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HLA DQB1*03 Genotypes and Susceptibility to Cervical Cancer in Indian Women

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ABSTRACT DNA from i) fresh tissues derived out of 89 women with CaCx (61 HPV16/18 positive) and ii) cervical scrapes from 213 cytologically normal women (73 HPV16/18 positive) were genotyped for HLA DQB1*03 by PCR and RFLP. The distribution of the genotypes of HLA DQB1*03 differed significantly between the malignant and control subjects (p= 0.002). The homozygous genotype was overrepresented among those having CaCx ($OR_{age-adjusted}$=2.94, 95% CI= 1.49-5.81, p=0.002), or HPV16/18 positive CaCx ($OR_{age-adjusted}$ =2.32, 95% CI= 1.07-5.01; p=0.033), when this was compared to the other genotypes (HLADQB1*03 non-homozygous, i.e. heterozygous and null together). Analysis restricted to the HPV16/18 positive CaCx and cytologically normal subjects failed to show such association. The heterozygous genotype, instead, showed a negative association with HPV16/18 positive CaCx over the null genotype ($OR_{age-adjusted}$ =0.42, 95% CI= 0.18-0.96; p=0.040) when HPV16/18 positive CaCx cases were compared with HPV negative cytologically normal controls. The association of HLA DQB1*03 homozygosity with CaCx was noted among those of age < 47 years. The genotypes of HLA DQB1*03 are likely to be important in determining the susceptibility to HPV or HPV16/18 related CaCx in Indian women.

INTRODUCTION

Exposure to high-risk human papillomavirus (HPV) is common among sexually active women (Villa 1997). However, only a small fraction develops persistent infection, which may progress to cervical cancer (CaCx) after a long latency. Therefore, additional factors seem to play important role in the pathogenesis of the infection and subsequent development of CaCx.

In recent times, identifying genetic factors that could influence the pathogenesis of human papillomavirus infection, specifically HPV16/18 related CaCx has gained importance for devising preventive strategies for the disease. Therefore, such studies are essential for targeting cervical screening to a high-risk population, particularly in the developing countries, where CaCx still poses a big threat.

A likely factor involved in the differential susceptibility to HPV infections seems to be immunogenetic. This has been indicated by the over representation of HPV-related cervical lesions in immunosuppressed individuals (Villa 1997). Human Leukocyte Antigens (HLAs) play a pivotal role in the recognition and presentation of foreign antigens to the immune system. Thus the ability of an individual to mount an immune response against viral infections and/or tumors strongly depends on the detection and elimination of infected/neoplastic cells by presentation of tumour specific and/or tumor associated antigens by the HLA molecules to T cells.

There are contradictory reports regarding the relation between polymorphic HLA genes and CaCx. Although various reports suggest a close link between certain HLA haplotypes and CaCx, the specific HLA alleles associated with it differ among various study groups. It has been suggested that women carrying HLA DQw3 antigen encoded by DQB1*03, are predisposed to develop CaCx (Wank and Thomssen 1991). While several reports have been put forward in support of this finding (Helland et al. 1992, 1994; Gregoire et al. 1994; Odunsi et al. 1995; Nawa et al. 1995; Montoya et al. 1998) there are contradictory reports that show no such association (Glew et al. 1992, 1993; Apple et al. 1994; Allen et al. 1996).

In India, CaCx ranks as number one among the female cancers. There is a lack of data on the prevalence of various HLA alleles and their association with CaCx, in this part of our country. Therefore, in this study, we restricted ourselves in determining the prevalence of the candidate HLA DQB1*03 allele (corresponding to HLA DQw3 antigen) together with the genotypes and

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analyzing their association, if any, with HPV16/18 related CaCx among Indian women.

MATERIALS AND METHODS

Samples and Subjects: We used DNA isolated from, (i) tissues that were derived from 89 women with suspected malignancy (sub-sequently confirmed by histopathology as squamous cell carcinoma) of age 26-75 years, who were attending a cancer referral hospital; and (ii) from 96 (HPV positive) and 117 (HPV negative) cervical scrapes. The scrapes (identified as normal from Pap smear tests) were derived from women aged 16-70 years (HPV positive) and 19-80 years (HPV negative), with no previous history of cervical dysplasia/malignancy. These women were attending a Reproductive and Child Health Clinic (Child in Need Institute, South 24 Parganas, West Bengal, India) for routine contraception and reproductive healthcare counseling. All samples (biopsy tissues and cervical scrapes) were collected from the participants with informed consent approved by the institutional ethical committee for human experimentation. A standardized questionnaire was canvassed on all by to obtain demographic and other relevant information.

All DNA samples were routinely typed for the presence of HPV infection. Among the 89 CaCx samples, 61 (68.54%) samples were found to be positive for HPV16 or 18, collectively termed the HPV16/18 group. Of the 96 HPV positive normal DNA samples, 73 (76.0%) were positive for HPV types16/18 out of which, 70 subjects were positive for HPV types 16 alone, 6 subjects for type 18 alone and 3 for both 16 and 18.

HLA Typing: HLA DQB1*03 typing was done following the method of Mehal et al. (1994) with modifications. The 39th and 40th codons of both HLA DQB1*03 and non- HLA DQB1*03 alleles are CGC and TTC respectively. A mutagenesis primer was used to mutate the first base of codon 40 from T to G. This creates a MluI site, specific for HLA DQB1*03 alleles having an A at the third base of codon 38, in contrast to G in case of non- HLA DQB1*03 alleles. PCR was thus carried out using the mutagenesis primer and an upstream primer DB130 (Bugawan and Erlich 1991) in 10 ml reaction volume using 1 ml of 10X supplied PCR buffer (without $MgCl_2$), 1.5 mM $MgCl_2$, 200 mM dNTPs, 20 ng of each primer, 0.5 U Taq DNA Polymerase and 100 ng DNA. Thirty cycles of amplification reactions were carried out, preceded by an initial denaturation at 94 °C for 8 min and followed by 5 min of final extension at 72 °C. Each cycle included 1 min denaturation at 94 °C, 1 min annealing at 60 °C and 1 min elongation at 72 °C. PCR products were digested overnight with MluI at 37 °C, separated on a 2% agarose gel by electrophoresis and visualized under UV light after staining with ethidium bromide.

Statistical Analysis: The histopathologically confirmed CaCx cases, either all (irrespective of HPV infection status) or HPV16/18 positive, were compared with the cytologically normal HPV negative controls. Similar analyses were also carried out within HPV16/18 positive cases and controls. Furthermore, since it is proposed that close to 99.7% CaCx cases are HPV positive (Walboomers et al. 1999), all such cases were also compared with HPV positive cytologically normal samples.

In this study, the CaCx cases (n= 89) were significantly older (mean age, 47.76 ± 11.91 years) than HPV negative cytologically normal controls (mean age: 37.41 ± 13.44 years). The mean age of HPV16/18 positive CaCx cases (n= 61) was also significantly higher (Mean age, 48.53 ± 12.78 years) than the cytologically normal controls, either HPV negative or HPV16/18 positive (mean age: 27.15 ± 8.67 years). Also, between the latter two groups, the HPV16/18 positive cytologically normal subjects were found to be significantly younger than those HPV negatives. Therefore multivariate logistic regression analyses were performed using the SPSS 7.5 statistical software package to compute the age-adjusted OR (95% CI) values (Hosmer and Lemeshow 1989), wherever necessary.

Analyses were also carried out with CaCx subjects stratified into cases with age < 47 years and ³ 47 years, respectively, based on the median age of onset (47 years) of CaCx, in this population.

RESULTS

The distribution of HLA DQB1*03 allele across various study groups is presented in Table 1. The prevalence of the allele was at the same level among CaCx cases, irrespective of HPV (78.0%, n=89) and the HPV negative controls (80.0%, n= 117). On testing for the equality of distribution of the HLADQB1*03 genotypes (homozygous, heterozygous and null) among

these cases (21.3%, 40.4% and 38.2%, respectively) and controls (19.7%, 58.1% and 22.2%, respectively), a significant deviation was observed between the two groups (df= 2, p= 0.022). Compared to the null genotype, the homozygous genotype of HLA DQB1*03 ($OR_{age-adjusted}$ = 2.50; 95% CI= 0.94-6.66; p= 0.066), but not the heterozygous genotype ($OR_{age-adjusted}$ = 0.59, 95% CI= 0.27-1.29; p= 0.187), was proportionately higher among CaCx cases, although not statistically significant as shown in Table 2. The overrepresentation of the homozygous genotype among those having CaCx was significant ($OR_{age-adjusted}$ = 2.94, 95% CI= 1.49-5.81, p= 0.002), when this genotype was compared to the other genotypes (HLADQB1*03 non-homozygous, i.e. heterozygous and null together). Similar analysis also revealed that

Table 1: Distribution of HLADQB1*03 genotypes within different cytological/histological groups with consideration of their HPV infection status

Characteristics of _subjects	*Total*	*HLA DQB1*03 Genotypes*		
		null	*HLA DQB1*03 Heterozygous*	*HLA DQB1*03 Homozygous*
		n (%)	*n (%)*	*n (%)*
Cytologically normal				
Irrespective of HPV infection	213	45 (21.1)	117 (54.9)	51 (23.9)
HPV negative	117	23 (19.7)	68 (58.1)	26 (22.2)
HPV positive	96	22 (22.9)	49 (51)	25 (26)
HPV16/18 positive	73	16 (21.9)	38 (52.1)	19 (26)
CaCx				
Irrespective of HPV infection	89	19 (21.3)	36 (40.4)	34 (38.2)
HPV 16/18 positive	61	17 (27.9)	24 (39.3)	20 (32.8)

Table 2: Association of HLA DQB 1*03 allele and its genotypes with CaCx in Indian women

Control	*Case*	*HLA DQB 1*03 allele/ genotypes compared*	*Age-adjusted OR (95% CI)*	*p-value*
HPV negative cytologically normal (n = 117)	CaCx (n =89)	HLA DQB1*03 allele positive vs. null	0.92(0.44-1.91)	0.822
		HLA DQB1*03 heterozygosity vs. null	0.59(0.27-1.29)	0.187
		HLA DQB1*03 homozygosity vs. null	2.50(0.94-6.66)	0.066
		HLA DQB1*03 homozygosity vs. non-homozygosity	**2.94(1.49-5.81)**	**0.002**
	HPV16/18 positive CaCx (n =61)	HLA DQB1*03 allele positive vs. null	0.60(0.28-1.30)	0.197
		HLA DQB1*03 heterozygosity vs. null	**0.42(0.18-0.96)**	**0.04**
		HLA DQB1*03 homozygosity vs. null	1.43(0.51-4.00)	0.492
		HLA DQB1*03 homozygosity vs. non homozygosity	**2.32(1.07-5.01)**	**0.033**
HPV Positive Cytologically normal (n =96)	CaCx (n =89)	HLA DQB1*03 allele positive vs. null	2.29(0.74-7.11)	0.153
		HLA DQB1*03 heterozygosity vs. null	1.39(0.46-4.24)	0.561
		HLA DQB1*03 homozygosity vs. null	**3.91(1.08-14.20)**	**0.038**
		HLA DQB1*03 homozygosity vs. non-homozygosity	**3.08(1.18-8.08)**	**0.022**
HPV16/18 positive Cytologically normal (n =73)	HPV16/18 Positive CaCx (n =61)	HLA DQB1*03 allele positive vs. null	0.97(0.29-3.22)	0.954
		HLA DQB1*03 heterozygosity vs. null	0.64(0.18-2.27)	0.493
		HLA DQB1*03 homozygosity vs. null	2.04(0.49-8.45)	0.327
		HLA DQB1*03 homozygosity vs. non-homozygosity	2.97(0.89-9.88)	0.076

* *Risk genotype vs. Non-risk genotype*

homozygosity of the allele was significantly associated with HPV16/18 positive CaCx ($OR_{age-adjusted}$ = 2.32, 95% CI= 1.07-5.01; p= 0.033). The heterozygous genotype, instead, showed a negative association with HPV16/18 positive CaCx over the null genotype ($OR_{age-adjusted}$ =0.42, 95% CI= 0.18-0.96; p= 0.040). Compared to the HPV positive cytologically normal subjects, HLADQB1*03 homozygosity was significantly higher among CaCx cases compared to those with null genotype ($OR_{age-adjusted}$ = 3.91, 95% CI= 1.08-14.20; p= 0.038) and HLA DQB1*03 non-homozygous genotypes ($OR_{age-adjusted}$ = 3.08, 95% CI= 1.18-8.08; p= 0.022). However, no significant association was observed when analysis was restricted within HPV16/18 positive subjects, cases and controls.

The distribution of the HLA DQB1*03 genotypes (null, homozygous and heterozygous) among CaCx cases of age groups <47 years and ³ 47 years is shown in Table 3. The proportion of HLA DQB1*03 homozygous genotype was found to be significantly higher (p-value= 0.033 from Fisher's Exact Test) for CaCx cases, whose age was < 47 years (47.7 %) compared to those of age ³ 47 years (28.9%). Further analyses of the data to test the association of HLA DQB1*03 allele/genotypes with CaCx cases (irrespective of HPV) classified on the basis of age has been presented in Table 4. The HLA DQB1*03 homozygous genotype was found to be proportionately higher over non-homozygous genotypes among CaCx cases of age <47 years) compared to HPV negative cytologically normal controls ($OR_{age-adjusted}$ = 3.20, 95% CI= 1.53-6.66; p=0.002). Overrepresentation of HLA DQB1*03 homozygosity, rather than non-homozygosity, was also prominent among CaCx cases of the similar age group when compared to HPV positive cytologically normal subjects ($OR_{age-adjusted}$ = 2.59; 95%CI=1.23-5.47; p= 0.011). There was no significant association between HLA DQB1*03 allele or the genotypes and CaCx cases, who were of age ³ 47 years.

Table 3: Distribution of HLA DQB1*03 genotypes within CaCx cases of different age groups

Case	*HLA DQB1*03*	*Age group (in years)*	
	genotype	*<47*	*≥ 47*
CaCx (n = 89)	null	7 (15.9)	12 (26.7)
	HLA DQB1*03 heterozygous	16 (36.4)	20 (44.4)
	HLA DQB1*03 homozygous	21 (47.7)	13 (28.9)
	Total	44	45

Table 4: Association of HLA DQB1*03 allele/genotypes with CaCx cases in different age groups

Control	*Case*	*Analyses**	*OR (95% CI) p-value*	
			< 47 yrs	*≥ 47 yrs*
HPV negative cytologically normal (n = 117)	CaCx (n =89)	HLA DQB1*03 allele positive vs. null	1.29(0.51-3.27) 0.586	0.67(0.30-1.50) 0.332
		HLA DQB1*03 heterozygosity vs. null	0.77(0.28-2.11) 0.616	0.56(0.24-1.33) 0.187
		HLA DQB1*03 homozygosity vs. null	2.65(0.95-7.38) 0.058	0.96(0.37-2.51) 0.193
		HLA DQB1*03 homozygosity vs. non-homozygosity	**3.20(1.53-6.66) 0.002**	1.42(0.65-3.10) 0.374
HPV positive normal cytology (n =96)	CaCx (n =89)	HLA DQB1*03 allele positive vs. null	1.57(0.62-4.01) 0.342	0.82(0.36-1.85) 0.784
		HLA DQB1*03 heterozygosity vs. null	1.03(0.37-2.85) 0.205	0.75(0.31-1.79) 0.143
		HLA DQB1*03 homozygosity vs. null	2.64(0.94-7.39) 0.061	0.95(0.36-2.52) 0.195
		HLA DQB1*03 homozygosity vs. non-homozygosity	**2.59(1.23-5.47) 0.011**	1.15(0.52-2.54) 0.722

DISCUSSION

HLAs play a significant role in host immunity as they participate in presenting intracellular and extracellular antigens to professional immune cells (Berkower 1996). Therefore, identifying specific HLA types associated with CaCx and the genes that encode them, are likely to be useful in predicting the susceptibility towards CaCx that is intimately associated with HPV infections. Associations between HLA DQw3 (class II type molecule) and CaCx have previously been observed among African- American (Gregoire et al. 1994), Caucasian (Wank and Thomssen 1991; Helland et al. 1992; Montoya et al. 1998), and Japanese ethnic populations (Nawa et al. 1995). No such report exists on Indian women inspite of the fact that CaCx appears to be the major cancer burden in these women. We therefore, undertook the present study in order to investigate the association, if any, of the immunogenetic factor HLA DQB1*03, with HPV16/18 related CaCx among Indian women from West Bengal.

We have earlier reported an overall prevalence of 85% HPV infection, the prevalence of HPV 16/18 being 82%, in invasive CaCx. In the normal population of this region we also recorded 8.3% prevalence of HPV16/18 infection, which was predominant among those of age £ 23 years (Duttagupta et al. 2002). In this report we identified a significant difference in the distribution of HLA DQB1*03 genotypes between malignant and cytologically normal subjects, HPV negative or positive. The current study therefore is highly relevant in this context, for identifying those at risk of CaCx or HPV16/18 related CaCx.

A significant association of HLA DQB1*03 homozygosity with CaCx or HPV16/18 positive CaCx (which accounts for 70% of the CaCx) was observed. This was in comparison to the non-homozygous genotypes of the allele. Mueller and Machulla (2002) also identified an increased frequency of homozygosity at HLA class II loci (DR/DQ) in female patients with chronic lymphocytic leukemia. As proposed by such researchers and others, homozygosity of HLA alleles are likely to provide a restricted repertoire of presented peptides, which could lead to impaired host defense. Similar phenomenon could therefore account for the susceptibility of subjects homozygous for HLA DQB1*03 alleles, towards CaCx or HPV16/18 positive CaCx, by members of HLA DQB1*03 family of antigens, which share a common epitope.

Among the studies that found HLA DQB1*03 to be associated with CaCx, Neuman et al. (2000) compared patients with family controls and reported that presence of DQB1*0303 allele increased the risk of CaCx in women who were HPV positive. Gregoire et al. (1994), on the otherhand, failed to show any association between HPV type in the tumor and HLA DQB1 *03 among African-American women. It may be noted that Mehal et al. (Mehal et al. 1994) have failed to show any association between HLA DQw3 and CaCx based on their study among women of United Kingdom.

In this study we have also noted a negative association of the HLA DQB1*03 heterozygosity, compared to null genotypes, with HPV16/18 positive CaCx. This probably reflects a protective effect of heterozygosity of the allele. In fact, some studies have observed a selective advantage due to heterozygosity at human MHC (HLA class I and II) against infectious diseases like HIV infection and persistent hepatitis B virus infection (Carrington et al. 1999; Thursz et al. 1997). Odunsi et al. (1995) also reported that the risk towards CIN was lower in case of heterozygosity compared to homozygosity of HLA DQB1*03, the association being positive for both genotypes.

The differential role of the two genotypes could be due to various reasons. Firstly, the presence of a different allele along with HLA DQB1*03 at the same locus in the heterozygous condition may counterbalance the deficiency in immune responsiveness of HLA DQB1*03 allele coded antigens. Similar situation could also help in eliciting an immune response against a different epitope of the tumor or HPV related antigen. This may result in the lack of positive association with CaCx of HLA DQB1*03 heterozygosity. Furthermore, the possibility of a gene in linkage disequilibrium with HLA DQB1*03 in the homozygous condition may enhance the susceptibility towards development of CaCx, of this genotype. Further work is therefore required to establish the differential role, if any, of the two HLA DQB1*03 genotypes.

It is also reflected in our study that the association between malignancy and HLA DQB1*03 homozygosity may not be independent of HPV16/18 infection. This is evident from the lack of any significant association between these two

factors by a comparison between cases and controls, both positive for HPV16/18. The observation may be indicative of the association of HLA DQB1*03 homozygosity with HPV16/18 induced host cell antigen(s), specifically expressed in malignant cells.

This study also indicated that the risk of HLA DQB1*03 homozygosity towards CaCx development, was restricted to those of age < 47 years and absent in those of age ³ 47 years. It is well established that early age of onset is a risk factor for familial cancers. Therefore, HLA DQB1*03 homozygosity may have such genetic links also, which need to be explored in our population.

In summary, this study revealed that HPV infected (particularly, HPV16/18) and HLA DQB1*03 homozygous women of this population in West Bengal, India, appear to be at an enhanced risk to develop CaCx. However, heterozygosity of HLA DQB1*03 may be protective for women infected with HPV16/18. Moreover, it also appeared that the genotypes of HLA DQB1*03, rather than the allele positivity, are likely to be important in determining the susceptibility to HPV infection and /or development of CaCx in these women. Further elucidation of the association of specific alleles of HLA DQB1*03 with HPV16/18 related CaCx is needed, which is underway. We conclude that the findings are likely to add new dimension to the epidemiological background of CaCx in this region.

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