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Variations in immunogenetics, human papillomavirus (HPV) infection & predisposition to cervical cancer in Indian women

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Background & objectives: **Human papillomavirus (HPV) is the main causative agent for cervical cancer. Variability in host immunogenetic factors is important in determining the overall cellular immune response to the HPV infection. This study was carried out to confirm the association between human leukocyte antigen (HLA) class II alleles and cervical cancer in HPV infected women.**

Methods: **Both low and high resolution methods were used to genotype HLA class II (DRB1 and DQB1) alleles in 75 women with cervical cancer (cases) and 75 HPV positive women and 100 HPV negative women with healthy cervix (controls). Odds ratio and 95% confidence interval were calculated. Co-occurring HLA alleles (haplotype) across cases and controls were also studied.**

Results: **Significant association was found for HLA-DRB1*03(*13:01) and - DQB1*02(*02:01) with increased risk for cervical cancer. Also, HLA-DRB1*13(*13:01); -DQB1*06 and -DQB1*03:02 were significantly associated with decreased risk for cervical cancer. Haplotype analysis highlighted the significant association of HLA- DRB1*07:01-DQB1*02:02 and HLA DRB1*10:01-DQB1*05:01 with cervical cancer, while HLA-DRB1*14:04-DQB1*05:03 and DRB1*15:01-DQB1*06:01 conferred decreased risk for cervical cancer. Multivariate analysis highlighted the association of specific alleles with cervical cancer after adjusting for confounding factor age.**

Interpretation & conclusions: **There were possible associations of specific HLA class II alleles either with risk of developing cervical cancer, or with its protection. Our results confirmed the assessment of DRB1*13 as a protective marker in HPV infection outcome. Our study also revealed protective association of homozygous haplotype DRB1*15- DQB1*06 with cervical cancer.**

Key words Cervical cancer - human leukocyte antigen (HLA) - human papillomavirus (HPV) - India - risk factors

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Cervical cancer is the second most common cancer among women worldwide, and it ranks as the first most frequent cancer among Indian women[1]. The major risk factor for cervical cancer is infection with specific high-risk types of human papillomavirus (HPV). There exists a link between HPV infections and cervical cancer[2]. However, majority of women infected with HPV do not develop cervical intraepithelial neoplasia (CIN) or cancer. Experimental and clinical evidences demonstrate that the immunological and genetic backgrounds of the host play an important role in the outcome of HPV associated infection. The increased rate of HPV related diseases in patients with cellular immunodeficiency suggests an important role of the immune response in control of HPV infection[3]. An effective host immune response to HPV infection prevents persistence of the virus, an important determinant in infection outcome. Identification of genetic factors that influence the pathogenesis of HPV infection is important to devise preventive strategies for the disease. Host genetic factors, specifically human leukocyte antigen (HLA) with an extraordinary degree of polymorphism have an important role in HPV infection and further contribute to the progression to cervical cancer[4]. HLA class I and II molecules play a critical role in presenting HPV derived peptides to T cells. Engagement of T-cell receptor with HPV peptide-HLA complex and a co-stimulatory signal are necessary to activate a T- cell response and this may vary with HLA type[5]. HLA class I molecules are found on most nucleated cells and present peptides from cytosol to cytotoxic T cells. HLA class II molecules are found on antigen presenting cells and present peptides degraded in intracellular vesicles to helper T cells[6]. Optimal peptide presentation by both class I and II molecules is required to activate efficient helper and effector T-cell responses against HPV.

Studies over past decade have examined associations between HLA and cervical cancer[7-14]. However; the information is limited with respect to HLA association with cervical cancer in India. One study from eastern region of India highlighted the association of HLA-B*40:06 with protection, and a positive association of HLA-B*13:01 and HLA-B*18:01 with cervical cancer[15]. This group also reported over-representation of HLA-DQB1*03 in cervical cancer cases[16]. HLA-DRB1*15allele/DRB1*15-DQB1*06 haplotype was reported to be associated with predisposition to cervical cancer, whereas the HLA-DRB1*04 allele/DRB1*04-DQB1*03 haplotype might exhibit susceptibility to cervical precancerous lesions in north Indian population. HLA-DRB1*13allele/DRB1*13-DQB1*06 haplotype was strongly protective against risk to HPV infection as well as cervical cancer[17]. These studies on significant association of HLA with cervical cancer highlighted the importance of further study for confirmation of these findings in more number of cervical cancer patients from other regions of India. Keeping these in mind, we undertook the present study to confirm the association of polymorphisms in HLA class II alleles (DRB1 and DQB1) with cervical cancer in HPV infected women.

Material & Methods

Subjects: Women attending the outpatient department of TATA Memorial Hospital, a tertiary referred cancer hospital in Mumbai, during 2009-2013, were informed about the study. Women, who consented to give a part of their biopsy specimen without interfering in their medical diagnosis were enrolled as cases (n=75). Their malignancy was confirmed by histopathology as squamous cell carcinoma (scc).

Similarly, for recruitment of controls with normal cervix and with or without HPV infection, women attending the Gynaecology outpatient department of Seth G.S. Medical College and KEM Hospital, Mumbai, during 2009-2013 for family planning advice were approached (two days/week).They were counselled on HPV infection and cervical cancer. A total of 855 women agreed for screening of HPV infection and for further evaluation. Written informed consent was taken from each of these women according to the protocol approved by the Institutional review board of National Institute for Research in Reproductive Health (NIRRH) and of the respective hospitals.

Specimen: A part of the cervical biopsy tissue was collected from established cancer cases in 0.1 M phosphate buffer saline (PBS) solution for HPV detection and typing. Papanicolaou (Pap) test was done in women in control group to record the morphology of the cervix. Cervical swab specimen was collected in 0.1 M PBS solution from each of these women for HPV detection and typing. Peripheral blood specimens were collected from both the groups in EDTA for HLA typing. DNA from blood, tissue and swab specimen was extracted by in-house standardized method[18]. Its quality was assessed by PCR for beta-globin gene.

HPV detection and typing: HPV infection was screened by PCR using L1 consensus primers MY011 and MY 09[19]. Each amplified product of 450 bp was further confirmed with hybridization with biotin labelled

generic HPV probe. Restriction fragment length polymorphism (RFLP) and sequence analysis of 450 bp was done to confirm the type of HPV infection. When amount of available DNA was less, the typing was done using southern hybridization with HPV type specific probes[19,20].

HLA typing: HLA typing was carried out using polymerase chain reaction - sequence specific oligonucleotide probe (PCR-SSOP) method (SSOP, Invitrogen). In brief, the test was carried out in three major steps: PCR target amplification, wherein, DNA specimen was denatured, separating the double stranded DNA and exposing the specific primer target sequences. As the mixture cools, the biotinylated primers anneal to their targets in the presence of DNA polymerase and deoxynucleotide triphosphates (dNTPs) to produce biotinylated DNA sequences. The PCR amplification was followed by hybridization reaction, wherein, the amplicons were chemically denatured to form single stranded DNA and was added to a nylon membrane that contains an array of immobilized sequence specific oligonucleotide probes. The amplicons hybridized with oligonucleotide probes, containing a complementary target sequence captured onto the membrane. After hybridization, the amplicon-probe complex was detected with addition of streptavidin-horseradish peroxidase (SA-HRP) conjugate and hydrogen peroxide (H_2O_2)-tetramethylbenzidine (TMB) substrate. The resulting probe signals were compared with the control probe intensity and the samples hit patterns were recorded for interpretation. Randomly selected specimens were reassessed to check the reproducibility of the analysis. At the initial stage of standardization, quality control analysis of HLA typing was done in the Department of Transplant Immunology and Immunogenetics, All India Institute of Medical Sciences, New Delhi.

HLA data processing: The data were analyzed using pattern matching computer software program (PMP, version 5.42, Invitrogen, USA).

Statistical analysis: Chi-square test was applied to compare the number of cases and controls who were positive for specific allele. Allelic frequencies; odds ratios (OR) with respective 95 per cent confidence intervals were estimated using the SPSS software version 19 (SPSS, Chicago, Il, USA). Binary logistic regression analysis was performed to see the effect of the confounding factors like age on association of significant alleles. For allele and haplotype analysis, Bonferroni correction was applied to determine significant level (0.05/ n where "n" is the sum of conditions in whole study).

Multilocus analysis: To have the analysis with meaningful sample sizes, we examined the co-occurring alleles that occurred in >4 per cent of cases or controls to limit spurious associations. The population genetics package, PyPop, developed by the Biostatistics Core for the Workshop was used to estimate the haplotypes[21].

Results

HPV typing: Of the 75 women (median age: 48 yr) with established squamous cell carcinoma, 74 (98.67%) were positive for HPV DNA. HPV type 16 was the most common type (24%); multiple infections were seen in 70.6 per cent of the cases. A total of 855 women were screened to enroll 75 (8.8 %) HPV positive women (median age: 40 yr) and 100 HPV negative women (median age: 38 yr) with healthy cervix as control group. HPV 16 was the most common HPV type (n=11, 14.6%). Of the remaining, 15 (20%) had co-infection with two or three different types of HPV, while 35 (46.7%) had HPV types other than 16/18/6/11. None of the women of any groups had family history of cancer.

HLA typing: HLA class II allele analysis was completed in all 75 cases. In the control group, typing was also completed in all 100 women without any reproductive tract/sexually transmitted infections (RTIs/STIs). Among the 75 HPV positive women with normal cervix in the control group, HLA DRB1 and DQB1 typing could be done in 68 and 73 women, respectively. In the remaining subjects HLA typing was not done due to less concentration of available DNA, hence HLA-DRBI in control was completed in 168 and HLA-DQBI was completed in 173 participants.

There were 14 HLA-DRB1 and 5 HLA-DQB1 identified alleles (Figs 1,2). Further analysis at high resolution revealed 40 HLA-DRB1 and 28 HLA-DQB1 alleles. HLA-DRB1*02, -*03, -*11 and HLA-DQB1*02 were observed to be associated with increased risk for cervical cancer, while HLA-DRB1*13, -*14,-*15 and HLA-DQB1*06 were associated with decreased risk for cervical cancer ($P<0.05$). However, after applying Bonferroni correction, HLA-DRB1*03 ($P<0.003$) and DQB1*02 ($P<0.001$) were found to be significantly associated with increased risk for cervical cancer. On the other hand, allele DRB1*13 ($P<0.003$) and DQB1*06 were significantly ($P<0.01$) associated with decreased risk for cervical cancer. Significantly associated specific alleles either with increased risk or with decreased risk were given (Tables I, II).

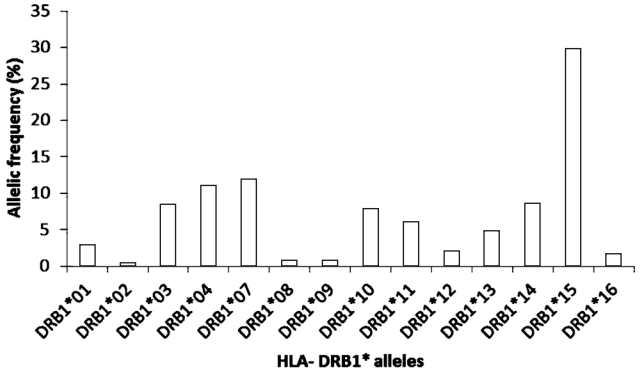

Fig. 1. Distribution of HLA- DRB1* alleles in study population.

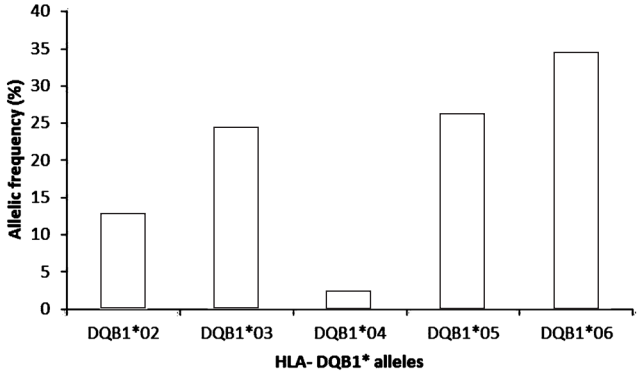

Fig. 2. Distribution of HLA- DQB1* alleles in study population.

Further analysis at high resolution revealed HLA-DRB1*07:01, -*11:65, -*15:02, -*15:18, -*03:01, -*04:49, -*02:01 and HLA-DQB1*02:02, -*02:01,-*03:07 and -*03:01 were associated with increased risk for cervical cancer. Alleles such as HLA-DRB1*15:01, -*14:04, -*13:01 and HLA-DQB1*03:02,-*06:01,-*06:03 and -*05:03 were associated with decreased risk for cervical cancer (P<0.05). However, after applying Bonferroni correction, HLA-DRB1*03:01 and DQB1*02:01 (P<0.001) alleles were found to be associated with the risk of cervical cancer. HLA-DRB1*13:01 and DQB1*03:02 were associated with decreased risk for cervical cancer (P<0.001) (Tables I, II).

In exploratory analysis only 5 co-occurring allele combinations/haplotypes occurred in >4 per cent of the cases or controls. HLA- DRB1*07:01-DQB1*02:02 and HLA DRB1*10:01-DQB1*05:01 were significantly associated with cervical cancer, while HLA-DRB1*14:04-DQB1*05:03 and DRB1*15:01-DQB1*06:01 conferred decreased risk for cervical cancer (P<0.05) (Table III).

When the data were stratified on the basis of HPV type, single type vs. multiple type infections, none of the alleles or haplotypes was significantly associated with the HPV type. In multivariate analysis, age was significantly associated with cervical cancer.

Discussion

The overall presence of HPV in cervical cancer cases was 98.67 per cent, while other reports suggested presence of HPV DNA in nearly 100 per cent of cervical cancers[22]. The aetiology of the woman (N=1) who had cervical cancer but was HPV negative might be due to absence of HPV DNA in these tumours, low viral load or with loss of viral genomes due to the tumour's own genetic instability[23]. High risk HPV 16/18 together as single or as co-infection was found in 95 per cent, as suggested earlier that high risk HPV as the major aetiological agent for the development of cervical cancer[24]. The presence of HPV in control population was 8.9 per cent similar to our earlier report (8.1%)[20].

Specific HLA alleles, HLA-DRB1*13 and DQB1*06 were associated with protection while HLA-DRB1*03 and DQB1*02 were with susceptibility to cervical cancer. Association of DRB1 *03 with increased risk for HPV 18 infected cervical lesions but not for HPV 16, 52 or 58 has been already reported in southern Chinese population[25]. However, our study showed its association as a risk factor both in HPV16 or HPV 16+18 positive women, indicating its association with cervical cancer was not type specific. Also increased frequency of DQB1*02 in cancer cases confirmed the observation in Venezuelan women[26]. This observation was in contrast to the reports from Argentine population, where they have shown a protective association of this allele in patients with CIN 1 (n=32) or CIN 3/invasive squamous cell carcinomas (n=44)[27]. This contradictory observation might be due to sample size. Our study was in75 established cancer cases with HPV infection, whereas in Argentine study only 44 had CIN 3/invasive SCC, highlighting the need for further study on this allele. Protective association of HLA-DRB1*13 confirmed the reports in different populations including an earlier study in Indian population[8,9,17,26-31]. Though only one study in Chinese population[11] revealed negative association of this allele with cervical cancer, another study in Han race of Chinese population supported the protective role of HLA-DRB1*13[11]. Hence, HLA-DRB1*13 can be considered as a universal marker of protection in HPV infected women. Further analysis at high resolution

Table I. HLA-DRB1* alleles associated with cervical cancer

Alleles	Controls (N =168)		Cases (N=75)		OR	CI	*P* value	Corrected *P* value
	n	Af (%)	n	Af (%)				
DRB1*01	12	3.57	3	2	0.55	0.12-2.13	0.57	
DRB1*02	0	0	3	2	ud	..	0.02	
DRB1*02:01	0	0	3	2	ud	…	0.02	
DRB1*03	18	5.35	24	16	3.37	1.69-6.73	0.0001	0.003
DRB1*03:01	18	5.35	24	16	3.37	1.69-6.73	0.0001	0.001
DRB1*04	39	11.6	16	10.67	0.91	0.47-1.75	0.76	
DRB1*04:49	0	0	5	3.34	ud	…	0.002	
DRB1*07	37	11.01	22	14.67	1.39	0.76-2.53	0.25	
DRB1*07:01	28	8.34	21	14	1.94	1.02-3.7	0.02	
DRB1*07:11	9	2.67	0	0	0	0-1.31	0.06	
DRB1*08	5	1.48	0	0	0	0-2.6	0.33	
DRB1*09	2	0.59	3	2	3.41	0.46-29.43	0.17	
DRB1*10	22	6.54	17	11.34	1.82	0.89-3.72	0.07	
DRB1*10:01	22	6.54	17	11.34	1.82	0.89-3.72	0.07	
DRB1*11	16	4.76	14	9.34	2.06	0.92-4.59	0.05	
DRB1*11:65	0	0	3	2	ud	…	0.02	
DRB1*12	7	2.08	4	2.67	1.29	0.31-4.99	0.74	
DRB1*13	24	7.14	0	0	0	0-0.44	0.0007	0.003
DRB1*13:01	24	7.14	0	0	0	0-0.44	0.0007	0.001
DRB1*14	38	11.3	5	3.34	0.27	0.09-0.74	0.004	
DRB1*14:04	25	7.44	4	2.67	0.34	0.1-1.06	0.04	
DRB1*14:93	9	2.67	0	0	0	0-1.31	0.06	
DRB1*15	110	32.73	36	24	0.65	0.41-1.03	0.05	
DRB1*15:01	99	29.46	30	20	0.6	0.37-0.97	0.02	
DRB1*15:02	11	3.27	0	0	0	0-1.04	0.02	
DRB1*15:18	0	0	3	2	ud	…	0.02	
DRB1*16	6	1.78	3	2	1.12	0.22-5.12	1	
DRB1*16:02	0	0	2	1.34	ud	…	0.09	

n, number of alleles; Af , allelic frequency; OR, odds ratio; CI, confidence interval; ud, undetermined
Pc<0.003 corrected *P* value (Bonferroni correction)

revealed DRB1*13:01 showing protective association for cervical cancer as seen in Costa Rica women (DRB1*13:01) and American women (DRB1*13:02)[9,10]. The other protective allele HLA-DQB1*06 observed in our study was also similar to that reported in a study on Puerto Rican women[32]. However, our observation was in contrast to reports from Chinese women with HPV/HPV 16[33] and in HPV 58 positive cervical neoplasia in Hong Kong women, but not associated with neoplasia caused by other HPV types[34]. Variation in frequency of HLA-DQB1*03 in women with or without cervical cancer was not significant, while a report from India revealed association of homozygous HLA-DQB1*03 with cervical cancer irrespective of HPV type[16]. HLA DQB1*02:01 association with increased risk for cervical cancer has been reported perhaps for the first time in Indian population, supporting the observation in Venezuelan women[26].

Co-dominance is a characteristic of HLA genes and it is plausible that the risk for HPV persistence

Table II: HLA-DQB1* alleles associated with cervical cancer

Alleles	Controls (N =173)		Cases (N=75)		OR	CI	*P* value	Corrected *P* value
	n	Af (%)	n	Af (%)				
DQB1*02	32	9.24	31	20.67	2.56	1.44-4.52	0.0004	0.01
DQB1*02:01	9	2.6	16	10.67	4.47	1.81-11.25	0.0001	0.001
DQB1*02:02	14	4.04	15	10	2.63	1.17-5.96	0.009	
DQB1*03	79	22.83	42	28	1.31	0.83-2.08	0.21	
DQB1*03:01	22	6.35	17	11.34	1.88	0.92-3.83	0.05	
DQB1*03:02	30	8.67	1	0.67	0.07	0-0.49	0.0007	0.001
DQB1*03:03	6	1.73	7	4.67	2.77	0.82-9.49	0.07	
DQB1*03:07	3	0.86	8	5.34	6.44	1.53-31.08	0.004	
DQB1*04	8	2.31	3	2	0.86	0.18-3.36	1	
DQB1*05	94	27.16	36	24	0.85	0.53-1.35	0.46	
DQB1*05:03	38	10.98	4	2.67	0.22	0.07-0.67	0.002	
DQB1*06	133	38.43	38	25.34	0.54	0.35-0.85	0.004	0.01
DQB1*06:01	84	24.27	21	14	0.51	0.29-0.88	0.01	
DQB1*06:03	15	4.33	1	0.67	0.15	0.01-1.08	0.04	

n, number of alleles; Af , allelic frequency; OR, odds ratio; CI, confidence interval;
Pc<0.01 corrected *P* value (Bonferroni correction)

Table III. HLA class II haplotypes associated with cervical cancer

Alleles	Controls (N = 168)		Cases (N=75)		OR	CI	*P* value
	n	Frequency (%)	n	Frequency (%)			
0701-0202	5	2.97	10	13.34	5.02	1.5-17.62	0.01
1001-0501	10	5.95	11	14.66	2.72	1.01-7.32	0.02
1101-0301	5	2.97	6	8	2.96	0.77-11.67	0.06
1404-0503	10	5.95	0	0	0	0-1.14	0.03
1501-0601	54	32.14	9	12	0.29	0.12-0.65	0.0009
15- 06 (homozygote)	26	15.47	3	2	0.23	0.05-0.83	0.01

n, number of alleles; OR, odds ratio; CI, confidence interval

attributable to these genes is an average of the effect of both alleles at each HLA locus, in addition to an overall effect produced by the interaction among alleles in different HLA genes. Hence, further analysis of haplotype revealed significant association of DRB1*15:01- DQB1*06:01 with decreased risk for cervical cancer, confirming the earlier observation (DRB1*15:01- DQB1*06:02) seen among British women[35]. However, this observation was in contrast to an earlier Indian report[17].

Though the current study observation was limited to HLA class II alleles, results confirmed significant difference in distribution of these alleles in women with or without cervical cancer. Multivariate analysis also highlighted the association of specific alleles with cervical cancer after adjusting for confounding factor age. Identification and confirmation of specific HLA alleles associated with cervical cancer and the polymorphism that encode these, may help in predicting the susceptibility of HPV infected individual towards cancer.

In summary, our findings suggest that HLA class II polymorphisms are involved in genetic protection or susceptibility to cervical cancer in Indian women. Though the analysis was limited to HLA class II

alleles, results confirmed significant association of HLA- DRB1*13:01 with protection, and could be used as a universal protective marker of cervical cancer. DRB1*15-DQB1*06 haplotype in homozygous condition was associated with protection, indicating the co-dominant effect of the HLA alleles in occurrence of cervical cancer.

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