

HLA B*1301 and B*1801 Alleles are Positively Associated with HPV Related Cervical Cancer in Women from Kolkata, Eastern India

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ABSTRACT The incidence of cervical cancer is high in Indian women. Human papillomavirus (HPV) infection is known to be the major risk factor for cervical cancer. Further both viral and host genetic factors have been postulated as important determinants. In the present study 31 suspected Indian women were analyzed molecularly for their HPV infection status, cervical cancer and HLA A, B and Cw allele associations. Among the 13 HPV-16 positive women the HLA analysis revealed a positive association for B*1301 and B*1801 alleles and a negative association with B*4006 in the six cervical cancer patients compared with seven histopathologically normal controls. Our study is the first report towards HPV related cervical cancer in Indian women which suggests that HPV type and HLA allele associations are population dependent.

INTRODUCTION

It has been demonstrated that human papillomavirus (HPV) infection, which is necessary for the development of cervical cancer (CaCx), is highly predominant among sexually active young women. Only a small fraction of such infected women ever develop persistent infections, which progress to CaCx. Hence, additional factors such as environmental and lifestyle together with host and viral genetic factors seem to be important in determining the risk of disease. Evidences in support of genetic predisposition towards CaCx have been noted in studies reporting higher prevalence of CaCx among women with familial history of the disease (Furgyik et al. 1986, Hildesheim and Wang 2002).

Host genetic factors are established to be multigenic (Aranjo de Sonya and Villa 2003). One such likely factor involved in the differential susceptibility to HPV infection seems to be immunogenetic as has been indicated by the over representation of HPV-related cervical lesions in immunosuppressed individuals (Konya and

Dillner 2001; Davidson et al. 2003). Such susceptibility could be due to presence or absence of specific HLA genes as evident from studies reporting associations between various HLA class I and class II alleles/haplotypes and HPV related CaCx (Duggan-Keen et al. 1996). Since HLA molecules participate in the presentation of foreign antigens to the immune system they play a central role in the immune recognition and subsequent clearance of virally infected cells (Evans et al. 2001).

Most of the studies done till date on HLA and cervical neoplasia, are based on HLA class II genes. There are only a few studies that have tested the role of HLA class I alleles in the pathogenesis of HPV infection related CaCx. Since down regulation of HLA class I molecules is a very common phenomenon in CaCx, such molecules may play a crucial role in the protection against HPV related disease. Therefore, identification of susceptible or protective alleles of the class I genes is of prime necessity. Among the population from the eastern part of India, there is a paucity of data on the genetic diversity of HLA alleles. Moreover, there is no report on the association of various HLA alleles with HPV related CaCx among Indian women. Therefore, this preliminary study was undertaken to identify the HLA class I alleles prevalent among women

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having high-risk HPV infection and to identify probable risk/protective alleles for HPV related CaCx in the eastern part of India. High-resolution genotyping methods were adopted for typing the class I loci A, B and C.

MATERIALS AND METHODS

Samples and Subjects: We used DNA isolated from tissues that were derived from women attending a cancer referral clinic of a hospital in Kolkata. Of the 31 fresh cervical biopsy tissues collected, histopathological identification revealed 6 subjects as having squamous cell carcinoma (moderately or poorly differentiating) of age 40-80 years (mean age 61.66 ± 13.29 years). Among the remaining 25 samples, three had histopathologically proven dysplasia and out of 22 histopathologically normals 19 were having chronic cervicitis, 2 had inflammatory cell infiltrates and one had koilocytotic atypia. All samples were collected from the participants with informed consent approved by the Institutional ethical committee for human experimentation. A standardized questionnaire was canvassed on all to obtain demographic and other relevant information.

DNA Isolation and HPV PCR: DNA was isolated from all the cervical biopsy tissues (malignant and non-malignant controls) according to the method of Miller et al. (1988). Samples were routinely screened for the presence of HPV by PCR amplification using L1 consensus primers (MY09/MY11) (Ting and Manos 1990) and HPV16/18 DNA using HPV 16 and 18 specific primers and RFLP (Haraf et al. 1996; Bhattacharaya et al. 2002), with modification (Duttagupta et al. 2002). In this sample set, all malignant tissues (n= 6), and the 2 moderate dysplasia samples were HPV16 positive. Among the 23 histopathologically normal samples, 18 were HPV negative and 5 were HPV 16 positive.

Genotyping of HLA Class I Alleles: Genomic DNA obtained from tissue samples were genotyped for their A*, B* and C* allele subtypes by polymerase chain reaction reverse line strip sequence specific oligonucleotide hybridization (PCR-RLS-SSOP) strips (Roche Molecular, Oakland CA, USA). Each strip for HLA A typing carried a total of 57 immobilized sequence specific oligos (SSOs), while the B carried a total of 84 immobilized SSOs. Genomic DNA was amplified using HLAA, HLA B or HLA

C locus specific biotinylated primers and hybridized with the SSO strips. Streptavidin conjugated alkaline phosphatase was used as a conjugate for positive color development using bromochloroindolyl phosphate/nitroblu-etetrazolium (BICP/NBT) in dimethylformamide (DMF) as substrate. The alleles were determined using the Pattern interpretation software supplied along with the kit.

Statistical Methods: HLAA, HLAB and HLA C allele frequencies and percent of subjects positive for the alleles were calculated and statistical difference (based on p values ≤ 0.05) between study groups were calculated by use of chi-squared test. The odds ratio (OR), etiological fraction (EF), preventive fraction (PF) and the P values were calculated as described earlier (Shankarkumar et al. 2002). In this study, controls were HPV negatives and histopathologically normal samples and cases were i) HPV 16 positive malignant samples (n=6), ii) HPV16 positive non-malignant samples (n=7) and iii) HPV16 positive samples, irrespective of histopathology (n=13), i.e. a combination of i) and ii).

RESULTS

Of the 13 HPV positive samples analyzed, 6 were malignant and positive for HPV16. Among the 7 non-malignant samples, 1 was positive for uncharacterized HPV type corresponding to HPV L1 typing, 4 were positive for HPV16, 1 for HPV18 and 1 for both HPV16 and 18. The most predominant type therefore appeared to be HPV16, followed by HPV18, as reported worldwide (Bosh et al. 1995) and in our earlier study (Duttagupta et al. 2002). This provided an opportunity to determine the HLA class I alleles predominant in case of HPV16/18 positivity and to identify those having the potential of being risk/protective alleles based on their prevalence among the cases and controls, respectively.

The HLA class IA, B and C alleles among the HPV negative controls and HPV 16/18 positive cases, irrespective of their histopathological status is presented (Table 1). Among the HPV positive cases, A*110101 and B*4006 was found to be present at higher frequencies (>20.00 %) while among the control group alleles A*110101, A*2402101, and B*4006 were found to be higher. Alleles found to be absent in the control samples were A*3204, B*1801, Cw*0102 and C*0708. HLA B*1801 and Cw*0708 showed statistical

significance when compared to the controls. However, A*3601 and C*120201 occurred at 3.7 and 2.5 fold higher frequencies respectively, compared to the controls. Also, B*4006 appeared to be 1.8 fold higher among the HPV negative controls, compared to the HPV positive cases. Further when the HLA allele frequencies were analyzed based on the histopathological status we found that 19 women with chronic cervicitis had increased frequencies of HLA A*110101

(26.3%), A*2402101 (21.05%) and B*4006 (23.68%); six women who had squamous cell carcinoma had A*110101 (41.66%) along with the B*1801 and B*1301 alleles; three women with dysplasia had increased frequency of A*2402101 (50%), B*3501 (50%), B*4006 (33.3%), Cw*0202 (33.3%), and Cw*070101 (33.3%); finally the remaining three women had frequencies of A*2402101 (33.3%), B*4006 (33.3%) B*510101 (50%) and Cw*0501 (33.3%), respectively.

Table 1: HLA class I allele frequencies in Eastern Indian women

HLA Allele	Total (n = 31)	Control group* (n = 18) No. (% AF)	Case group** (n = 13) No. (% AF)	p- value
A*0101	4(6.45)	2(5.55)	2(7.69)	0.72
A*020101	8(12.90)	5(13.88)	3(11.53)	0.76
A*030101	3(4.83)	2(5.55)	1(3.85)	0.75
A*110101	17(27.41)	9(25.00)	8(30.76)	0.53
A*2402101	15(24.19)	10(27.77)	5(19.23)	0.73
A*310102	5(8.06)	4(11.11)	1(3.85)	0.27
A*3204	1(1.61)	0(00.0)	1(3.85)	0.23
A*3303	6(9.67)	3(8.33)	3(11.53)	0.65
A*3601	3(4.83)	1(2.77)	2(7.69)	0.36
B*0702	5(8.06)	4(11.11)	1(3.85)	0.27
B*1301	5(8.06)	3(8.33)	2(7.69)	0.92
B*1536	5(8.06)	4(11.11)	1(3.85)	0.27
B*1801	2(3.22)	0(0.00)	2(7.69)	0.08 ***
B*3501	7(11.29)	4(11.11)	3(11.53)	0.95
B*3704	3(4.83)	1(2.77)	2(7.69)	0.36
B*4006	14(22.58)	8(22.22)	6(23.07)	0.92
B*4406	7(11.29)	4(11.11)	3(11.53)	0.95
B*510102	11(17.74)	7(19.44)	4(15.38)	0.64
B*8101	3(4.83)	1(2.77)	2(7.69)	0.36
C*0102	1(1.61)	0(0.00)	1(3.85)	0.23
C*020101	7(11.29)	4(11.11)	3(11.53)	0.95
C*0303012	2(3.22)	2(5.55)	0(0.00)	0.21
C*040101	9(14.51)	6(16.66)	3(11.53)	0.53
C*0501	3(4.83)	3(8.33)	0(0.00)	0.12
C*0502	1(1.61)	1(2.77)	0(0.00)	0.38
C*0602	8(12.90)	5(13.88)	3(11.53)	0.76
C*070101	8(12.90)	5(13.88)	3(11.53)	0.76
C*0702	1(1.61)	1(2.77)	0(0.00)	0.38
C*0708	2(3.22)	0(0.00)	2(7.69)	0.08 ***
C*0801	1(1.61)	1(2.77)	0(0.00)	0.38
C*120201	6(9.67)	2(5.55)	4(15.38)	0.17
C*150201	7(11.29)	3(8.33)	4(15.38)	0.35
C*1507	6(9.67)	3(8.33)	3(11.53)	0.65

%AF = percentage allele frequency

*Control subjects are those having normal histology and are HPV negative

** Cases are HPV positive samples irrespective of histology

*** Significant P value

Further analysis of the data was done by distributing the HPV16/18 positive cases into two groups of those without cervical malignancy (case 1, n= 7) and those with the disease (case 2, n= 6) and comparing with the HPV negative controls (n= 18), as shown (Table 2). It was observed that among the case group 1, A*0101, A*3601, B*3501, B*4006, B*8101, C*120201 and C*150201 alleles were present at higher frequencies compared to the HPV negative controls, but such differences were not statistically significant. Likewise, the allele frequencies of A*110101, A*3303, B*1801, C*020101 and C*1507 were higher among the case group 2, compared to the controls as well as the case group 1. Statistical analysis of the data identified HLA alleles B*1301 (OR=14;

EF=0.15; Pvalue 0.09), B*1801 (OR=14; EF=0.15; P value 0.09) to be significantly increased when compared with HPV positive non-malignant controls and B*1301 (OR= 2.50; EF= 0.19; P value 0.38) B*1801 (OR = 36; EF = 0.32; P value 0.01) increased when compared with HPV negative controls. The allele frequency of B*4006 (OR=0.20; PF= 0.30; P value 0.048) was found to be significantly lower among the case group 2, compared to the case group 1. Further statistically non-significant increase of HLA A*110101 (OR=6.66; EF=0.34), A*3303 (OR=3; EF=0.10), B*4406 (OR=3; EF=0.10), Cw*020201 (OR=3; EF=0.10), Cw*070101 (OR=3; EF=0.10) and Cw*1507 (OR=3; EF=0.10) were observed when compared to the HPV positive controls. Moreover, the allele frequency of A*3601, B*8101

Table 2: HLA class I allele frequencies in HPV positive CaCx cases (case 2), HPV positive normal (case1) and HPV negative controls (control)

Allele groups	No. (%AF)			p- values		
	Control(n=18)	Case 1(n=7)	Case 2(n=6)	p- values		
				p ¹	p ²	p ³
A*0101	2(5.55)	2(14.29)	0(00.0)	0.35	0.36	0.15
A*020101	5(13.88)	2(14.29)	1(8.33)	0.89	0.63	0.79
A*030101	1(2.77)	0(0.00)	1(8.33)	0.53	0.45	0.26
A*110101	9(25.00)	3(21.43)	5(41.66)	0.75	0.16	0.13
A*2402101	10(27.77)	4(28.57)	1(8.33)	0.75	0.16	0.13
A*310102	4(11.11)	0(0.00)	1(8.33)	0.15	0.68	0.26
A*3204	0(00.0)	0(0.00)	1(8.33)	-	0.09	0.26
A*3303	3(8.33)	1(7.14)	2(16.66)	0.79	0.47	0.31
A*3601	1(2.77)	2(14.29)	0(00.0)	0.35	0.53	0.15
B*0702	4(11.11)	0(0.00)	1(8.33)	0.22	0.91	0.26
B*1301	3(8.33)	0(0.00)	2(16.66)	0.22	0.47	0.09
B*1536	4(11.11)	0(0.00)	1(8.33)	0.15	0.68	0.26
B*1801	0(0.00)	0(0.00)	2(16.66)	-	0.015/0.06^a	0.42
B*3501	4(11.11)	2(14.29)	1(8.33)	0.89	0.91	0.79
B*3704	1(2.77)	1(7.14)	1(8.33)	0.53	0.45	0.90
B*4006	8(22.22)	5(35.71)*	1(8.33)	0.22	0.24	0.04
B*4406	4(11.11)	1(7.14)	2(16.66)	0.57	0.70	0.41
B*510102	7(19.44)	3(21.43)	1(8.33)	0.80	0.35	0.30
B*8101	1(2.77)	2(14.29)	0(00.0)	0.35	0.53	0.15
C*020101	4(11.11)	1(7.14)	2(16.66)	0.79	0.47	0.41
C*0303012	2(5.55)	0(0.00)	0(00.0)	0.33	0.36	-
C*040101	6(16.66)	1(7.14)	2(16.66)	0.27	0.86	0.41
C*0501	3(8.33)	0(0.00)	0(00.0)	0.33	0.36	-
C*0502	1(2.77)	0(0.00)	0(00.0)	0.49	0.53	-
C*0602	5(13.88)	2(14.29)	1(8.33)	0.89	0.63	0.79
C*070101	5(13.88)	1(7.14)	2(16.66)	0.57	0.70	0.41
C*0708	0(0.00)	1(7.14)	1(8.33)	0.12	0.09	0.90
C*0801	1(2.77)	0(0.00)	0(00.0)	0.49	0.53	-
C*120201	2(5.55)	3(21.43)	1(8.33)	0.10	0.80	0.30
C*150201	3(8.33)	3(21.43)	1(8.33)	0.23	0.91	0.30
C*1507	3(8.33)	1(7.14)	2(16.66)	0.79	0.47	0.41

p¹ corresponds to control versus case 1

p² corresponds to control versus case 2

p³ corresponds to case 1 versus case2

* indicates significantly higher by test of proportion (comparison between case1 and case2)

and C*120201 was noted to be higher among the HPV positive normal group (case 1) by 4.6, 4.6 and 3.4 folds respectively, compared to the HPV negative controls.

DISCUSSION

In this study, we determined the contribution of the HLA class I alleles (corresponding to the loci A, B and C) to the risk of developing HPV16/18 positive CaCx in Indian women. The comparison between cases (HPV16/18 positive CaCx or HPV16/18 positive samples irrespective of the histopathological status) and controls indicated several alleles, some of which could impart risk towards HPV related disease development, while some could act to be protective. However, because of the small sample size analyzed, statistical significance of association (positive or negative) could not be identified for most of the alleles. HLA B*1301 and B*1801, revealed a significant positive association with HPV16/18 positive CaCx, compared to the HPV negative controls. Likewise, among the HPV positive subjects, a comparison between the normal and those with CaCx showed that B*4006 could have a protective effect on disease development, which was statistically significant. It may further be noted that among the 13 HPV positive samples, only one was found to be HPV18 positive and another sample turned out to be positive for both HPV16 and HPV 18. Both these samples carried HLAA, B and C alleles, which were common to that found in HPV16 positive samples. A study based on a larger sample size should be able to indicate whether there exists any correlation between HPV type specificity and HLA class I allele frequencies.

Studies on the HLA class I alleles and disease associations in case of CaCx are mainly focussed on Caucasian populations in Europe (Heilmann and Kreinberg 2002) and have used serology-based methods. Some of these have identified a protective effect of B15, which was not confirmed in other studies (Glew et al. 1993; Krul et al. 1999; Bontkes et al. 1998). Further another study reported B44 as a risk allele for disease progression (Brady et al. 2000). However, B7 in combination with the HLA class II allele DQB1*0302 was shown to impart elevated risk towards cervical disease in two distinct populations in North and Central America (Wang et al. 2001; Hildesheim et al. 1998). In a population

based study on normal women from West Bengal, India, we have identified HLA B*07 to be a risk allele for HPV infection, specifically HPV16/18 (Sengupta et al. 2004).

Recent studies on HLA disease associations are mostly based on allele specific high-resolution genotyping methods. HLA DRB1*1602 allele is positively associated with HPV infection in Bolivian Andean women (Cervantes et al. 2003). Frequencies of DRB1*1501 and DQB1*0602 increased among Japanese patients with HPV 16 E6 variants and may be related with cervical carcinogenesis (Matsumoto et al. 2003). Increased frequencies of HLA B63 in HPV positive patients other than HPV16/18 and HLA B55 in HPV negative patients from Netherlands have been reported (Krul et al. 1999). Swedish women with HLA B*44, B*51 or B*57 and infected with HPV 16 E6 variant had approximately four to five fold increased risk of cancer (Zehbe et al. 2003). Also there are reports that HLA B18 associated Cytotoxic T lymphocytes are recognized by HPV 16 E6 and E7 protein epitopes (Bourgault et al. 2000). Furthermore, two homologous antigenic peptides specific for B*3901 allele have been identified from L1, HPV-16 and HPV-18 proteins (Monroy-Garcia et al. 2002). The first study conducted in three populations in Central and North America reported HLA C*0202 to be negatively associated with the risk of cervical disease (Wang et al. 2002). Ours is the first of its kind, which employed such a methodology to determine the contributions of various HLA class I alleles towards the risk (HLA B*1301 and B*1801) or protection (HLA B*4006) towards HPV related CaCx in Indian women. This study can be regarded as a baseline report to implement future studies in this direction on a larger scale from India.

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