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Original Paper

Prevalence of Serum Antibodies to Synthetic Peptides to HPV16 Epitopes Among Indian Women with Cervical Neoplasia

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Serum samples from 38 Indian women with cervical intraepithelial neoplasia (CIN) and cancer along with 20 control women were analysed for IgG antibodies against a panel of four synthetic peptides derived from early and late gene open reading frames of HPV16. The cervical tissue samples from these patients were also subjected to PCR in order to detect the presence of human papilloma viruses (HPVs) type 6, 11 and 16 DNA, using a set of primers for the E6 open reading frames in order to establish the acquisition of HPV infection. E2, E6 and L1 peptides of HPV16 were found to be highly reactive with the patients' sera, and a significant prevalence of antibodies to E6, E2 and L1 ($P < 0.01$) was observed among women with CIN, while antibodies to E6 peptide were only significantly elevated ($P < 0.01$) among women with cervical invasive carcinoma. Furthermore, there was good agreement between the development of the E6 antibody response and the presence of HPV16 E6 DNA among women with CIN and cervical invasive carcinoma as shown by high values of the kappa coefficient (0.7–1.0). The study clearly revealed a strong agreement between two assay methods defining unambiguous immunogenic B-cell epitopes on synthetic peptides to early and late gene open reading frames of HPV16 which could be used in HPV peptide serology. As such, single or combination of peptides in HPV peptide serology can be used as a screening tool to identify HPV-associated cervical lesions. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

CANCER OF uterine cervix is the commonest malignancy among Indian women, with 90000 new cases every year. Numerous clinico-epidemiological studies have implicated the venereally transmitted agent human papilloma virus in human cervical carcinoma [1–6]. HPV16 is the predominant type in cervical uterine tumours and is often integrated into the host cellular DNA [7, 8]. Molecular studies worldwide have shown that 80–85% of human cervical carcinomas contain HPV 16/18 DNA, suggesting that this parameter is important for identifying women at risk [5,7,9–15].

E6 and E7 proteins are the transforming proteins of oncogenic HPVs and are consistently expressed in HPV-associated cervical cancer. Similarly, E2 gene function is pleiotropic and affects a number of viral functions including transformation, replication and plasmid maintenance [16]. Natural viral E2 protein has not yet been clearly identified. The expression and detection of HPVs are modulated by the immune status of the host. To date, the development of serological assays has been hampered by a lack of appropriate target antigen. A serological assay could provide valuable information on transmission and the natural history of HPV-associated disease.

Several studies have reported prevalence of serum antibodies to transforming (E6/E7) and capsid (L1/L2) open reading frames to human papilloma virus in patients with cervical neoplasia [17–21]. Dillner and associates [22] mapped linear epitopes of human papilloma virus type 16E1, E2, E4, E5, E6 and E7 open reading frames, and also showed that, although 87% of patients with cervical carcinoma had antibodies to E2 peptides in their serum, only a few had antibodies to E7 peptides. However, the interpretation of

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these studies remained inconclusive as the serum donors were not fully characterised in terms of their viral status.

We used synthetic peptides representing epitopes on E2, E6, E7 and L1 open reading frames of HPV16 as target antigens in a serological assay (ELISA), capable of measuring human serum antibodies in a type-specific manner. An attempt was made to demonstrate the serum antibody response to HPV 16 infection among Indian women with cervical intraepithelial neoplasia as well as invasive cervical carcinoma in order to identify serological markers for HPV-associated cervical lesions.

MATERIALS AND METHODS

Study subjects

A total of 58 subjects were randomly selected from an existing study on the natural history of cervical neoplasia being carried out at the Institute of Cytology and Preventive Oncology (ICMR), New Delhi. Five millilitres of blood were collected at the time of enrolment of subjects into study and sera were separated. The patients were diagnosed using standard histopathological criteria as chronic cervicitis (20 cases), various grades of cervical intraepithelial neoplasia (CIN I, II and III) (19 cases) as well as invasive cervical carcinoma (19 cases). All cervical tissues were indexed for HPV 6, 11 and 16 DNA sequence in paraffin embedded tissues by polymerase chain reaction (PCR) assay.

Synthetic peptides

Four synthetic peptides (Table 1) derived from B-cell immunoreactive epitopes of E2, E6, E7 and L1 open reading frames of HPV16 (kind gift of Dr Raphel Visicidi, the Johns Hopkins School of Medicine, Baltimore, Maryland, U.S.A.) were evaluated as serological reagents in an enzyme-linked immunosorbent assay (ELISA).

PCR analysis for HPV DNA

(i) *Treatment of sections.* A single 5–6 μm thick paraffin section was placed in a 500 μl Eppendorf tube. The average surface area of the section was 3 mm^2. Each section was deparaffinised with 400 μl of octane, vortexed and then pelleted by centrifugation. The octane was decanted and its residues were removed by two washes with 400 μl ethanol. The specimens were resuspended in 50 μl of 50 mM Tris–1 mM EDTA containing 0.54% Tween-20, 0.45% NP40 and 200 μg/ml Proteinase K (Boehringer Mannheim), and incubated overnight at 37°C. The samples were heated at 100°C in a water bath for 7 min to inactivate proteinase K.

(ii) *PCR procedure.* Five microlitres of the supernatant of the test sample was used for each reaction. The PCR reaction mixture contained 200 μM of dATP, dCTP, dGTP and dTTP, 10X buffer (50 mM KCl, 15 mM $MgCl_2$), 0.01% gelatine w/v and 10 mM Tris–HCl, pH 8.3; 0.25 μM of each oligonucleotide primer and 1 unit of thermostable Taq polymerase (Perkin–Elmer Cetus Instruments, U.S.A.). The primers were type specific and were designed to amplify a 134-239 base pair region of the E6 open reading frame of HPV 6, 11 and 16 (Table 2). The PCR was performed with the following cycle profile; denaturation at 94°C/min, annealing at 42°C/min, extension at 72°C/min for 45 cycles of amplification.

(iii) *Identification of PCR products.* The PCR products were identified by hybridisation in Southern transfer and slot blot formats as reported by Sharma and associates [14].

Enzyme-linked immunosorbent assay for synthetic peptides to HPV16 E2, E6, E7 and L1 open reading frames

All four synthetic peptides were first titrated in order to determine the optimum concentration of each peptide for use in the ELISA. Accordingly, 50 μl of optimally (25 μg/ml) diluted peptides in phosphate-buffered saline (PBS, pH 7.4) or carbonate buffer (pH 9.6) was adsorbed on to Nunc Immunoplates by overnight incubation at 4°C. PBS or carbonate buffer were also used to monitor the non-specific adsorption of sera in the immunoplate. Serum specimens were tested in duplicate in ELISA. Human sera diluted 1:25 in PBS with 3% BSA was added to each well and incubated for 1 h at 37°C. After three washings with PBS containing 0.05% Tween-20, plates were treated with anti-human goat Immunoglobulin-G (IgG) conjugated with horseradish peroxidase enzyme (Sigma), diluted 1:1000 in PBS containing Tween-20 for 1 h at room temperature. Excess conjugate was removed by washing three times with PBS containing 0.05% Tween-20, and the reaction was visualised with 100 μl of ABTS enzyme substrate. Absorbance of the colour reaction was read at 405 and 490 nm. Using a mean optical density (OD) with 2 S.D.s of the background absorbance for each peptide as a cut-off point, the percentage of positive sera among each group was calculated.

Statistical analysis

Sensitivity and specificity of the two tests, that is, HPV DNA by PCR versus seropositivity to synthetic peptides by ELISA, were estimated. The kappa statistic was used to test for agreement between the two tests. The differences in the positivity proportions between different groups of subjects were tested using χ^2-test of significance.

RESULTS

Prevalence of HPV antibodies

In the present study, serum samples from women characterised for HPV DNA by polymerase chain reaction (PCR), having various grades of cervical intraepithelial neoplasia,

Table 1. Immunoreactive peptides of HPV 16

Open reading frames	Peptide sequence	Length of peptide
E2	HKSAIVTLTYDSEWQRDQC	19 aa
E6	MHQKRTAMFQDPQERPRKLPQLC	23 aa
E7	PTLHEYMLDLQPETTDLYCYEQLNDSSEEE	30 aa
L1	IACQKHTPPAPKEDPLKKYTFWEVNLK	27 aa

aa, amino acids.

Table 2. Oligonucleotide sequences of all primers and probes for HPVs for E6 open reading frames and human β-globin gene of PCR

Primer/probe	Nucleotide/sequence	G/C (content) (%)	Length of amplimer (base pair)
Primer			
HPV 6-1	CACCTAAAGGTCCTGTTTCG	50	
HPV 6-2	CGGTTTGTGACACAGGTAGC	50	183
Probe			
HPV6	AGGCGGCTATCCATATGCAG	55	
Primer			
HPV 11-1	GTTGCTTAGAACTGCAAGGG	50	
HPV 11-2	CGGCTTGTGACACAGGTAAC	55	134
Probe			
HPV 11	GCTGCATATGCACCTACAGT	50	
Primer			
HPV 16-1	ACAGTTACTGCGACGTGA	55	
HPV 16-2	TTTGTTCAGGACACAGTGGC	50	239
Probe			
HPV 16	GAGATGGGAATCCATATGCT	45	
Primer			
β-Globlin-1	GAAGAGCCAAGGACAGGTAC	55	
β-Globin-2	CAACTTCATCCACGTTACACC	50	268
Probe	CTGACTCCTGAGGAGAAGTC		
β-Globin	TGCCGTTACTGCCCTGTGGG	60	

invasive carcinoma along with chronic cervicitis as control women were tested for IgG antibody reactivities to synthetic polypeptides, encoded for transforming (E6/E7), transactivating (E2) and capsid (L1) open reading frame derived from HPV16, utilising enzyme-linked immunosorbent assay (ELISA).

It was observed that HPV16 E6 encoded polypeptide was more reactive among 42% cases of cervical intraepithelial neoplasia (CIN) as compared to 36% cases of invasive cervical carcinoma. None of the 20 cases of chronic cervicitis showed any seropositivity to HPV16 E6 polypeptide. The differences observed in the positivity rate between chronic cervicitis versus CIN and chronic cervicitis versus invasive cervical carcinoma were found to be statistically significant ($P = 0.0012$ and 0.032, respectively). Surprisingly, a poor responsiveness of antibodies to E7 encoded peptide to HPV16 was observed among CIN (11%) as well as invasive cervical carcinoma (15%). No seropositivity to E7 peptide was found among control women. Seropositivity to E7 peptide among CIN and invasive cervical carcinoma was not found to be statistically significant as compared to controls.

It was interesting to observe a high seroprevalence to the L1 peptide of HPV16 among women with CIN lesions (58%), but cases of invasive cervical carcinoma and controls revealed almost similar seroprevalence to L1 peptide (11 and 10%, respectively). The differences in seropositivity to L1 peptide between CIN and control subjects were observed to be statistically highly significant ($P < 0.001$) (Table 3).

In contrast to this, it was most striking finding of the study that IgG antibodies to E2 peptide of HPV16 were more common in women with CIN (68%) as compared to controls (20%) (Table 3). The differences in the seropositivity rate was found to be significant ($P < 0.002$). Further the proportion of women with invasive cervical carcinoma who had IgG antibodies to E2 (31%) did not increase significantly when compared to controls ($P > 0.30$). The data also indicated an inverse relationship of HPV16E2 transactivation protein to antibody response to E6 and L1 among women with CIN. As such, it is tempting to speculate that loss of E2 function may lead to integration, transformation and malignancy.

Detection of HPV DNA by polymerase chain reaction (PCR)

HPVs are commonly known to establish subclinical or latent infection of genital tract epithelium. Therefore, it was essential to use an independent measure of HPV16 infection by PCR in order to establish the specificity of antibody production in response to prolonged antigenic stimuli of HPV16. As such, cervical tissues from above subjects were analysed for HPV 6, 11 and 16 DNA by PCR.

Interestingly none of the control women revealed the positivity for E6 HPV16 DNA while 57% women with CIN lesions and 68% cases of invasive cervical cancer showed positivity to HPV 16 DNA by PCR.

As regards HPV 6/11 DNA status, 25% control women revealed positivity for the same while none of the cases of CIN and cervical carcinoma were positive for 6/11 DNA.

Sensitivity, specificity and serum antibody response to synthetic peptides to HPV16 open reading frames

The sensitivity, specificity and kappa coefficient for agreement between the two assay methods are shown in Table 4.

Comparison of HPV16 DNA status with seropositivity to synthetic peptides of HPV16 ORF revealed a high sensitivity (ranged from 73 to 100%) and specificity ranging from 75 to 100% with regard to antibodies to E6, E2 and L1 synthetic peptides amongst CIN lesions. Although E7 peptide had a high specificity (100%), it showed a poor sensitivity (18%). The agreement between the two assay system revealed a high kappa coefficient value ranging 0.7 to 1.0.

With regard to invasive cervical carcinoma, seropositivity to

Table 3. Prevalence of serum IgG antibodies to synthetic peptides of HPV16 epitopes among Indian women

Study group	Positivity for E6 open reading frames HPV DNA 6+11	16	Seropositivity of synthetic peptides to HPV16 E2	E6	E7	L1
Chronic cervicitis ($n = 20$)	5/20 (25%)	0/20	4/20 (20%)	0/20	0/20	2/20 (10%)
CIN (I/II/III) ($n = 19$)	0/19	11/19* (58%)	13/19 (68%)*	8/19 (42%)*	2/19 (11%)	11/19 (58%)*
Invasive carcinoma ($n = 19$)	0/19	13/19 (68%)*	6/19 (32%)	7/19 (37%)*	3/19 (16%)	2/19 (11%)

*Probability estimated by exact test for distribution in disease categories as compared to control women. Significant P value = <0.01.

Table 4. Comparison of sensitivity and specificity of seropositivity to synthetic peptides derived from early and late open reading frames of HPV16 with DNA status of HPV16

Cytodiagnosis	Type of HPV	Synthetic peptides to open reading frames of HPV16	HPV16 DNA status: Positive — Serum antibodies: Positive	Positive — Serum antibodies: Negative	Negative — Serum antibodies: Positive	Negative — Serum antibodies: Negative	Kappa coefficient (KQ)	Sensitivity	Specificity
Chronic cervicitis ($n = 20$)	6/11	E2	4	1	0	15	0.83	4/5 (80%)	15/15 (100%)
		L1	2	3	0	15	0.50	2/5 (40%)	15/15 (100%)
CIN ($n = 19$)	16	E2	11	0	2	6	0.77	11/11 (100%)	6/8 (75%)
		E6	8	3	0	8	0.69	8/11 (73%)	8/8 (100%)
		E7	2	9	0	8	0.14	2/11 (18%)	8/8 (100%)
		L1	11	0	0	8	1.0	11/11 (100%)	8/8 (100%)
Invasive cervical carcinoma ($n = 19$)	16	E2	6	7	0	6	0.35	6/13 (46%)	6/6 (100%)
		E6	6	0	1	12	0.87	6/6 (100%)	12/13 (92%)
		E7	3	3	0	13	0.70	3/6 (50%)	13/13 (100%)
		L1	2	11	0	6	0.12	2/13 (15%)	6/6 (100%)

E6 alone indicated a high sensitivity and specificity with DNA status of HPV16. Although there was a high specificity for E2 and L1 polypeptides, a low sensitivity was found for E2 and L1 peptides. Similarly, E7 revealed a sensitivity of only 50% for HPV16 DNA but with high (100%) specificity.

Further, the control group of women showed a high sensitivity and specificity between HPV6/11 and E2 peptide. However, a poor sensitivity was observed for L1 peptide, although specificity was very high. The kappa coefficient (KQ) also revealed a high agreement between HPV6/11 DNA and E2 peptide.

DISCUSSION

The findings of the study revealed a high sensitivity as well as specificity for serum antibody response to synthetic peptides to E2, L1 and E6 open reading frames of HPV16 by ELISA with DNA status of HPV16 among CIN and invasive cervical carcinoma. Seroprevalence of IgG antibodies to E2, E6 and L1 peptides of HPV16 among CIN and cervical cancer of the present study also revealed a strong agreement between the two assay methods used, that is serum antibody response by ELISA and HPV DNA status by PCR. The study also clearly showed an independent immunoregulation of antibody response to E2, E6 and L1 synthetic peptides among women with CIN and cervical cancer as revealed through the high sensitivity and specificity which is perhaps related to viral gene activity in the tumour. The development of HPV antibodies parallels the acquisition of HPV infection as determined by HPV DNA detection, as evident by high values of the kappa coefficient (0.7–1.0). Antibody response to HPV16 E6 synthetic peptide is intriguing because E6 gene is involved in HPV-mediated cellular transformation. Based on the findings of the present study, these antibodies may serve as a tumour marker for HPV-associated cervical lesions. E6 peptides have been shown to contain several different epitopes to which an antibody response has been reported by Dillner [23]. Muller and associates [24] have also described several immunoreactive epitopes of E6 that preferentially react with cervical cancer patient's sera although with low sensitivity. Surprisingly, we observed a poor responsiveness anti-peptide antibody to E7 open reading frames of HPV16 among CIN and cervical cancer. This peptide has previously been shown to be reactive with IgG in the sera of 37% of cervical cancer patients but only 9% of control sera [25]. It may possibly be due to differences in antigenic epitope reactivity of the E7 peptide used in this study which may not be similar to that of peptide E7/2 [26] and E7/2 [22].

The most striking finding of the study is a highly elevated

seropositivity to E2 peptide of HPV16 among CIN patients, which is in good agreement with earlier reports [16, 17, 27, 28]. Interestingly, the seropositivity to E2 peptide, which significantly decreased in patients with invasive carcinoma to the level observed in control women, suggests that the E2 gene is an important factor in viral function particularly transformation. The association of antibodies to HPV16 E2 in CIN patients does, therefore, suggest that the natural history of cervical cancer at some point of time involves exposure to E2 antigen from HPV16 or related virus types.

The high seropositivity (58%) to L1 peptide of HPV16 revealed a preferential response among CIN patients as compared to invasive cervical carcinoma (11%). L1 open reading frames encodes the major structural component of the virion which is higly conserved among HPV types. Several studies have reported that serological reactivity to the major capsid protein L1 peptide is strongly age-dependent [27], which does not remain in infected individuals in a persistently active form.

This study clearly revealed that a synthetic protein derived from HPV16, utilising single or combination of several peptides to HPV16, can be used in HPV serology to define immunoreactive B-cell epitopes. As such, demonstration of antibody responses to individual human papilloma virus (HPV) epitopes by serology could be employed as a screening tool to diagnose women harbouring HPV-associated cervical lesions. However, in order to utilise this tool for screening the general population, the study needs to be carried out in well-defined larger population groups.

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