Significance of multiple HPV infection in cervical cancer patients and its impact on treatment response

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Received July 21, 2008; Accepted September 10, 2008

DOI: 10.3892/ijo_00000148

Abstract. Human papilloma virus (HPV) is the major cause of invasive cervical cancer (ICC). The study aim was to determine the prevalence of HPV genotypes and to correlate HPV types with response to radiotherapy. A total of 43 cervical biopsies collected from sequentially enrolled patients were analyzed by DNA amplification with MY09/MY11 primers and sequenced to determine the HPV genotype. Samples with multiple infections were resolved by multiplex PCR, combined with array primer extension (APEX). HPV DNA was detected in 40 of 43 (93%) samples. Nine different HPVs, including the most common types -16 (53%) and -18 (13%) were detected. Other types were HPV 31, 33, 45, 52, 58, 66 and 68. Single HPV types were found in 33 of 40 samples (82%) and multiple types in 7 of 40 samples (18%). The following significant predictors were identified: a) HPV 58 was most significant (p=0.02), followed by HPV 18 (p=0.04) associated with lack of treatment response; b) tumor size (p=0.042) and treatment response (p=0.025) elicited association with HPV infection type; c) treatment failure were found to be nearly 5-fold higher in case of multiple infections than of single infection (57% versus 12%) (odds ratio = 9.66; 95% CI 1.6-6.00). d) Multiple HPV infections correlated most prominently with lack of treatment compared with single type infection (p=0.005). Hence, patients with multiple infections, large tumor size, and HPV 58 and/or 18, are at risk of treatment failure and need to be followed for response and suitable inter-ventions done for a favorable outcome.

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Key words: human papilloma virus, cervical cancer

Introduction

Cervical cancer is second to breast cancer in its incidence and mortality among women worldwide. It is estimated that of the half million cervical cancer cases reported in the world every year, at least 80% occur in developing countries (1). Cervical cancer is the most common malignancy among women in India, accounting for 26% of female cancers, with 125,000 women developing the disease and 90,000 dying of cancer annually (2). Most studies worldwide have demonstrated the association between human papilloma virus (HPV) and cervical cancer. More than 100 types of HPVs are known; mucosal types are generally classified according to their potential to induce malignant transformation into high-, intermediate- and low-risk HPVs. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 are considered 'high-risk' types because they are detectable in cervical carcinomas and dysplasias whereas HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81 are regarded as 'low-risk' because they are more common in mild or severe dysplastic lesions than in carcinomas. Among the high-risk strains, HPV 16 and 18 are the most closely associated with cervical carcinoma being found in 50% and <15%, respectively of all cervical cancer cases (3,4). Functionally, high-risk HPV infection contributes to carcinogenesis and tumor progression predominantly through the actions of two viral oncogenes, E6 and E7, which interact and inhibit the activities of critical components of cell cycle regulatory system, represented by p53, which is the main E6 target and Rb, which is target of E7 (5).

Prospective studies have shown that infection with high-risk HPV precedes the development of cervical neoplasia. It is now accepted that HPV may be necessary but not sufficient to cause of cervical cancer since other established co-factors appear to be or are necessary for progression from HPV infection to cancer. These factors include long-term use of hormonal contraceptives, tobacco smoking, co-infection with *Chlamydia trachomatis* (CT) and herpes simplex virus type-2 (HSV-2), immunosuppression, and certain dietary deficiencies. Genetic and immunological host factors and viral factors such as type, variants of type, viral load and viral integration are also important but their role has not been clarified yet (6).

Low educational and socioeconomic level, marriage or first sexual intercourse at young age, high parity and male partner's sexual behavior are other most consistently reported risk factors.

Although the fact that cervical cancer burden in India is high, there are very few large-scale studies describing either HPV prevalence or type distribution in invasive cervical cancer. It is important to describe the distribution of HPV genotypes in cervical cancer samples that represent multiple high-risk populations before these data can be generalized for application in national cancer prevention strategies. Most studies in India have identified HPV 16 and 18 as the most prevalent types in cervical cancer (7-10). The aim of the study was to determine the HPV genotype present in cervical tumor tissue of women attending the Regional Cancer Center in Chennai, India, using highly sensitive polymerase chain reaction (PCR). Rapid PCR-based amplification of HPV DNA with L1 consensus primer systems (e.g. MY09/11 and/or GP5/6) detects as few as 10-100 molecules of HPV targets from a single sample (11). A novel E7 PCR-based assay developed by Gheit *et al* (12) has been used to detect a large spectrum of multiple high-risk HPV types in a single sample. This assay takes advantage of multiplex PCR methods, i.e., high sensitivity and the ability to perform multiple amplifications in a single reaction with an array primer extension (APEX) assay.

We also tried to determine the association between infection with single or multiple HPV types and the response to radiotherapy.

Patients and methods

Patients and sample collection. Fifty consecutive patients with invasive cervical carcinoma, were recruited from the out patients division at the Cancer Institute, Chennai, India. Criteria for enrolling the patients were clinical diagnosis with invasive cancer of cervix, no prior treatment history with chemotherapy or radiation, and willingness for participation. A written consent was taken from all patients participating in the study. Since consecutive patients were enrolled they can be considered as random sample. Seven of 50 later withdrew from the study. The study group of 43 included 40 patients with invasive squamous cell carcinoma (SCC) and 3 with adenosquamous cell carcinoma (ADC). The study was approved by the Institutional Ethics Committee of the Cancer Institute, Chennai and the University of Louisville Institutional Review Board (IRB).

Patients were treated with the standard protocol of 6 MeV X-ray beam therapy to deliver 50-60 Gy to the pelvis, followed by intra-cavitary applicator to deliver 16 or 18 Gy to point A. After 6 weeks gap, they were reviewed every 3 months.

Tumor biopsies were collected in cryovials on ice and immediately stored at -70˚C for subsequent DNA extraction. Clinical staging was evaluated according to the classification of the International Federation of Gynecology and Obstetrics (FIGO 1994). Detailed clinical history, diagnosis and demographics of the patients were gathered from the tumor registry division. Complete case history with socio-demographic status, histopathology and clinical details including nodal involvement, local spread and treatment details were recorded.

DNA isolation. DNA was isolated by solvent extraction method involving enzymatic removal of RNA and proteins with RNase and proteinase K, respectively, followed by sequential extractions with phenol, phenol-sevage (chloroform: isoamyl alcohol 24:1) and sevage. DNA was recovered by precipitation with absolute ethanol and re-suspended in HPLC-grade water. All enzymes were stored in aliquots at -20˚C (13,14).

Detection of HPV DNA. Purified DNA was subjected to PCR amplification using consensus primers MY09/MY11 to amplify a 450-bp fragment in the HPV L1 gene: forward, 5'-CGT CCM ARR GGA WAC TGA TC-3'; reverse, 5'-GCM CAG GGW CAT AAY AAT GG-3' (15). Presence of human genomic DNA and quality of the sample were verified by amplification of a 268-bp fragment of ß-globin gene by using primers PCO4/GH2: forward, 5'-CAA CTT CAT CCA CGT TCA CC-3'; reverse, 5'-GAA GAG CCA AGG ACA GGT AC-3' (16). PCR conditions were as follows: preheating for 2 min at 94˚C was followed by 30 cycles of 30 sec at 94˚C, 30 sec at 55˚C and 60 sec at 68˚C, and a final extension of 10 min at 68˚C. PCR was performed in a final volume of 50 μl, which consisted of 1 μl extracted DNA as template, 5 μl 10X PCR buffer, 1 U Platinum Taq polymerase high fidelity (Invitrogen, CA), 20 pmol each primer and 0.2 mM dNTPs. The final concentration of $MgCl_2$ was 2.5 mM.

The amplicons were checked on 1.5% agarose (Nusieve, Cambrex, ME) and gel-purified using QIAquick gel-extraction kit. HPVs were typed by DNA sequencing, using the same primers of the PCR. The online Blast server was used for sequence analysis. In some samples the results of PCR sequencing suggested multiple infections with two or more HPV types. These were further typed using multiplex PCR by Gheit *et al* method of PCR followed by hybridization on specific probes (12). In brief, multiplex PCR was performed with primers for E7-region gene and included the following HPV 18, 45, 51, 35, 58, 39, 68, 56, 66, 59, 33, 52, 16, 31, 26, 53, 70, 73, and 82. Oligonucleotides were mixed to obtain a 10X solution containing 2 μM of each primer. Two primers for the amplification of ß-globin were added to provide a positive control for the quality of the template DNA. Some primers were used for more than one type (e.g., HPV18R/HPV45R) due to the high similarities between the E7-region genes.

PCRs were performed with the Qiagen multiplex PCR kit according to the instructions of the manufacturer. dUTP was added to a final concentration of 50 μM, to allow PCR product fragmentation. The PCR products ranged from 210 to 350 bp in size. The presence and the sizes of PCR products were systematically checked on agarose gels.

The DNA chip with two 5'-C-6 amino-linker-modified oligonucleotides (C-6 oligonucleotides) covering two 30-bp regions of each E7-region gene designed, synthesized by MWG Biotech, and spotted onto silanized slides was used. All C-6 oligonucleotides were designed in order to incorporate only uracyl (cyanine 5-ddUTP) during the extension reaction.

In APEX protocol, the PCR products were purified, concentrated and fragmented to facilitate the hybridization reaction with the arrayed oligonucleotides. The fragmented PCR products were added to a reaction mixture containing cyanine 5-ddUTP and cyanine 3-ddATP, -ddCTP, and -ddGTP

(4x50 pmol); 10X buffer; and 4 U of Thermo Sequenase (Amersham Biosciences, Uppsala, Sweden) and placed onto the chip and incubated at 58˚C for 10 min. After hybridization of the PCR products to the chip, the extension reaction was performed to allow incorporation of the cyanine 5-ddUTP. The slides were washed to remove the traces of the non-hybridized PCR products and the unused labeled dideoxy-nucleoside triphosphates (ddNTPs). The slides were imaged by Genorama-003 four-color detector (Asper Biotech, Tartu, Estonia). The signal for specific HPV types in the APEX method was considered positive only if both APEX probes gave a signal in the U channel, as the probes were designed to extend only U. The fluorescence intensities at each position were measured and converted to base calls according to the Genorama image analysis and genotyping software (Asper Biotech).

Rolling circle PCR amplification was performed using Templiphi amplification kit (Amersham Biosciences, UK) as per vendor's instructions to identify episomal forms of HPV DNA in the cervical specimens. The product of rolling circle amplification was restricted with *Eco*RI, *Bam*HI, *Hin*dIII and *Pst*I enzymes.

Statistical method. There are two main aims in this study - first, finding association between HPV infection type (none, single and multiple); demographic (age, class), and disease related variables and second, finding predictors of the clinical response. Descriptive statistics such as frequency, percentages, 95% confidence intervals and odds ratios were calculated. The association was estimated using a χ^2 test (Fisher's exact test for 2x2 tables, Pearson's χ^2 for other tables) and Univariate logistic regression model. If p-values were close to 0.10 a more accurate procedure based on StatXact software was used for precise estimation. We chose a p-value of ≤0.05 to declare significant results. Since the sample size is small, results should be interpreted with caution. Statistical analyses were performed using SAS V9 and StatXact V8.

Results

A total of 43 invasive cervical tumor samples were analyzed. The mean age at histological diagnosis was 55 years (range 28-65). Squamous cell carcinoma was diagnosed in 40 patients (93%) and adenocarcinoma in 3 (7%). High-risk HPV DNA was identified in 40 of 43 specimens (93%) (Fig. 1). We did not find low-risk HPV types in our specimens. Three samples were PCR-negative.

Table I represents the associations between socio-demographic, clinicopathological variables and single versus multiple HPV infection in the study group. Tumor size (p=0.042) and clinical response (p=0.025) are two factors that were associated with HPV infection type. Those with multiple infections were more likely to have larger tumor size and negative clinical response. No other clinicopathological factors (*viz*, stage, grade, nodal status and histological type of the tumor) were found to be associated with HPV infection. Socio-demographic factors like education, sanitary habits, location etc. (Table I) had no significant association with HPV infection.

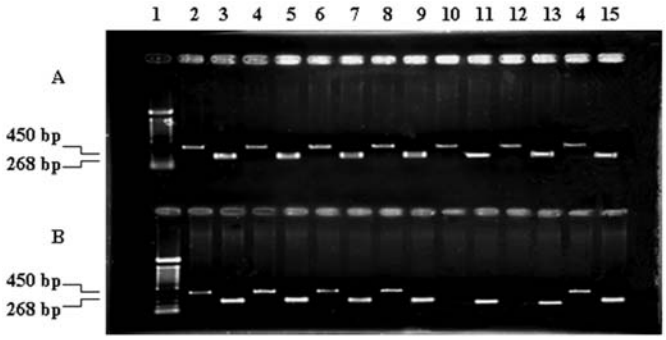

Figure 1. PCR product of HPV L1 gene amplification: Row A and B represent PCR products of 14 DNA samples, even number lanes represent L1 gene product (450 bp) and odd number lanes represent house keeping gene ß-globin (268 bp). Lanes 10 and 12 of row B are negative for L1 gene. Lane 1, DNA ladder.

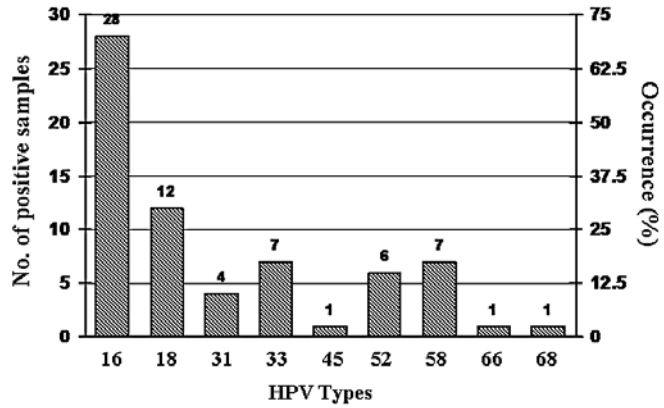

Figure 2. Human papillomavirus (HPV) distribution among 40 positive cervical specimens with both single and multiple infections.

In 33 of 40 cases (82%), single infection with different HPV types were detected. Seven patients (18%) were infected with 4 or more different genotypes. Three patients diagnosed with SCC were negative by PCR; this could be because of low HPV copy number in these samples to be detected by PCR sensitivity. Among patients infected with single HPV type, HPV 16 was by far the most prevalent type (53%) followed by HPV 18 (13%), HPV 33 (10%) and HPV 58 (8%). Seven multiple infection specimens were analyzed by multiplex PCR (12), as results obtained from PCR using consensus primer MY09/11 for L1 gene were not conclusive. These specimens had 4 or more different HPV types and HPV 16 and 18 as common infections (Table II). There was no association between HPV type and clinical histology.

A total of 9 high-risk HPVs were identified in this population i.e., HPV 16, 18, 31, 33, 45, 52, 58, 66 and 68 (Fig. 2), with HPV 16 showing highest incidence (70%), followed by HPV 18 (30%). Both HPV 16 and 18 were present in all multiple infection cases.

The prominent significant predictor of the clinical response was HPV infection (single vs. multiple, p=0.015). Nine of 43 patients (21%) were non-responsive to treatment (Table III). Four of 33 with single-infection (12%) and 4 of 7 (58%) with multiple infection did not respond to treatment [odds ratio (OR) = 9.66; 95% CI 1.6-60.0]. Among the HPV types, 58

Table I. Associations of socio-demographic, clinical-pathologic variable with single versus multiple HPV in cervical patients (n=43).

	No. of patients (%)	No. of negative HPV types (%)	No. of single HPV types (%)	No. of multiple HPV types (%)	χ^2	p-value
Socio-demographic parameters						
Age (years)					2.46	0.29
≤50	31 (72.1)	1 (3.2)	25 (80.6)	5 (16.1)		
>50	12 (27.9)	2 (16.7)	8 (66.7)	2 (16.6)		
Education					0.68	0.41
No	40 (93.0)	3 (7.5)	30 (75.0)	7 (17.5)		
Yes	3 (7.5)	0	3 (100)	0		
Marital status					0.72	0.40
Previously married	7 (16.28)	0	5 (71.43)	2 (28.57)		
Currently married	36 (83.72)	3 (8.33)	28 (77.78)	5 (13.89)		
Consanguinity					0.46	0.50
Yes	18 (41.86)	2 (11.11)	14 (77.78)	2 (11.11)		
No	25 (58.14)	1 (4)	19 (76)	5 (20)		
Age at marriage					0.15	0.69
<17	28 (65.12)	1 (3.57)	25 (89.29)	2 (7.14)		
≥17	15 (34.88)	2 (13.33)	8 (53.33)	5 (33.33)		
First pregnancy					1.95	0.16
≤18	28 (65.12)	3 (10.71)	19 (67.86)	6 (21.43)		
>18	15 (34.88)	0	14 (93.33)	1 (6.67)		
No. of births					1.04	0.31
≥3	27 (62.39)	2 (7.41)	22 (81.48)	3 (11.11)		
<3	16 (37.21)	1 (6.25)	11 (68.75)	4 (25)		
Abortions					0.72	0.40
≥1	7 (16.28)	0	5 (71.43)	2 (28.57)		
None	36 (83.72)	3 (8.33)	28 (77.78)	5 (13.89)		
Menopause attained					0.11	0.75
Yes	28 (65.12)	3 (10.71)	21 (75)	4 (14.29)		
No	15 (34.88)	0	12 (80)	3 (20)		
Addictions					0.10	0.75
Yes	7 (16.28)	2 (28.57)	4 (57.14)	1 (14.29)		
No	36 (83.72)	1 (2.78)	29 (80.56)	6 (16.67)		
Family history					0.00	0.95
Yes	6 (13.95)	0	5 (83.33)	1 (16.67)		
No	37 (86.05)	3 (8.11)	28 (75.68)	6 (16.22)		
Location					0.33	0.57
Rural	34 (79.07)	3 (8.82)	25 (73.53)	6 (17.65)		
Urban	9 (20.93)	0	8 (88.89)	1 (11.11)		
Clinical-pathological parameters						
FIGO stage					3.67	0.45
Ia+Ib	5 (11.6)	0 (0.0)	4 (80.0)	1 (20.0)		
IIa+IIb	25 (58.1)	3 (12.0)	17 (68.0)	5 (20.0)		
IIIa+IIIb	13 (30.2)	0 (0.0)	12 (92.3)	1 (7.7)		
Nodal status					1.83	0.40
Positive	7 (16.2)	1 (14.3)	4 (57.1)	2 (28.6)		

Table I. Continued.

	No. of patients (%)	No. of negative HPV types (%)	No. of single HPV types (%)	No. of multiple HPV types (%)	χ^2	p-value
Clinical-pathological parameters						
Histological differentiation					5.07	0.28
Grade I	4 (9.3)	0	3 (75.0)	1 (25.0)		
Grade II	3 (6.9)	1 (33.3)	1 (33.3)	1 (33.3)		
Grade III	36 (83.7)	2 (5.5)	29 (80.5)	5 (13.9)		
Histological type					0.84	0.66
SCC[a]	40 (93.0)	3 (7.5)	31 (77.5)	6 (15.0)		
ADC[b]	3 (6.9)	0 (0.0)	2 (66.6)	1 (33.3)		
Tumor size					5.89	0.04
≤4 cm	10 (23.2)	2 (20.0)	5 (50.0)	3 (30.0)		
>4 cm	33 (76.7)	1 (3.0)	28 (84.8)	4 (12.1)		
Response					7.37	0.02
Yes	34 (79.1)	2 (5.9)	29 (85.3)	3 (8.8)		
No	9 (20.9)	1 (11.1)	4 (44.4)	4 (44.4)		

[a]SCC, squamous cell carcinoma; [b]ADC, adenocarcinoma.

Table II. Distribution of single and multiple HPV types in 40 HPV-positive women.

Infection type	No. (%)
Single infection	
16	21 (52.5)
18	5 (12.5)
33	4 (10.0)
58	3 (7.5)
Total single infections	33 (82.5)
Multiple HPVs	
16/18/31/52	1 (2.5)
16/18/31/52/58	2 (5.0)
16/18/52/58/68	1 (2.5)
16/18/33/58/66	1 (2.5)
16/18/31/33/52	1 (2.5)
16/18/31/33/45/52	1 (2.5)
Total multiple infections	7 (17.5)
Total	40 (99.6)[a]

[a]Rounding error produces a sum <100.

was the most significant (p=0.02) (OR=8.27) followed by 18 (p=0.047) (OR=4.82) for negative clinical response (Table III). One PCR-negative patient was not responsive to treatment and showed residual/radio-resistant tumor. We also analyzed the physical state of HPV infection, i.e., if, episomal or integrated form were present in cervical carcinoma specimens and found all of the samples to be negative for episomal forms.

Discussion

Epidemiological and molecular studies over the past two decades have convincingly demonstrated that certain types of HPVs have etiological relevance in development of cervical cancer (17). HPV is considered to be a necessary although not a sufficient cause of cervical cancer (18).

Infection with oncogenic high-risk HPV types, of which HPV 16 is the most prevalent, is a major factor for the development of pre-invasive cervical intraepithelial neoplasia (CIN) and invasive cervical carcinoma. HPV DNA, originating from oncogenic types, has been detected in up to 95-99.7% of the cervical carcinomas (18). Due to existence of diversity of HPV types found in cervical cancer, development of effective vaccines would require a detailed study on HPV genotypes in different regions of the world. Prophylactic vaccines composed of HPV type-specific L1 proteins that self-assemble into non-infectious, recombinant virus like particles (VLPs) have been recently approved by FDA and have been proven to be extremely effective in preventing HPV-type specific infection as well as cervical neoplasia. Countries like Australia, Canada, Mexico, New Zealand, Togo, and the United States have approved the use of this vaccine (19). UK plans to start immunization program in September 2008. However, in developing countries, the price of the vaccine represents one of the greatest barriers to its introduction (20). Geographic variations of HPV infection and their possible impact on the effectiveness of the vaccination program in Greece, studied by Mammas *et al*, revealed that HPV 18 and 16 were present in 29.7% and 23% in Crete versus 13.1% and 34.2% in Central

Table III. Summary of risk factor analysis for clinical response - logistic regression (n=43).

	Clinical response			Univariable model	
Predictors	No. of yes (%)	No. of no (%)	No. of total (%)	Odds ratio (95% CI)	p-value
Age					
≤50	27 (87.0)	4 (12.9)	31 (72.1)	1.39 (0.29-6.75)	0.68
>50	7 (58.3)	5 (41.6)	12 (27.9)		
FIGO stage					
I	4 (80.0)	1 (20.0)	5 (11.6)		0.58
II	21 (84)	4 (16.0)	25 (58.1)	1.78 (0.15-21.39)	0.90
III	9 (69.2)	4 (30.7)	13 (30.2)	2.33 (0.48-11.45)	0.50
Nodal status					
Negative	4 (57.1)	3 (42.8)	7 (16.2)	3.75 (0.66-21.25)	0.135
Positive	30 (83.3)	6 (16.6)	36 (83.7)		
Histological differentiation					0.33
Grade I	4 (100)	0	4 (9.3)	NR[b]	
Grade II	3 (100)	0	3 (6.9)		
Grade III	27 (75.0)	9 (25.0)	36 (83.7)	NR	
Histology type					
SCC	32 (80.0)	8 (20.0)	40 (93.0)	2.00 (0.16-24.92)	0.59
ADC	2 (66.6)	1 (33.3)	3 (6.9)		
Tumor size					
≤4 cm	8 (80.0)	2 (20.0)	10 (23.2)	1.08 (0.19-6.26)	0.93
>4 cm	26 (78.7)	7 (21.2)	33 (76.7)		
HPV status					
No infection	2 (66.6)	1 (33.3)	3 (6.9)		0.047
Single infection	29 (87.8)	4 (12.1)	33 (76.7)	9.66 (1.56-60.00)	0.048
Multiple infection	3 (42.8)	4 (57.1)	7 (16.2)	2.67 (0.16-45.13)	0.91
HPV16 status					
No vs. yes	22 (78.5)	6 (21.4)	28[a]	1.09 (0.23-5.16)	0.91
HPV18 status					
No vs. yes	7 (58.3)	5 (41.6)	12[a]	4.82 (1.02-22.84)	0.047
HPV31 status					
No vs. yes	3 (75.0)	1 (25.0)	4[a]	2.95 (0.41-21.13)	0.28
HPV33 status					
No vs. yes	6 (85.7)	1 (14.2)	7[a]	1.66 (0.26-10.39)	0.59
HPV45 status					
No vs. yes	1 (100)	0	1[a]	NR	0.98
HPV52 status					
No vs. yes	3 (60.0)	2 (40.0)	5[a]	5.17 (0.83-32.00)	0.078
HPV58 status					
No vs. yes	5 (83.3)	1 (16.6)	6[a]	8.27 (1.41-48.52)	0.019
HPV66 status					
No vs. yes	0	1 (100)	1[a]	NR	0.98

Response to treatment is determined on the smear cytology report for abnormal/malignant cells post-radiation treatment. [a]HPV as single and co-infection, [b]NR, results are not reliable due to small n.

Table IV. Distribution of HPV types in the current study versus study by Bosch *et al* (30) (1995) involving 22 countries.

Infection type	Current study No. (%)	95% CI	Africa No. (%)	Central and South America No. (%)	Southeast Asia No. (%)	Europe No. (%)	North America No. (%)
Single infection							
16	21 (52.5)	37.2-68.5	79 (42.5)	255 (50.5)	42 (42.9)	56 (65.1)	33 (57.9)
18	5 (12.5)	5.1-25.8	33 (17.7)	48 (9.5)	31 (31.6)	7 (8.1)	9 (15.8)
33	4 (10.0)	3.5-22.7	5 (2.7)	18 (3.6)	2 (2.0)	1 (1.2)	0 (0.0)
58	3 (7.5)	2.1-19.3	5 (2.7)	11 (2.2)	2 (2.0)	1 (1.2)	0 (0.0)
Total single infections	33 (82.5)		-	-	-	-	-
Multiple HPs[a]							
16/18/31/52	1 (2.5)		-	-	-	-	-
16/18/31/52/58	2 (5.0)		-	-	-	-	-
16/18/52/58/68	1 (2.5)		-	-	-	-	-
16/18/33/58/66	1 (2.5)		-	-	-	-	-
16/18/31/33/52	1 (2.5)		-	-	-	-	-
16/18/31/33/45/52	1 (2.5)		-	-	-	-	-
Total multiple infections	7 (17.5)		-	-	-	-	-
Total	40 (99.6)		-	-	-	-	-

Rounding error produces a sum <100. CI, confidence interval. [a]Multiple type infections were not described in detail in the Bosch *et al* study (30).

Greece. This study re-emphases the need to investigate the prevalence of different HPV types to further investigate the effectiveness of HPV vaccination (21).

In this study, HPV 16 predominated (70%), followed by HPV 18 (30%), 33 (17.5%), 52 (12.5%), 31 (10%), 58 (7.5%), 45 (2.5%), 66 (2.5%), 68 (2.5%), considering both single and multiple infections (Fig. 2). The distribution of HPV types found in this study was similar to a recent study reported from India with a sample size of 205 cases and consistent with the most common types found in South East Asia (22).

Various studies demonstrated the presence of integrated HPV 16 and 18 genomes in the vast majority of cervical cancers and in cell lines isolated from cervical malignancies. Our findings reconfirm that HPV in cervical cancer is mainly integrated since we could not detect the episomal form in any of the positive samples. The integration of HPV DNA into the host genome leading to loss of function of E1/E2 proteins and subsequent constitutive expression of the oncoproteins E6 and E7 represent two activation mechanisms for the progression of pre-invasive lesions to cervical carcinoma (23). E6 and E7 proteins deregulate cell-cycle control through interaction with different cell proteins, for example, tumor suppressor gene products such as p53 and retinoblastoma protein (Rb), thereby initiating the immortalization and transformation of HPV-infected cells (24).

Park *et al* concluded that integrated HPV DNAs were detected in patients with far-advanced stage of cervical cancer, with no episomal forms (25). However, Cullen *et al* observed pure integrated HPV DNA, mixed forms and episomal forms in HPV 16 containing cancers, whereas all HPV 18 containing cancers revealed only integrated form (26); Matsukura *et al* detected exclusive episomal forms of HPV 16 DNA in infected cervical epithelium, albeit this form was present in 70% of investigated cervical malignancies (27-29).

We compared prevalence of HPV types in the current study cohort with different ethnic populations reported in the large survey published by Bosch *et al* (30) with 95% Blyth-Still-Casella Exact Confidence intervals (Table IV). This study involved 32 hospitals from 22 countries with 1050 samples in total. It was conducted by International Biological Study on Cervical Cancer (IBSCC) towards understanding the relationship between HPV and cervical cancer. Most prevalent HPV types in single infection types were 16 (52.5% with 95% CI 37.2-68.5%) and 18 (12.5%, with 95% CI 5.1-25.8), with-out any significant difference among the ethnic groups. Single infection types 33 and 58 were somewhat overly represented in our study population. Infection types 16 and/or 18 were present in 33 (82.5%) of the patients and infection type 16 single or 16 with multiple other infection types are present in 28 (70%). Our study sample though small represented the HPV distribution observed in the larger worldwide studies which is noteworthy.

One of the most interesting observation was the presence of multiple infections with 4-6 HPV types in 7 (18%) of 40 cervical cancer patients. This finding is consistent with previous studies with cohort from France that reported 22% of multi-type HPV infections (17). A meta-analysis of 85 studies however, reported multiple infection in only 3.7% of all ICC cases (31). Consensus primer PCR assays using MY09/11 are not optimized for amplifying multiple types in the same mixture and some HPV types are amplified less effectively than others. Underestimation of the number of types for those

who develop cervical lesions could underestimate the effect of multiple types. Studies from India have reported infection with 2-4 HPV types in invasive cancers, Franceschi *et al* (22) reported multiple infections in 30 of 190 (15.7%) cases of cervical carcinoma whereas 18% multiple infections were described by Peedicayil *et al* (32). The multiple infections reported in these studies included HPV 16/39/52/58, 16/58/52/33, 16/31/33, 16/33/45, 16/18 etc. In our study, multiple infections always included both HPV 16 and 18, in association with other high-risk HPVs like 31, 33, 45, 52, 58, 66 and 68. In other studies, infections with multiple high-risk HPV types were found in 33.3%, 41.8% and 40.4% of samples with borderline, mild and high grade dyskaryosis, respectively (33). Multiple HPV genotypes were found to be a risk factor for persistent infection in healthy young women and co-infections involving HPV 16 and 58 seemed particularly prone to increased risk. Multiple HPV types seem to act synergistically in cervical carcinogenesis (34). A recent study also indicated that the presence of HPV co-infections might contribute to the development or progression of cervical dysplasia (35). In our study, 4 of 7 (58%) patients with multiple HPV types did not respond to radiation treatment, whereas only 4 of 33 (12%) patients with single HPV types were non-responsive. Although the sample size is small this observation suggests that cancers with multi-type HPV could be more resistant to therapy than those with a single infection. Multi-type infections were found to be common among high-risk populations and conferred greater risk for untoward outcomes. The true biological significance of multiple HPV genotypes in cervical carcinoma is still to be clarified. It has been presumed that HPV-associated cervical cancer is monoclonal in origin and that a single HPV type is responsible for the tumor development. It would be interesting to study the physical status of the papilloma virus in multi-type infection and the role of E6, E7 and E2 gene expression in these patients.

HPV vaccines have been found effective in preventing cervical neoplasia and are currently available for public health programs in developed countries. However, these vaccines only prevent the infection from the 2 most common oncogenic types (HPV 16 and 18). Our data in conjunction with the recent findings in South Asian region (36,37), who reported significant contribution of HPV types 45, 33, 35 and 58 for 20% in occurrence of cervical cancer suggests a need for a second generation of vaccine for optimal cervical cancer prevention in this region. Also to be determined is whether prevention of the most common high-risk HPV types would lead to the emergence of other HPV types as the predominant oncogenic viruses. Identification of novel types and HPV variants by sequencing would also be of importance.

In summary, this study was an attempt to show the distribution of various HPV types in cervical cancer patients attending the regional cancer center of Chennai, Southern, India and to reveal a possible correlation of HPV infection with clinical and histopathological characteristics, severity of the disease and response to treatment. We observed that the rate of patients not responding to treatment was nearly 5-fold higher in patients with multiple infections in comparison with single infection (58% versus 12%, respectively). Similar observations were made by Bachtiary *et al* (38) in a cohort from Austria where cervical cancer patients with multiple HPV infections had a significantly shorter progression-free survival and cervical cancer-specific survival. Hence, more emphasis and studies are warranted in patients with multiple infections, large tumor size and infection with HPV types 58 and 18 need to be followed for treatment response and suitable interventions given as required.

Acknowledgements

We thank Dr G. Selvaluxmy, Department of Radiation Oncology and staff of the Out-patient Division, Gynaecology section and Female ward of Cancer Institute (WIA), Chennai, India, for their extended cooperation during specimen collection. We are very grateful to Dr Massimo Tommasino and Dr Tarik Gheit, Infections and Cancer Biology Group, International Agency for Research on Cancer, Lyon, France for analyzing sample with multiple infections as a goodwill gesture. The work was supported, in part, by Duggan Endowment Funds.

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