Nonavalent strains of Human papilloma virus in the cervical cancer patients from western India

Ashi R. Thobias[1], Kruti A. Mehta[2], Kinjal A. Patel[3], Jayendrakumar B. Patel[4], Prabhudas S. Patel[5]

[1,2]PhD scholar-Gujarat University, [3]Junior Research Associate, [4]SSO & Head-Molecular Diagnostic & Research Laboratory-3, Former Professor and Head; Chairman K.H. Hospital
[1]Cancer Biology Department,
The Gujarat Cancer & Research Institute,
Asarwa, Ahmedabad -380 016, Gujarat, India
[1]Department of Life Science, Gujarat University
Navrangpura, Ahmedabad-380009, Gujarat, India

Abstract:
Purpose: Since the release of HPV vaccines in 2006, 100 countries have included the HPV vaccines in their vaccination programs. In India, the data on prevalence of HPV types other than HPV 16 and 18 are lacking especially from the western region. Therefore, the present investigation aimed to evaluate the prevalence of the HPV strains included in the current nonavalent vaccine.
Method: DNA was isolated from 400 samples and endpoint TS-PCR was conducted to detect HPV 16, 18, 31, 33, 45, 52, 58, 6 and 11. The data were analyzed by chi square test using SPSS software version 20.
Result: The total HPV prevalence in this studied cohort was 77.8%. All the HPV types except HPV 11 were present in the studied cohort. HPV 16 (74.2%), HPV 18 (14.8%) and HPV 45 (4%) were observed to be comparatively higher than the other HPV type. Moreover, HPV 16 was noted to be significantly higher in cases with lymph node metastasis (p=0.015)
Conclusion: The HPV infections are prominently prevalent in the cervical cancer cohort from the western region of India. The data in the present study will be useful in strengthening the HPV vaccination programs in India.

Keywords: HPV strains, cervical cancer, screening, TS-PCR

Introduction:
Cervical cancer is the second most common cancer among Indian women. Globocan estimated 6,04,127 new cases and 3,41,831 deaths in the year 2020 for cervical cancer [1]. Infection with the high-risk human papilloma viruses (HPV) have been implicated to causing cervical cancer since 1970s when Zur Hausen discovered the presence of HPVs in genital warts and cervical cancer [2]. According to studies conducted across the globe, HPV-16 and 18 are the most highly oncogenic types found in invasive cervical cancer, and out of these two, HPV-16 has been found to be most prevalent [3]. Several epidemiological studies have confirmed that one or more of the oncogenic HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 should be considered as a cause for cervical neoplasia [4].
HPV is one of the most widely occurring sexually transmitted disease in the world. Worldwide, 4.5% of all cancers (630,000 new cancer cases per year) are attributable to HPV; 8.6% in women and 0.8% in men. Of these cancer cases, cervical cancer accounts for 83% of HPV-attributable cancer [2]. Till date, more than 150 types of HPVs have been classified. To battle these HPV strains, HPV vaccinations have been introduced since 2006 with the launch of Gardasil and Cervarix. In, 2014, Merck launched an improved version of its previous product, Gardasil 9 which is known to target nine types of HPV strains i.e HPV 16, 18, 31, 33, 45, 52, 58, 6 and 11. Many countries have adapted the HPV vaccine in their vaccination programs [5]. Studies from these regions have shown promising results in curbing the incidence of cervical cancer post vaccination. [6,7]. Approximately 90% of cervical cancers occur in low-income and middle-income countries that lack organized screening and HPV vaccination programs. While in developed countries, the incidence and mortality have more than halved during the past 30 years since the introduction of formal screening programs [5]. Very few studies on HPV types other than these two highly prevalent strains have been conducted in the country. Furthermore, the studies reporting the prevalence of different HPV strain from western region of India is lacking. To implement the HPV vaccines in the vaccination programs in India, data regarding the prevalence of the strains targeted by these vaccines need to be collected to evaluate the need of vaccination programs. Hence, the present study evaluated the prevalence of HPV 16, 18, 31, 33, 45, 52, 58, 6 and 11 strains in 400 cervical cancer patients from western region of India.

Materials and Methods:
Histopathologically confirmed and previously untreated 400 cervical cancer patients from the out patients' department of The Gujarat Cancer & Research Institute, Ahmedabad, India, were enrolled for the study after informed consent. Approval for the study was granted by the Ethics Committee of Gujarat Cancer Society & The Gujarat Cancer Institute (approval no. EC/31/2018). Biopsy tissue samples were collected from the patients. As soon as the tissue samples were obtained, they were washed with phosphate buffer saline (pH 7.4) and immediately stored at -80˚C until analysis.

Genomic DNA isolation and PCR for HPV detection

DNA was isolated from the cervical cancer tissues using commercially available DNA mini kit (Qiagen, Valencia, CA, USA). The DNA was quantified using spectrophotometer (Spectrophotometer UV-1800, Shimadzu, Japan) and integrity of the isolated DNA was checked on 0.8% agarose gel.
The DNA samples were amplified by type-specific (TS) PCR for HPV 16, 18, 31, 33, 45, 52, 58, 6 and 11. The thermocycler conditions for the same are given in table 1.

Table 1: Thermocycler conditions of HPV 16, 18, 31, 33, 45, 52, 58, 6 and 11

HPV type	Initial denaturation	Denaturation	Annealing	Extension	Final Extension
16	94 °C 3 min	94 °C 1 min	52.1 °C 30 sec	72 °C 30 sec	72 °C 5 min
18			51.2 °C 1 min		
31	94 °C 3 min	94 °C 15 sec	61 °C 30 sec	72 °C 10 sec	72 °C 1 min
33			65 °C 30 sec		
45			60 °C 30 sec		
52			60.3 °C 1 min		
58			60.6 °C 1 min		
6		94 °C 1 min	60 °C 30 sec	72 °C 30 sec	72 °C 3 min
11			61 °C 30 sec		

The reaction was performed on a thermal cycler (ThermoFischer Scientific, Proflex PCR System). A total reaction mixture of 25 µl was prepared. The mixture contained 12.5 µl of master mix, 10 pmole of forward and reverse primers, 200 ng DNA sample and nuclease free water. For the internal control, β globin gene was used while HPV 31, 33, 45, 52, 58, 6 and 11 positive cervical cancer samples were used as positive controls. Post the thermocycler reaction, the samples were loaded in 2% gel with 100 bp ladder (HPV 16,18, 52, 58, 6, 11) or 50 bp ladder (HPV 31, 33, 45) to evaluate presence of infection.

The data obtained were analyzed using the SPSS (Statistical Package for the Social Sciences) software version 20 and Medcalc. Correlation between clinicopathological parameters and HPV infection was carried out using Pearson chi-square test. $p < 0.05$ was considered as statistically significant for all the analysis.

Results:

Clinical details of the cervical cancer patients:

Total 481 cervical cancer patients were enrolled. Out of that, 81 patients were excluded due to unavailability of biopsy report, no confirm malignancy, co-infection with other viruses (HIV/HsBg/HCV), previous treatment and poor DNA quality.

The Clinical details of patients were collected from hospital records which are summarized in table 2.

Table 2: Clinical parameters of the cervical cancer patients

CHARACTERISTICS	PATIENT PERCENTAGE
AGE	
<50	233 (58.25)
>50	167 (41.75)
AGE AT MARRIAGE	
<21	117 (29.25)
>21	95 (23.75)
MENOPAUSAL STATUS	
Premenopausal	66 (16.5)
Perimenopausal	24 (6)
Postmenopausal	185 (46.25)

The cervical cancer patients were in the age range of 21-80 years with median age of 50 years. Of the total 400 patients, 233 (58.25%) females were below the age of 50 years; while 167 (41.75%) women were above the age of 50 years. The age at marriage ranged from 44 years with median of 21 years. Of these, larger group of women (29.25%) were married at a younger age, while 23.75% patients were married at an older age. The menopausal status of 275 patients were known; of which 16.5% women were in premenopausal status, 6% patients were in perimenopausal status, while a large group of women (46.25) were postmenopausal status.

Clinicopathological details of cervical cancer cohort:

The clinicopathological parameters of the cervical cancer cohort are given in table 3.

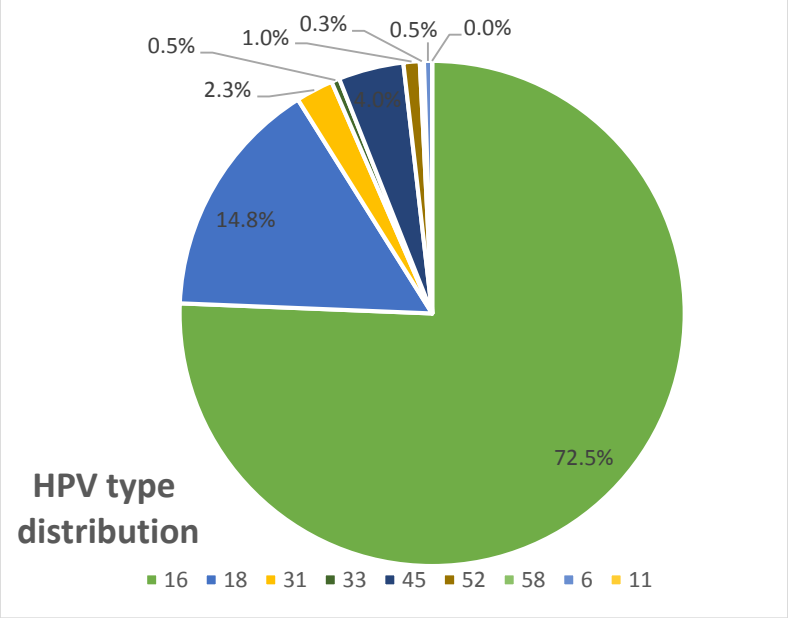
Table 3: Clinicopathological details of the cervical cancer cohort

CHARACTERISTICS	**PERCENTAGE PATIENTS**
HISTOLOGY	
Squamous Cell Carcinoma	365 (91.25)
Adenocarcinoma	30 (7.5)
Unknown	05 (1.25)
DIFFERENTIATION	
Well	14 (3.5)
Moderate	254 (63.5)
Poor	75 (18.75)
Unknown	57 (14.25)
STAGE	
Stage I	31 (7.8)
Stage II	62 (15.5)
Stage III	234 (58.5)
Stage IV	11 (2.75)
Unknown	62 (15.5)
LYMPH NODE METASTASIS	
Lymph node positive	56 (14%)
Lymph node negative	187 (46.75%)
Unknown	157 (39.25%)

Of the 400 subjects, 365 (91.25%) patients had squamous cell carcinoma, 30 (7.5%) patients had adenocarcinoma. In majority of the patients, the tumor was moderately differentiated (63.5%), while 75 (18.75%) patients had poorly differentiated and 14 (3.5%) patients had well differentiated tumors. The stage statistics of the cohort were as follows: higher number of patients were in stage III (234), 62 cases were of stage II, 31 cases were of stage I and 11 patients were at stage IV of disease progression. The data on the lymph node metastasis was available for only 243 patients of which 56 patients had lymph node metastasis while 187 cases show no metastasis in the lymph node.

HPV investigation by type specific primers for HPV 16, 18, 31, 33, 45, 52, 58, 6 and 11:

In this cohort, 311 (77.75%) patients were HPV positive. Of these HPV-positive cervical cancer patients, majority harbored HPV 16 infection (72.5%) and 14.8% were positive for HPV 18 infection. Co-infection of HPV 16 and 18 in cervical cancer patients was found in 31 (2.3%) patients. The details of the HPV infection harbored in the studied cohort are given in fig. 1.



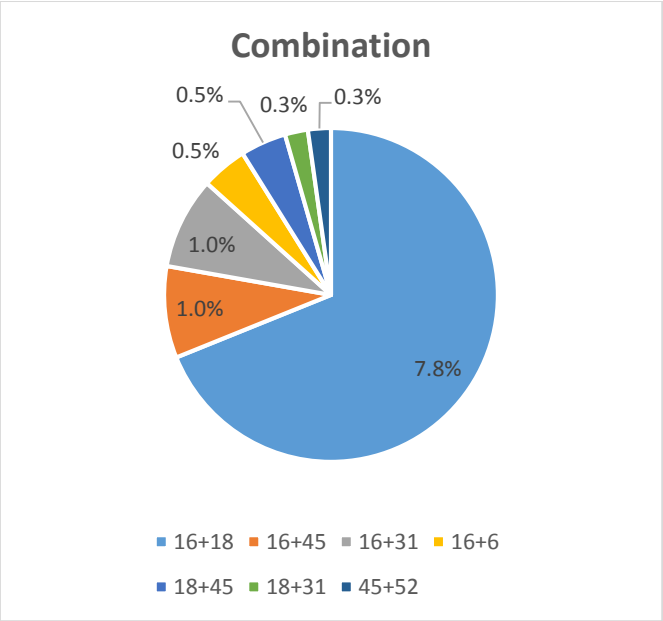


Figure 1: HPV Type Distribution in the Cervical Cancer Cohort

Association of different HPV type infections with clinical and clinicopathological features of the cervical cancer cohort:
The association between HPV types and clinical and clinicopathological parameters of the patients is documented in table 4.

Table 4: Association of HPV strains with Clinical and Clinicopathological parameters

Characteristics	Category	No. of patients	HPV 16 +ve	HPV 18 +ve	HPV 31 +ve	HPV 33 +ve	HPV 45 +ve	HPV 52 +ve	HPV 58 +ve	HPV 6 +ve
Age at diagnosis	<50	233	173 (74.2)	37 (15.9)	4 (1.7)	2 (0.8)	8 (3.4)	0 (0)	1 (0.4)	1 (0.4)
	>50	167	117 (70.1)	22 (13.2)	5 (3.0)	0 (0)	8 (4.8)	4 (2.4)	0 (0)	1 (0.6)
Age at Marriage	<21	177	91 (51.4)	17 (9.6)	1 (0.6)	1 (0.6)	2 (1.1)	2 (1.1)	0 (0)	2 (1.1)
	>21	95	70 (73.7)	14 (14.7)	2 (2.1)	1 (1.1)	2 (2.1)	1 (1.1)	1 (1.1)	0 (0)
Menopausal Status	Premenopausal	66	52 (78.8)	11 (16.7)	2 (3)	0 (0)	2 (3)	0 (0)	0 (0)	0 (0)
	Perimenopausal	24	19 (79.2)	1 (4.2)	0 (0)	1 (4.2)	0 (0)	0 (0)	1 (4.2)	0 (0)
	Postmenopausal	185	132 (71.3)	26 (14.0)	3 (1.6)	1 (0.5)	10 (5.9)	2 (1.1)	0 (0)	2 (1.1)
Histology	SCC	365	268 (73.4)	48 (13.1)	8 (2.2)	2 (0.5)	13 (3.6)	4 (1.1)	1 (0.3)	2 (0.5)
	ADC	30	18 (60)	10 (33.3)	1 (3.3)	0 (0)	2 (6.7)	0 (0)	0 (0)	0 (0)
Differentiation	Well	14	11 (78.6)	3 (21.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Moderate	254	188 (74.0)	37 (14.6)	6 (2.4)	2 (0.8)	7 (2.7)	2 (0.8)	1 (0.4)	1 (0.4)
	Poor	75	57 (76)	9 (12)	0 (0)	0 (0)	4 (5.3)	0 (0)	0 (0)	1 (1.3)
Stage	I	31	23 (74.2)	4 (12.9)	1 (3.2)	0 (0)	1 (3.2)	0 (0)	0 (0)	0 (0)
	II	62	47 (75.8)	11 (17.7)	1 (1.6)	0 (0)	3 (4.8)	1 (1.6)	0 (0)	1 (1.6)
	III	234	171 (73.1)	30 (12.8)	4 (1.7)	2 (0.8)	10 (4.3)	3 (1.3)	1 (0.4)	1 (0.4)
	IV	11	9 (81.8)	3 (27.3)	0 (0)	0 (0)	1 (9.1)	0 (0)	0 (0)	0 (0)
LN Metastasis	LN +	56	48 (85.7)	8 (14.3)	0 (0)	0 (0)	2 (3.6)	0 (0)	0 (0)	0 (0)
	LN -	187	132 (70.6)	25 (13.4)	6 (3.2)	1 (0.5)	7 (3.7)	3 (1.6)	1 (0.5)	1 (0.5)

None of the cases was found to have HPV 11 infection. The present investigation noted that higher number of patients exhibited HPV 16 (74.2%), 18 (15.9%), 33 (0.8%) and 58 (0.4%) infection in the younger age group (< 50 years) compared to the older age group (> 50 years) while the HPV 31 (3%), 45 (4.8%), 52 (2.4%) and 6 (0.6%) positivity was higher in older age group. The status for the age at marriage was available for only a small segment of patients (272). The median of age at marriage was 21 years and groups were made based on it. All the HPV infections i.e. HPV 16 (73.7%), 18 (14.7%), 31 (2.1%), 33 (1.1%), 45 (2.1%), 52 (1.1%) and 58 (1.1%) were higher in women married at older age, except HPV 6 (1.1%) which was observed exclusively in patients married in younger age. In the 275 cases whose menopausal status of the patients known, HPV 16 (79.2%), 33 (4.2%) and 58 (4.2%) was higher in perimenopausal women, HPV while HPV 18 (16.7%), 31 (3%) and 58 (4.2%) was higher in premenopausal women. Whereas, HPV 52 (1.1%) and 6 (1.1%) was observed higher in postmenopausal patients.

Higher percentage of patients had squamous cell carcinoma followed by adenocarcinoma. A trend of higher prevalence of HPV 16 positivity was noted in patients with squamous cell carcinomas (73.4%; p=0.088), while 13.1 % were positive for HPV 18. In patients with adenocarcinoma, HPV 16 positivity was lower (60%) while HPV 18 positivity was comparatively higher (33.3%). In the case of tumor differentiation, well differentiated tumors showed higher HPV 16 (78.6%) and HPV 18 (21.4%) infection rate, moderately differentiated tumors showed exclusive prevalence of HPV 31 (2.4%), 33 (0.8%), 52 (0.8%) and 58 (0.4%) infections; while poorly differentiated tumors showed higher HPV 45 (5.3%) and 6 (1.3%).

On the association of HR-HPV with stage of the disease, it was observed that HPV 16 (81.8%), 18 (27.3%) and 45 (9.1%) infection rates were higher in patients with stage IV disease i.e. advanced stage. It was also observed that omitting stage III (HPV 18 rate=12.8%), HPV 18 infection rate increased with the advancement of the disease. HPV 31 (3.2%), HPV 52 (1.6%) and 6 (1.6%) infection rates were higher in early stage (stage I and stage II). While positivity of HPV 33 (0.8%) and 58 (0.4%) were observed exclusively in stage III. The status for lymph node metastasis was available for 243 patients. Of these patients HPV 16 (85.7%, p=0.015) and HPV 18 (14.3%) infection rates were observed to be higher in lymph node positive cases whereas HPV 31 (3.2%), 33 (0.5%), 45 (3.7%), 52 (1.6%), 58 (0.5%) and 6 (0.5%) were observed to be higher in cases which had lymph node negative status. Interestingly, HPV 31, 33, 45, 52, 58 and 6 (0.6%) were present exclusively in patients with no lymph node metastasis.

Discussion:

In the present study we have screened cervical cancer patients from western India with nonavalent HPV types to denote the efficacy of the Gardasil 9 vaccine in curbing the HPV associated cervical cancer burden in India**.** The HPV analysis for the cohort revealed 77.8% cases to be positive for HPV infection. Prevalence of HPV 16 (86.2%) and HPV 18 (19.0%) infections was higher while HPV 31 (2.3%), 33 (0.5%), 45 (4%), 52 (1%), 58 (0.3%), and 6 (0.5%) were also present No presence of HPV 11 infection was noted in the cohort. A study conducted in Odisha revealed 87.28% cases with HPV 16 infection and 24.56% of HPV 18 infection. They also noted HPV 45 (1.7%), HPV 58 (1.1%), HPV 52(.57%), HPV 6/11 (0.57%), while the overall HPV infection rate was observed to be 60.33% [8]. Moreover, HPV 31 was observed to be 6.1% in a multicentric study [9]. While another study from Nepal reported notable infection rate of HPV 16 (72.2%), HPV 18 (14.8%), 31 (1.8%), 33 (2.4%), 45 (4.8%), 52 (2.4%), 58 (2.4%) and 6 (0.6%) [10].

When we observed the cases with multiple infections, we noted that there was higher prevalence of cases of HPV 16 with 18 infection (7.8%), followed by HPV 16 with 45 (1%) infection and HPV 16 with 31 infection (1%). Increased prevalence of these combinatorial infections has also been observed in other studies which noted 1.8% cases of HPV 16 with HPV 18 infection and 1.2 % of HPV 16 with HPV 45 cases. [10]

HPV 16 (74.2%), 18 (15.9%), 33 (0.8%) and 58 (0.4%) infection were higher in the younger age group (< 50 years) while the HPV 31 (3%), 45 (4.8%), 52 (2.4%) and 6 (0.6%) positivity was higher in older age group. Thus, a higher load of HPV infected cases was observed in the younger age group. A study from Odisha noted a bimodal peak in the prevalence of various HPV types with peaks observed in 36-35 years age group and >55 age group. The frequency of HPV infection with multiple types increases with age which explains cumulative lifetime exposure [8], relative incompetency in viral clearance and insufficient adaptive immune responses at this age caused by hormonal changes at menopausal transition, contributing to HPV persistence or reactivation of latent HPV infections [11,12].

In addition, HPV 16 (73.7%), 18 (14.7%), 31 (2.1%), 33 (1.1%), 45 (2.1%), 52 (1.1%) and 58 (1.1%) were higher in women married at older age, except HPV 6 (1.1%) which was observed exclusively in patients married in younger age. Also, HPV 16 (79.2%), 33 (4.2%) and 58 (4.2%) was higher in perimenopausal women, while HPV 18 (16.7%), 31 (3%) and 58 (4.2%) was higher in premenopausal women. Whereas, HPV 52 (1.1%) and 6 (1.1%) was observed higher in postmenopausal patients. Thus, most of the HPV infection bulk was observed before or during menopause. Similar to this, a bimodal HPV prevalence has been noted in several countries, with the highest peak among young women around the age of sexual debut and a second peak around the age of menopause [13]. The reason for such a pattern could conceivably reflect new sexual exposures, reactivation of latent infection due to perimenopausal hormone fluctuations, or the combination of both. [14]

It was also observed that 73.4% patients with squamous cell carcinomas showed HPV 16 positivity while 13.1 % were positive for HPV 18. In contrast, patients with adenocarcinoma, the HPV 16 (60%) infection rate decreased and HPV 18 (33.3%) prevalence increased. This result is in consensus with earlier reports depicting prominent expression of HPV in SCC while HPV 18 has shown to play the pathogenic role in ADC [15].

On association with the tumor differentiation, we found HPV 16 (78.6%) and HPV 18 (21.4%) infection rate to be higher in well differentiated tumors while HPV 31 (2.4%), 33 (0.8%), 52 (0.8%) and 58 (0.4%) were exclusively present in moderately differentiated tumor, while in poorly differentiated tumors, infection rate of HPV 45 (5.3%) and 6 (1.3%) was higher. It is interesting to note that only HPV 16 and 18 infections were present in well differentiated tumors. Similarly, frequency of HPV infection was observed to be higher in well differentiated tumors than in poorly differentiated tumors [16]. Other researchers including Liang et

al and Seraj et al have also found that HPV 16 was more prevalent in well differentiated than in moderately or poorly differentiated tumors in oral cancer [17,18].

In relation with the stage of disease, HPV 16 (81.8%), 18 (27.3%) and 45 (9.1%) infection rates were higher in patients with stage IV disease. Moreover, HPV 18 infection rate increased with advancement of the disease. HPV 31 (3.2%) infection rates were higher in stage I, while HPV 52 (1.6%) and 6 (1.6%) were higher in stage II. Positivity of HPV 33 (0.8%) and 58 (0.4%) were observed exclusively in stage III. Furthermore, HPV 16 (85.7%, p=0.015) and HPV 18 (14.3%) infection rates were observed to be higher in lymph node positive cases whereas HPV 31 (3.2%), 33 (0.5%), 45 (3.7%), 52 (1.6%), 58 (0.5%) and 6 (0.5%) were observed to be higher in cases which had lymph node negative status. This hints at the more aggressive nature of HPV 16, 18 and 45 as compared to the other analyzed HPV types. Similar results were obtained by Chen et al when they observed HPV infection rates in pelvic lymph nodes. HPV16 positivity in cervical lesions has been shown to be an independent prognostic risk indicator of disease relapse (19). Interestingly, HPV 31, 33, 52, 58 and 6 were present exclusively in patients with no lymph node metastasis. This supported by previous studies which have noted a greater range of HPV DNA positivity from 35.7% to 90.1% in patients with non-metastatic lymph nodes. [19].

The data shows the prominent prevalence of HPV strains in the cervical cancer population of western India. In our study we have observed differential prevalence of each HPV strain when associated with the clinicopathological parameters. This hints at the distinct behavior of each HPV strain and their variegated effect on cervical tumors. HPV strains 16, 18 and 45 were observed to be more prevalent in cases with advanced stage of the disease and lymph node metastasis showing their potential for aggressive disease and distance metastasis. While strains 31 and 52 were observed in cases with early stage and no lymph node involvement which hints their docile potential for aggressiveness of the malignancy.

Conclusion:

A noteworthy prevalence of the HPV strains included in the nonavalent vaccine was observed in the study. HPV 16 infection was significantly higher in patients with lymph node metastasis. The HPV strains differ in the potential for disease progression and metastasis. The data will be useful in strengthening HPV vaccination program in India.

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