most common among all the cancers. Approximately 528,000 new cases of CC recorded in 2012.[1] Cervical cancer death estimated to be 266,000 and it accounts for almost 7.5% of all female cancer deaths worldwide.[1] In India 123,000 new cases of CC with 67,000 deaths recorded in 2012.[1] Carcinoma cervix of uterus (CaCx) is the second most common malignancy affecting women aged between 15 to 44 years, in terms of both incidence and mortality rates worldwide.[2]

Human papillomavirus (HPV) is double-helical DNA structure, which taxonomically is placed in family Papillomaviridae.[3] They were first discovered, isolated, and sequenced from cervical tumor specimens and have been attributed to be one of the official causative agents for the development of cervical cancer by zur Hausen et al.,[4,5] which subsequently led him to win the Nobel Prize of Physiology and Medicine for the year 2008. Following its discovery in cervical tumor specimen, its presence in cutaneous and mucosal tissues of the oral cavity, upper gastrointestinal tract, anogenital tract, and skin of hands and feet was also discovered.[4] Till date, several HPV types have been linked with malignancy of both genital tract and nongenital tract and put under the high risk (HR) category. These types include - HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73, 82, 85, and IS39.[6] while low-risk (LR) types of Human papillomavirus (HPV), including 6, 11, 32, 40, 42, 44, 54, 55, 61, 62, 64, 71, 72, 74, 81, 83, 84, 87, 89, and 91.[6] In the present study, an attempt was made to evaluate the presence of high risk HPV Type 16 and 18 in cervical cancer (CC) lesions among the specimen obtained from the patients.

MATERIALS AND METHODS

Tissue specimens were obtained from 96 patients of cervical cancer attending the OPD, Department of Obstetrics & Gynecology of ERA's Lucknow Medical College & Hospital, Lucknow & Department of Obstetrics & Gynecology - King George's Medical University, Lucknow. Institutional ethical clearance was obtained, and written informed consent was taken from all the patients. Size of the tumor and severity were achieved from pathology reports and clinical notes. Staging was done according to the International Federation of Gynecology and Obstetrics (FIGO).[7] Cervical biopsy from the lesion was sent for histopathological (HPE) examination as sample A and for real-time polymerase chain reaction (RT-PCR) analysis as sample B. For HPE examination, the tissue was collected in 10% formalin and sent to HP laboratory. The tissue was processed through histokinette. Sections up to 4–5 micron thickness were cut on leica microtome and stained with hematoxylin and eosin (H and E) staining. Other specialized stains such as H and E and Van Gieson were used, wherever necessary for diagnosis. All these methods are time-honored and standardized in histopathology laboratory Department of pathology, ERA's Lucknow Medical College & Hospital, Lucknow (ELMC&H).

For conducting RT-PCR analysis tissue biopsy samples were collected in normal saline i.e. sample B. Extraction of DNA and detection of HPV 16, 18 was done by using HPV 16, 18 RT-PCR kit (Liferiver, Shanghai), according to manufacturer's protocol. DNA extraction buffer is supplied in the kit, thoroughly thaw the buffer and briefly spin down in the centrifuge. Crushed the tissue and mixed in 1.0ml normal saline and vortex vigorously. Centrifuge at 13000rpm for 5 minutes and then remove and discard supernatant carefully from the tube without disturbing the pellet. After that Add 1.0ml normal saline and suspend the pellet with vortex vigorously. Centrifuge at 13000rpm for 5 minutes. Remove and discard supernatant carefully from the tube without disturbing the pellet. After that Add 50µl extraction buffer (DNA extraction buffer) and close the tube, vortex for 10 seconds. Spin down briefly in a centrifuge. Incubate the tube for 10 minutes at 100°C and then again centrifuge at 13000rpm for 5 minutes. Then collect the supernatant i.e. DNA extracted and can be used for PCR as a template.

Real time-polymerase chain reaction (RT-PCR) was performed in a 40 µl reaction mixture (RM) containing approximately 4 µl of extracted DNA (template) and 36 µl of master mix (MM). The master mix for each reaction was prepared through pipetting 35 µl of reaction mix (HPV serotype 16 and 18 reactions mix), 0.4 µl of enzyme (DNA polymerase), and then 1 µl of internal control (IC) ending up with a total of 36.4 µl of master mix. The RT-PCR cycling conditions included were initial one cycle at 37°C for 2 min, then one cycle denaturation at 94°C for 2 min, followed by 40 cycles at 93°C for 15 s and 60°C for 60 s. Amplified HPV-16, 18 DNA fragments detection was performed in fluorimeter channel FAM and HEX/VIC/JOE with the fluorescent quencher BHQ1 at 60°C (Fig 1). Data were analyzed by using SPSS-15 (Statistical Package for Social Sciences, Version 15.0). Chi-square test was planned to be employed to find out the association between HPV positivity, patient characteristics, clinical presentation, stage and severity of disease.

RESULTS

All the characteristics of patients were shown in table 1. Age of the patients ranged from 18 to 75 years. Parity in the cases were 15 (15.62%), 67 (69.80%) and 14 (14.58%) while 26%, 50% and 24% in controls. Majority of cases were from upper middle social class (91.67%) as compared to controls, and were uneducated up to 5^{th} standard (45.84%) while 37.50% were 8^{th} standard or above (16.66%) [Table 1]. Most of the cases were belong to rural place of residence 77 (80.20%) and Hindu religion 73 (76.04%) in comparison to control samples. Most of the cases were active smoker as compared to passive smokers.

Histopathologically, majority of the patients had keratinizing SCC (85.42%) followed by non-keratinizing SCC (10.42%) and adenocarcinoma (4.16%) summarized in table 2. Some patients had mild dysplasia (10.42%) followed by those having moderate dysplasia (2.08%). None had severe dysplasia. Staging of cervical cancer patients was outlined by the International Federation of Gynecology and Obstetrics (FIGO) discussed in table 2. Maximum patients were of Stage II (57.30%). A total of 21 (21.87%) were of Stage I. Advanced stages (Stages III and IV) were seen in 15 (15.62%) and 5 (5.21%) patients. Out of 96 patients, majority (60%) of the lesion size was 2–4 cm2. A total of 19 (19.79%) had lesion size <2 cm2 while 17 (17.71%) had lesion size >4 cm2. In 3 (3.12%) patients, lesion size could not be specifically measured. Detection of HPV 16 & 18 was done by using RT-PCR and results were discussed in table 3. Most of the cases were HPV16 positive 65.63% as compared to controls. While the identification of HPV18 positive were 18.75% in cases and 6% in controls. Majority of the cases were HPV 18 negative 81.25% as compared to HPV 16 [Table 3]. Distribution of high risk HPV 16, 18 positive and negative were discussed in table 4.

Table 1: Showing demographic profile of cervical cancer cases and controls.

CASES N= 96			CONTROLS N=100		Chi squared test	P-value
Age (years)	**No.**	**%**	**No.**	**%**		
21-40	21	21.87	34	34		
41-60	60	62.50	59	59		
>60	15	15.63	7	7		
Parity						
≤2	15	15.62	26	26		
3 to 5	67	69.8	50	50		
≥ 6	14	14.58	24	24		
Socioeconomic status						
Upper middle	88	91.67	82	82	4.889	0.046
Lower middle	8	8.33	18	18		
Age at marriage						
<18	32	33.33	42	42	1.667	0.219
18-30	64	66.67	58	58		
≥ 31	0	0	0	0		
Religion						
Hindu	73	76.04	48	48		
Muslim	22	22.92	38	38		
Sikh	1	1.04	14	14		
Educational status						
<5th	44	45.84	30	30		
5th-8th	36	37.5	22	22		
≥ 8th	16	16.66	48	48		
Place of residence						
Rural	77	80.2	63	63	7.185	0.007
Urban	19	19.8	37	37		
Smoker						
Active smoker	18	18.75	8	8	5.266	0.026
Passive smoker	78	81.25	92	92		

Table 2: Histopathological analysis, stage and size of tumor.

Histopathological analysis	No.	%
Non-keratinizing SCC	10	10.42
Keratinizing SCC	82	85.42
Adenocarcinoma	4	4.16
Dysplasia		
Mild	10	10.42
Moderate	2	2.08
Severe	0	0
Stages		
I	21	21.87
II	55	57.3
III	15	15.62
IV	5	5.21
Size of tumor		
<2cm	19	19.79
2-4 cm	57	59.38
>4cm	17	17.71
Not specified	3	3.12

Table 3: HPV 16, 18 analysis in cases and controls.

Human Papilloma Virus	N	%	N	%	Chi squared test	P- value
HPV 16 +ve	63	65.63	37	37	16.06	**0.0006**
HPV 16 -ve	33	34.37	63	63		
HPV 18 +ve	18	18.75	6	6	**7.410**	**0.007**
HPV 18 -ve	78	81.25	94	94		

Table 4: HPV 16, 18 analysis in different age group in cervical cancer cases.

High Risk HPV 16	**21-40**	**41-60**	**61-80**	**>80**	**Total**
Positive	11	43	8	1	63
Negative	10	17	6	0	33
Total	21	60	14	1	96
High Risk HPV 18					
Positive	1	10	6	1	18
Negative	20	50	8	0	78
Total	21	60	14	1	96

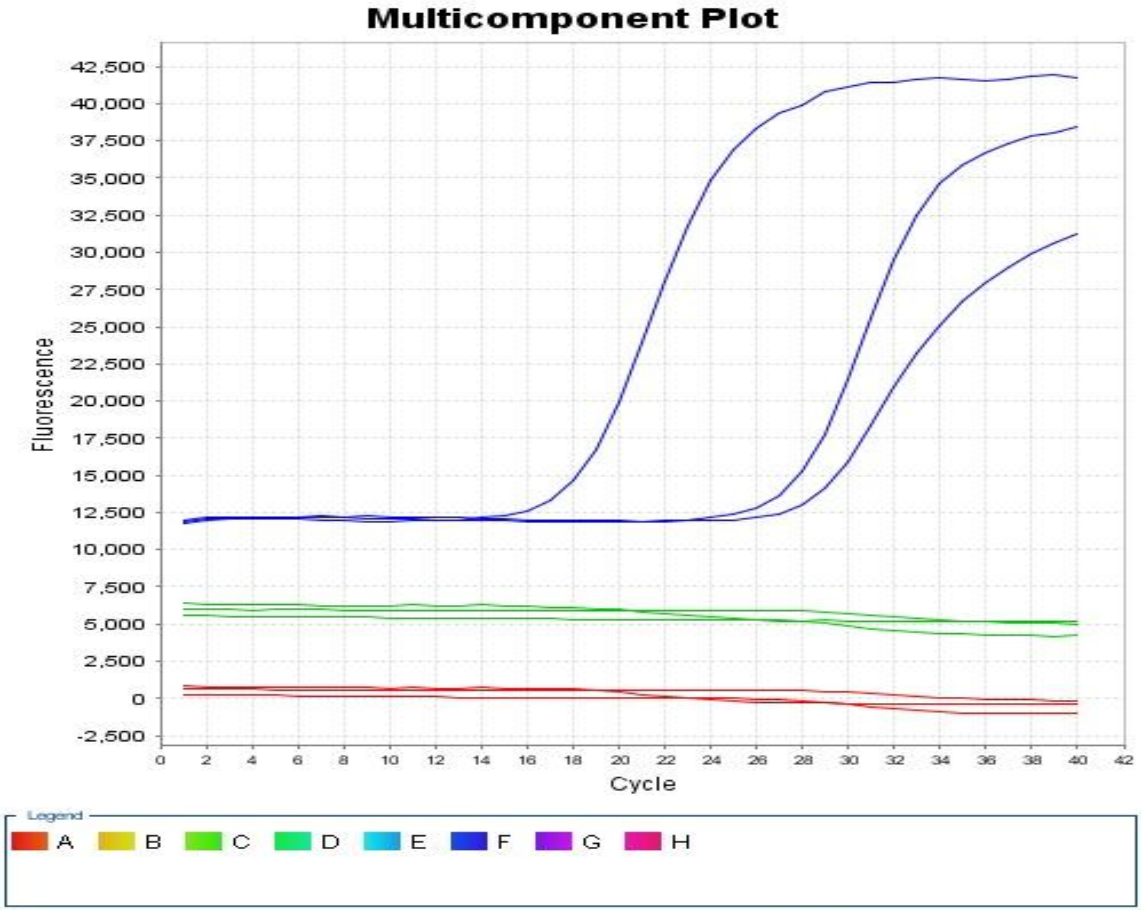

Figure 1: Real-time PCR multicomponent graph showing HPV 16 positive (blue line), 18 negative (green line) & internal control (red line) expression cervical cancer samples.

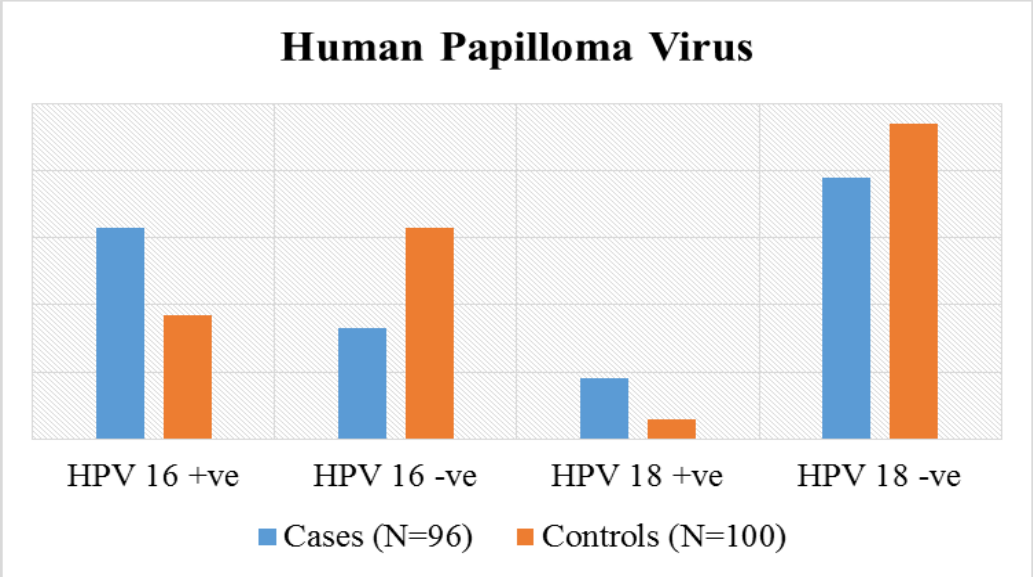

Figure 2: Showing Human Papilloma Virus type 16 and 18 positive and negative in cases versus controls.

DISCUSSION

Certain types of hr-HPV were identified in cervical cancer that seem to carry diverse oncogenic risks and prospective for malignant transformation.[8] High-risk HPV infection was demonstrated as an indicator of malignant lesions like cervical cancer,[9] with a magnitude of 84%, by Riou et. al., and 90% by zur Hausen.[10] In this study we observed a highly significant association between high-risk type HPV 16 (hr-HPV) infection and development of cervical cancer (P= 0.0006). Results of our data were also revealed that HPV 16 was the most predominant type in cervical lesions, specifically invasive cancer. Beside HPV 16, hr-HPV 18 is also showed significant association in the development and progression of cervical cancer (P= 0.007). A study by Alfaro et al., revealed that identification of HPV 16 was 50.9% of the out of 462 women with cervical cancer.[11] The findings of this study support this viewpoint. In this study, HPV 16 was identified in 65.63 % of cervical cancer out of 96 patients. A study published by Dong et al., The detection of HPV 16 DNA in the blood of one of the women with no cervical HPV infection or cervical pathology was an unexpected finding, although this has been previously reported.[12] In the current study, the identification of high risk HPV 16 was found to be 44.80% in cervical cancer patients belonging to age group between 41-60 years. But HPV 18 was 10.42% in same age group. Gnanamony M. et al, evaluated that HPV 16 DNA detection in plasma samples is a marker of advancing cervical disease. HPV DNA was detected in women with high grade lesions and invasive cervical cancer while not in healthy women. However, absolute viral load of HPV 16 in plasma was not related to cervical disease and stage or on HPV 16 viral load in tissue.[13]

Cervical cancer is associated with low socioeconomic indices, presenting a higher prevalence in regions with high poverty, high illiteracy rates and precarious hygiene habits.[14,15] The prevalence of HPV in invasive cervical cancer samples can range from 70 to 100%, which may be associated with different techniques used to detect the virus.[16-18] HPV 16 is the most prevalent in cervical cancer worldwide, followed by HPV 18. However, the HPV type's frequency may vary according to the geographic region of the population under analysis.[19] The precise mechanism through HPV DNA enters in the bloodstream and role it in the pathogenesis of HPV related disease remains undistinguishable. One mechanism proposed is that the HPV plasma viremia is due to presence of disseminated tumor cells in circulation. This has been supported by two studies from Taiwan, which has shown that mRNA of HPV was seen in patients with early and late stage CC.[20,21] Since naked mRNA of HPV can't survive in bloodstream. However, they proposed that the presence of mRNA indicated the presence of circulating feasible tumor cells. Another study published by Bodaghi S et al, has also reported, the presence of DNA of HPV in PBMCs suggesting that PBMCs may serve as carriers for transmission of HPV.[22] Whatever the mechanism, the presence of DNA of HPV in peripheral blood has been strongly linked to metastasis of disease.[23]

In this study, HPV identification was done in both mild and moderate dysplasia groups. In this study, the significant association was found to HPV 16, 18 identification with clinical and HPE severity. In the present study findings further established the fact that high risk type HPV 16, 18 has been recognized as a significant risk factor for cancerous lesions.

CONCLUSION

Analyzing the results of the various prevalence studies, it is absolutely clear that there exist provincial variations in the prevalence of hr-HPV type 16 and 18 infections in India. The persistent hr-HPV infection has been considered to be the primary etiological factor in

causation of cervical cancer. The results of the current study are definitely helpful to know the hr-HPV infection in the development and progression of cervical cancer in central region of India. In conclusion our study showed that high risk type human papilloma virus, HPV 16 and 18 identification was greater in cervical cancer cases as compared to controls. However, hence an effective vaccination program based on regional HPV epidemiological profile along with the cervical cancer screening is necessary to reduce the cervical cancer burden in India.

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Conflicts of interest

All authors declare that they have no conflict of interest.

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