Original Article

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Genetics Section

Screening of High and Low-risk Human Papillomavirus Variants in Cervical Cancer Patients by Polymerase Chain Reaction: A Cross-sectional Study

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ABSTRACT

Introduction: Cervical cancer is the second most common gynaecological malignancy worldwide. Persistent Human Papillomavirus (HPV) infection is the principal risk factor leading to cervical carcinogenesis and causes a large number of casualties.

Aim: To estimate the risk factors and symptoms related to cervical carcinoma and to check for mono-infection or co-infections of low-risk and high-risk HPVs in Northern Indian females.

Materials and Methods: This cross-sectional study was conducted from January 2019 to January 2021 on 110 confirmed cervical cancer tissue samples collected from the Department of Obstetrics and Gynaecology at Pandit BD Sharma University of Health Sciences, Rohtak, Haryana, India. Information about risk factors and symptoms was collected using a semistructured proforma. Detection of HPV infection and HPV genotyping (16, 18, 33, 58, 6, and 11) was done by using type-specific Polymerase Chain Reaction (PCR). Fisher's exact test was applied to determine the association of high-risk HPV infection with other risk factors using GraphPad Prism Version 6 (La Jolla, California, USA). A p-value of ≤0.05 was considered significant.

Results: Of all the studied cases, 68 (62%) were above 55 years old, 67 (61%) were in a postmenopausal state, and 86 (78%) were from a rural background. Irregular menstruation was observed in 99 (90%) cases, bleeding after menopause in 59 (54%) cases, and early age at first intercourse in 57 (52%) cases were the most common symptoms. HPV infection was found in all cases of cervical carcinoma. The incidence of high-risk-HPV16 (84%) and HPV18 (73%) was the most prominent, while the incidence of low-risk-HPV6 (7.2%) and HPV11 (6%) was the lowest. Co-infection of HPV16/18 was the Highest in 60 (54.5%) cases, followed by HPV16/33 in 13 (11.8%) cases. Multi-infection of HPV types 16/18/33 was found in 10 (9.1%) cases, and multi-infection of HPV types 16/18/33/6/11, except HPV58, was found in one patient.

Conclusion: Higher age, postmenopausal status, early age at intercourse, and poor menstrual hygiene were significantly associated with high-risk HPV co-infection. Educating women about the risk factors and symptoms of cervical cancer and screening for high-risk HPVs in rural women are required to reduce the prevalence of cervical cancer in Northern India.

Keywords: Co-infection, Human papillomavirus genotyping, Mono-infection, Risk factors, Symptoms

INTRODUCTION

Cervical cancer is the 3rd most common cancer in women worldwide. Among all gynaecological cancers, it ranks 2nd only after breast cancer and is the most manageable if diagnosed early [1]. In developed countries, a decrease in cervical cancer incidence is observed with increased screening programs, but in developing countries, there is little or no awareness of screening programs, leading to a high incidence [2]. In India, more than 123,000 women were diagnosed with cervical cancer, contributing 9.4% to all cancer cases, out of which 77,348 women died due to this disease in the year 2020. The major causes of the higher mortality rate of cervical cancer in India include the lack of awareness regarding its preventive measures, risk factors, and screening programs, unequal distribution of cancer care facilities, and the late presentation of symptoms [3,4]. Research studies involving cervical cancer have shown that the likelihood of developing cervical cancer increases with an increase in the number of sexual partners and at a younger age at sexual intercourse. Mostly middle-aged women (aged 40-55 years) are affected by this cancer, especially those with Poor economic status who are unable to access regular health checkups due to the monetary issues. Poor hygiene and an imbalanced lifestyle also play important roles in causing this cancer [5,6].

The relationship between cervical cancer and HPV is well established, and the association of HPV in cervical cancer is as high as the association of smoking in lung cancer [7]. More than 100 types of HPV have been reported and classified as high-risk (16, 18, 31, 33, 45, 52, and 58) and low-risk (6, 11, 42, 43, 70, and 90), which are responsible for causing cancer of the cervix uteri. Epidemiological studies suggest that more than 95% of cervical cancer cases worldwide are associated with HPV infection [8,9]. Molecular trials have identified that the two most prevalent extremely oncogenic HPV types observed in invasive cervical carcinoma are HPV16 and 18 [10]. Co-infection and multiple HPV type infections are often related to the development of cervical cancer, but the prevalence of combinations of HPV genotypes is not well known. Some studies suggest that co-infection with HPV types increase the risk of cervical cancer, while multiple HPV infections decrease the survival rate by reducing the response to therapy. Therefore, co-infection or multiple infections are considered prominent risk factors that drive cervical carcinogenesis [11,12]. Studies on different HPV types and their specific co-infections in different populations are necessary to validate this. To the best of the authors' knowledge, this was the first study on the North Indian (Haryana) women population that aimed to estimate the risk factors and compare the status of high-risk as well as low-risk HPVs and their co-infection in tumour tissue of cervical cancer.

MATERIALS AND METHODS

This cross-sectional study was conducted from January 2019 to January 2021 on 110 confirmed cervical cancer tissue samples

collected from the Department of Obstetrics and Gynaecology of Pandit BD Sharma University of Health Sciences, Rohtak, Haryana, India. Ethical permission for sample collection was obtained from the Institutional Human Ethical Committee (IHEC), Maharshi Dayanand University, Rohtak, Haryana, India, with letter number IHEC 119/06 dated 15.01.2019. The subject of the study was verbally explained to the patients or their parents/guardians in the local language. Written information consent was obtained from all enrolled cases (educated) and their parents/guardians (illiterate).

Inclusion criteria: Pathologically confirmed cervical cancer cases were included in the study.

Exclusion criteria: Patients with a record of any other synchronous malignancy or who had received any therapy were excluded from the study.

Histological grading was assigned to samples based on the observed changes in cervical epithelium regarding histological types and disease progression (poor differentiation, moderate differentiation, and well differentiation) [13], and this information was recorded from the patient data sheet. Relevant parameters were noted in the performa from the medical records. Responses for some confidential factors could not be recorded due to patients' denial.

Genomic DNA isolation: All the collected samples were stored immediately at -80°C and processed in a Biosafety Level II laminar flow hood in the Department of Genetics, Maharshi Dayanand University, Rohtak, Haryana, India. For DNA isolation, tissues were crushed in liquid nitrogen resuspended in STE buffer (100 mM NaCl, 10 mM Tris, and 1 mM EDTA), and incubated with 100 µg of proteinase K at 55°C for 16 h. The purity and concentration of genomic DNA were checked by a Nanodrop spectrophotometer (mySPEC, Sigma-SVi).

HPV genotyping in cervical biopsies: In the present study, HPV genotyping of high-risk HPV16, HPV18, HPV33, and HPV58 and low-risk HPV6 and HPV11 was carried out using PCR with specific primers shown in [Table/Fig-1]. Patients found infected with one type of HPV were placed under the mono-infection category, and those with more than one type were placed under the co-infection category.

S. No.	HPV type	Primer sequence	Size (bp)
1.	HPV16	F 5′-GGAGTACCTACGACATGGGG-3′ R 5′-GGGGATCATCTTCTTTAGGTGCT-3′	250
2.	HPV18	F 5′-ACACTGGGCTAAAGGCACTG-3′ R 5′-TAGCAAAAAGCTGCTCACGC-3′	280
3.	HPV33	F 5′-GCACATGGTGGTGTTTTAAC-3′ R 5′-AGTCAGGATCAGGAGCAGGT-3′	935
4.	HPV58	F 5′-GTATACTGGTTATGCACATGGT-3′ R 5′-AAAAAAGCAGGGTCAACAAC-3′	847
5.	HPV6	F 5′-TAGTGGGCCTATGGCTCGTC-3′ R 5′-TCCATTAGCCTCCACGGGTG-3′	280
6.	HPV11	F 5′-GGAATACATGCGCCATGTGG-3′ R 5′-CGAGCAGACGTCCGTCCTCG-3′	360

[Table/Fig-1]: List of High-risk (16, 18, 33 and 58) and Low-risk (6 and 11) HPV primers (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).
HPV: Human papillomavirus; bp: Base pairs

A total of 10 µL PCR mixture was prepared by mixing 10× PCR buffer, 2 mM dNTPs, 10 pmol of each forward and reverse primer, 100 ng DNA, Taq DNA Polymerase, and nuclease-free water. Reactions were performed as follows: Initial denaturation for four minutes at 94°C followed by 35 cycles with denaturation for 45 seconds at 94°C, primer annealing for 45 seconds at 53-61°C, extension for one minute at 72°C, and final extension for seven minutes at 72°C. The PCR products were analysed with gel electrophoresis using a 2% agarose gel stained with the EtBr dye and were seen under a UV transilluminator.

Parameters studied under the study: Risk factors and symptoms observed by the gynaecologist during the investigations, like age, residential background, educational status, age at intercourse, menopause, and menstrual hygiene, were studied. Symptoms like irregular menstruation, postmenopausal bleeding, vaginal odour, genital tract infection, bleeding during intercourse, white discharge, pelvic pain, and discomfort during urination were also noted.

STATISTICAL ANALYSIS

Responses to individual questions were documented, and the data were analysed using Microsoft Excel. The data are expressed in percentage frequencies calculated from the total number of enrolled cases of cervical cancer. Fisher's exact test was applied to determine the association of high-risk HPV infection with other risk factors using GraphPad Prism Version 6 (La Jolla, California, USA). A p-value of ≤0.05 was considered significant.

RESULTS

Cervical cancer cases were categorised into different age groups. The youngest patient was 28 years old, and the eldest was 82 years old. Most cases fell within the age group of 56-65 years. The mean age of the registered cases was 55.3, and the median was 56.5. A 68 (62%) patients were above 55 years old, while 42 (38%) were below 55 years old. The age at first intercourse in the studied subjects was less than 18 years in 57 (52%) cases. Many of the cases (67, 61%) were in their postmenopausal stage, and 72 (65%) were practicing poor menstrual hygiene. In this study, 93 (84.5%) women suffering from cervical cancer were illiterate or just literate, as most of them were from rural backgrounds (86, 78%), lacking access to education and other awareness systems at their time. Only 17 (15.5%) enrolled cancer cases had completed high school.

Histological grading: Out of the 110 enrolled cases, 106 (96%) belonged to squamous cell carcinoma, while 2 (2%) cases each of adenocarcinoma and adenosquamous carcinoma were observed in biopsies. Sixty-five (59%) cases were moderately differentiated, with 8 (7%) cases each classified as well and poorly differentiated. For 29 (26.3%) cases, no degree of differentiation was found.

Symptoms: The most common symptoms in enrolled cases were irregular menstruation (99, 90%), bleeding postmenopause (60, 54%), strong foul vaginal odour (52, 47%), genital tract infection (47, 43%), bleeding during intercourse (42, 38%), white discharge (39, 35%), pelvic pain (39, 35%), and discomfort during urination (11, 10%).

Human Papillomavirus (HPV): All 110 cancerous biopsies were found positive for HPV DNA.

- **Detection of High-risk HPVs in cervical cancer cases**

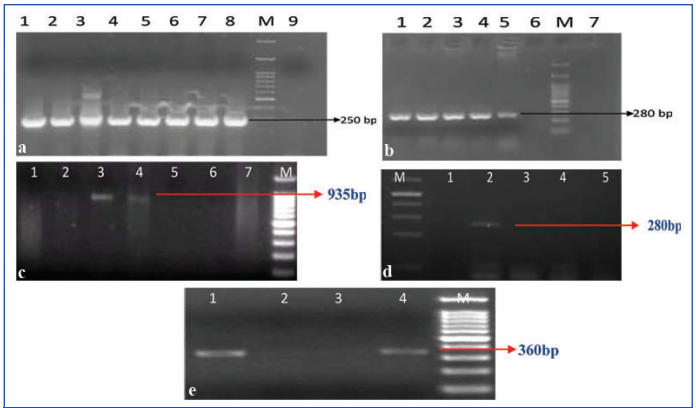
Out of the 110 HPV-positive cervical cancer cases, 92 (84%) samples were found to be HPV16 positive, while 80 (73%) samples were found positive for HPV18 and 15 (14%) samples were found positive for HPV33 as shown in [Table/Fig-2a-c, 3a]. No sample was found to be infected with HPV58 type.

- **Detection of Low-risk HPVs in cervical cancer cases**

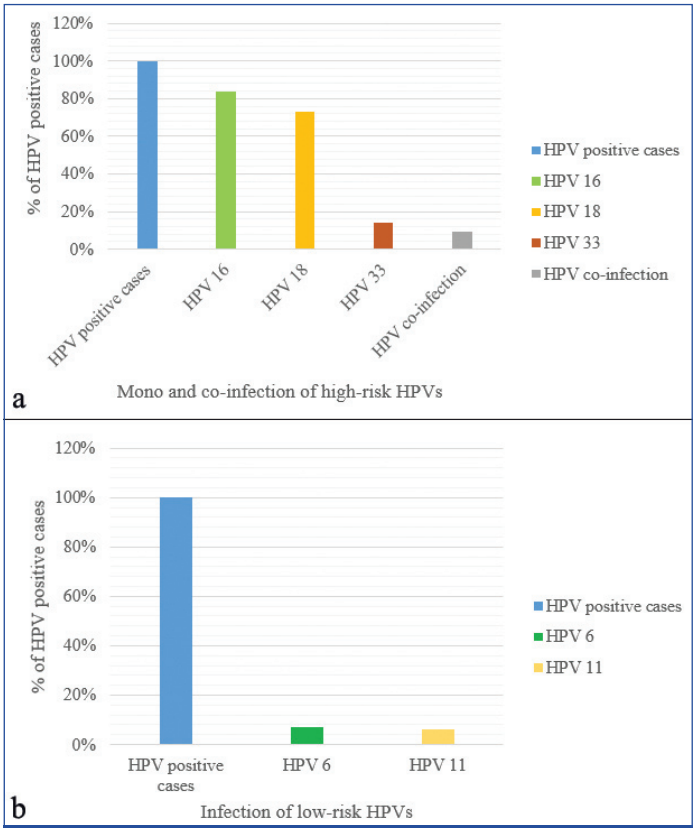
Among the 110 HPV-positive cervical cancer cases, 8 (7.2%) samples were found to be HPV 6 positive, and 7 (6%) cases were found positive for HPV11 as shown in [Table/Fig-2d,e,3b].

- **Co-infection and multi-infection of High-risk HPVs and Low-risk HPVs in cervical cancer cases**

The percentage of patients co-infected with different HPV types in cervical cancer patients is shown in [Table/Fig-4]. Women had a higher prevalence of HPV16/18 co-infection (54.5%), followed by HPV16/33 co-infection (11.8%) and HPV18/33 co-infection (10.9%). Multi-infection of HPV types 16/18/33 was found in 9.1% of cases, and multi-infection of all HPV types except HPV58 was found in only one patient. Patients infected with high-risk HPV types were more prevalent compared to those infected with low-risk HPV types or co-infected with both (low- and high-risk HPV).



[Table/Fig-2a-e]: Representative image of 2% agarose gel showing Lane M: Upto 1 kb DNA ladder: (a) HPV 16-Lane 1 to 9 shows cervical samples, sample 9 is HPV 16 negative; (b) HPV 18- Lane 1 to 7 shows cervical samples; (c) HPV 33-Lane 1 to 7 shows cervical samples; (d) HPV 6-Lane 1 to 5 shows cervical samples; (e) HPV 11-Lane 1 to 4 shows cervical samples.



[Table/Fig-3a-b]: Mono-infection and co-infection of HPVs in the enrolled cervical cancer patients (a) High-risk HPV prevalence; (b) Low-risk HPV prevalence.

Co-infection	n (%)
HPV16/18	60 (54.5)
HPV16/33	13 (11.8)
HPV16/6	7 (6.4)
HPV16/11	5 (4.5)
HPV18/33	12 (10.9)
HPV18/6	4 (3.6)
HPV18/11	6 (5.5)
HPV16/18/33	10 (9.1)
HPV16/18/33/6/11	1 (0.91)

[Table/Fig-4]: Percentage frequency of co-infection of High-risk and low-risk HPVs in cervical cancer patients.

In the current study, the association of high-risk HPV co-infection was also examined with other risk factors. HPV co-infection of HPV16/18 was associated with higher age, younger age at intercourse, post-menopausal stage, and poor menstrual hygiene in women, while HPV16/33 and 18/33 co-infection were found to be statistically associated with the early age of intercourse in women as shown in [Table/Fig-5].

DISCUSSION

A very high prevalence of HPV infection was observed in all cases. Patients infected with high-risk HPVs were more numerous compared to those infected with low-risk HPVs or co-infected with both. Mono-infection of HPV16 was the most common, followed by the infection of HPV18. The incidence of HPV6 and HPV11 mono-infection was also found to be low in the present cervical cancer samples. Co-infection or multiple infections of HPV types may increase the development and progression of precancerous lesions compared to mono-infection. A recent epidemiological study indicates that around 50% of HPV-positive females were co-infected with more than one type of HPV [14]. In another study, it was found that co-infections with low-risk HPV types increase the risk of low-grade squamous intraepithelial lesions [15]. The incidence of mono-infection and co-infection varies from area to area and country to country. In some areas, HPV mono-infection was more frequent than co-infection, while in other areas, a higher prevalence of co-infection was observed [16-18]. In the women from the western Mexican population, the co-infections reported with a high prevalence were HPV51/52 and HPV16/51/52. Although single infections with these HPV genotypes were also commonly observed [19]. In the present study, a higher incidence of HPV16/18 (54.5%) co-infection and lower incidence of HPV16/33 (11.8%) and HPV18/33 (10.9%) co-infection were found. Multi-infection of HPV16/18/33 was found in 9.1% of the cervical cancer cases. Co-infection of high-risk HPVs was statistically associated with higher age, early age at intercourse, postmenopausal status, and poor menstrual hygiene of the patients. These observations were similar to other related studies [20-22]. However, further details on HPV co-infection and multi-infection in association with cervical cancer are required to draw fruitful results.

More than 60% of the cases were above 55 years of age and in their postmenopausal stage, showing that the chances of cervical cancer increase with age, and postmenopausal women are at a higher risk of being affected. Most of the patients in this study population belonged to a rural background 86 (78%) and were illiterate (90%). The role of residential background and education comes into play when determining the incidence of cervical cancer, as people in rural areas and those who are illiterate are less aware of screening and preventive measures than the people in urban areas and educated individuals. The scenario is somewhat different for women in urban areas compared to rural areas, where a high level of awareness has been observed [23]. A national representative survey conducted by Dikshit R et al., has also shown that cervical cancer incidence has decreased in women in urban areas due to an increased level of awareness [24]. Previous studies have shown that women with an early age of intercourse and poor menstrual hygiene have a greater risk of carcinoma cervix [25-27]. The present study also looked at these risk factors and found that 57 (52%) of the enrolled cases had their first intercourse before the age of 18 years, and 72 (65%) of the women were practicing poor menstrual hygiene by using the same cloth repeatedly to manage menstrual bleeding.

In one of the authors' previous studies conducted on rural and urban populations, it was observed that irregular painful menstruation, smelly white discharge, pain, and bleeding during or after intercourse were the major symptoms in most cases [23]. In the present study, irregular menstruation was observed as the most common symptom. Moreover, the participants had been experiencing these problems for a long time, but they were ignorant of the symptoms and felt ashamed to contact a medical advisor. If they were aware of the consequences of these symptoms in the future, they could be diagnosed early and provided with better treatment.

Risk factors	Number of patients (N)	HPV16/18 (60)	p-value	HPV16/33 (13)	p-value	HPV18/33 (12)	p-value
Age (years)							
>55	68	19	0.0001*	5	0.07	4	0.055
≤55	42	41		8		8	
Residential background							
Rural	86	49	NS	12	NS	10	NS
Urban	24	11		1		2	
Age at first intercourse							
<18	57	8	0.0001*	1	0.0007*	1	0.001*
≥18	53	52		12		11	
Menopause							
Pre	43	18	0.04*	5	NS	4	NS
Post	67	42		8		8	
Menstrual hygiene							
Poor	72	47	0.0025*	7	NS	8	NS
Good	38	13		6		4	

[Table/Fig-5]: Association of HPV co-infection with risk factors.
(N: Number; Fisher's-exact test to determine association of HPV infection with risk factors; *indicates the significant p-value of ≤0.05) NS: Not significant

Vaccines targeting high-risk HPVs may prove beneficial in the timely eradication of cervical cancer. However, there is a need to find out whether vaccines designed for high-risk types can also cure infections of low-risk HPVs. The upcoming vaccines should be designed in ways that are effective against low-risk as well as the other most common genotypes of HPV.

Limitation(s)

Since the sample size for the present study was limited, further studies with larger sample sizes are needed to evaluate the role of underestimated HPV genotypes (in mono- or co-infection forms) in cervical cancer. Moreover, this study was conducted using the PCR method; further advanced techniques such as Real-Time PCR and Methylation-Specific PCR can be applied to unveil the underlying molecular mechanisms involving these HPV mono- and co-infections. Thirdly, as the control samples could not be accessed, future studies can be planned to compare the status of mono- and co-infections of different high-risk and low-risk HPV types in normal and cervical cancer patients.

CONCLUSION(S)

In the present study, high-risk HPV infection was found to be more frequent than low-risk HPV infection, and co-infection of HPV16/18 was found to be high compared to other co-infections or multi-infections. Despite the high incidence of different HPV infections in women of Haryana, there is very little or no knowledge of HPV infection and its vaccination. Activities like the training nurses and local workers, holding seminars in rural schools and colleges, involving local panchayats and social workers like Anganwadi and ASHA workers, engaging women representatives, and educating dispensaries about cervical cancer will help in educating all women.

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