Indian J Med Res 156, September 2022, pp 554-559
DOI: 10.4103/ijmr.ijmr_1145_21

Quick Response Code:

Correspondence

HLA association with biliary atresia in north India

Sir,

Biliary atresia (BA), though rare, is a life-threatening neonatal cholestatic disorder. It is characterized by inflammatory and progressive fibrosis of the biliary apparatus that results in their obliteration, thereby leading to cirrhosis and liver failure. Its reported incidence varies from five to 32 per 100,000 live births[1,2]. Its frequency is higher in East Asian countries, including India, where it comprises almost one-third of cases of neonatal cholestasis[3-5].

As murine and human studies suggested, possible aetiological factors for BA include viral infections, environmental exposures, developmental defects, abnormal fibrogenesis, genetic abnormalities and aberrant neonatal immune responses causing autoimmunity. The latter is thought to be the most recent and widely accepted trigger among these[6-8]. The HLA genes are linked to various autoimmune disorders[9]. A few researchers have attempted to analyze the role of HLA Class I and II in the aetiopathogenesis of BA. However, the data obtained thus far are contradictory and inconclusive. Geographic, racial and demographic differences and analysis of small sample sizes in these studies could be the reason. However, this may also be attributed to a relatively low frequency of this disorder[2,10-14].

In this prospective study, 73 infants with BA who attended the clinics of Paediatric Surgery and Paediatric Gastroenterology departments of Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, and were operated on with Kasai portoenterostomy between January 2016 and July 2020, were enrolled. Their diagnosis was based on the suggestive clinical history, intervention and laboratory findings (ultrasonography, Mebrofenin hepato-biliary scan, pre-operative cholangiogram, histopathology of scrapping and liver biopsy). None of these cases had any associated syndrome, *e.g*. BA splenic malformation syndrome. After a thorough evaluation, patients with alternative causes of neonatal cholestasis were excluded, such as neonatal hepatitis, congenital hepatic fibrosis, patent vitellointestinal duct, choledochal cyst, cystic fibrosis, α-1 antitrypsin deficiency and metabolic abnormalities. For the controls, 73 unrelated, healthy donor children for bone marrow transplant patients were randomly chosen. About 300 µl blood sample in ethylenediaminetetraacetic acid was obtained from each patient and each of the 73 unrelated control infants. Before the children were selected, the Institute Ethics Committee approved the study, and the parents or guardians gave their written informed consent.

DNA extraction was done using commercially available kits (Qiagen, USA) as per the manufacturer's instructions. The purity and concentration of extracted DNA were quantified on a calibrated spectrophotometer (Genova Nano, Janeway, UK). The profile of extracted DNA was checked on 0.8 per cent agarose gel, and after confirming the purity (absorbance 260/280 ratio=1.7-2.0) and quality, the extracted DNA was stored in aliquots at −80°C till further use.

LIFECODES sequence-specific oligonucleotide HLA typing kits (Immucor, USA) were used in a multiplexing platform to type HLA-A and HLA-B (Luminex 200, Immucor, USA). HLA-A and HLA-B alleles were amplified in a thermocycler (Veriti, 96-Well, Thermo Fisher Scientific, USA). The PCR product was denatured and hybridized with Luminex microspheres pre-coated with probes specific for the complementary sequences of HLA-A and HLA-B (Immuncor GTI Diagnostics Inc., Waukesha, Winconsin, USA). The red fluorescent protein R-Phycoerythrin (R-PE)-conjugated streptavidin secondary antibody was used to label the hybridized result. The analysis was performed on a multiplexing platform (Luminex 200), and the results were interpreted with the help of MATCH IT DNA software (Immucor Inc., Norcross, GA, USA).

Table I. *HLA-A*, *HLA-B* and *HLA-DRB1* frequencies in study groups

HLA alleles	Patients (%) (n=73)	Controls (%) (n=73)	P_u	P_c	OR	95% CI
A*01	19.2	16.4	0.665	-	0.829	0.369-1.960
A*02	35.6	34.2	0.862		0.942	0.482-1.830
A*03	13.7	27.4	0.041†	0.574	2.380	1.0-5.490
A*11	37.0	56.2	0.020†	0.280	2.180	1.120-4.170
A*23	1.37	1.4	1.000	-	1.000	0.052-19.20
A*24	32.9	26.0	0.364		0.718	0.361-1.487
A*26	9.6	8.2	0.771		0.844	0.265-2.432
A*29	1.4	0	1.000		0	0-9.0
A*30	0	1.4	1.000		-	-
A*31	11.0	2.7	0.097		0.229	0.047-1.0
A*32	1.4	6.8	0.209		5.290	0.689-63.10
A*33	16.4	9.6	0.219		0.539	0.211-1.440
A*68	20.5	9.6	0.064		0.410	0.162-1.110
A*69	1.4	0	1.000		0	0-9.130
B*07	19.2	9.6	0.099		0.447	0.176-1.120
B*08	12.3	16.4	0.479		1.40	0.568-3.320
B*13	8.2	1.4	0.116		0.155	0.013-1.0
B*15	11.0	8.2	0.574		0.728	0.233-2.320
B*17	0	2.7	0.497		-	-
B*18	1.4	2.7	1.000		2.030	0.231-29.80
B*27	4.1	6.8	0.719		1.720	0.428-6.660
B*35	17.8	31.5	0.055		2.120	1.0-4.580
B*37	1.4	4.1	0.620		3.090	0.448-40.60
B*38	1.4	1.4	1.000		1.000	0.052-19.20
B*39	0	2.7	0.497		-	-
B*40	35.6	37.0	0.863		1.060	0.552-2.050
B*41	4.1	1.4	0.620		0.324	0.024-2.230
B*44	9.6	6.8	0.547		0.693	0.237-2.130
B*48	1.4	0	1.000		0	0-9.0
B*49	1.4	0	1.000		0	0-9.0
B*50	8.2	6.8	0.754		0.821	0.268-2.990
B*51	34.2	11.0	0.001†	0.023‡	0.236	0.102-0.574
B*52	9.6	23.3	0.026‡	0.598	2.860	1.110-7.210
B*55	1.4	8.2	0.116		6.450	1-74.90
B*56	0	1.4	1.000			-
B*57	4.1	8.2	0.494		2.090	0.552-7.830
B*58	13.7	8.2	0.289		0.564	0.192-1.530
DRB1*01	9.6	5.5	0.533		1.830	0.512-6.541
DRB1*03	21.9	24.7	0.695		0.858	0.398-1.850
DRB1*04	6.8	16.4	0.071		0.374	0.125-1.122
DRB1*07	27.4	24.7	0.706		1.153	0.550-2.417
DRB1*08	2.7	5.5	0.681		0.486	0.086-2.739

Contd...

HLA alleles	Patients (%) (n=73)	Controls (%) (n=73)	P_u	P_c	OR	95% CI
DRB1*09	5.5	0	0.120		2.058	1.738-2.437
DRB1*10	9.6	12.3	0.596		0.754	0.265-2.146
DRB1*11	21.9	16.4	0.400		1.427	0.622-3.276
DRB1*12	1.4	0	1.000		2.014	1.709-2.373
DRB1*13	19.2	15.1	0.510		1.337	0.562-3.181
DRB1*14	17.8	27.4	0.166		0.574	0.261-1.265
DRB1*15	52.1	47.9	0.619		1.179	0.616-2.257
DRB1*16	4.1	4.1	1.000		1.000	0.195-5.126

[†]Significant. P_u, uncorrected *P* value; P_c, corrected *P* value; OR, odds ratio; CI, confidence interval

Commercially available HLA-DRB sequence-specific primer typing kits (Morgan, TBG Biotechnology Corp., Germany) were used to type the Class II locus DRB. The PCR master mix was prepared with 260 µl PCR ready mix, 5 U/µl Taq polymerase (2.2 units) and 50 µl (30 ng/µl) of DNA. Thermocycling conditions were set as 96°C for two min, 96°C for 15 sec and 65°C for 60 sec for 10 cycles, followed by 96°C for 15 sec and 61°C for 50 sec and 72°C for 30 sec for 20 cycles. The amplified products were subjected to two per cent agarose gel electrophoresis, followed by band visualization under ultraviolet illumination to detect the individual HLA-DRB1 alleles.

The Statistical Package for the Social Sciences (SPSS) software (version 20.0, IBM Corp., Chicago, IL, USA) was utilized for statistical analysis. The strength of the association between HLA-A, HLA-B and HLA-DRB1 and BA was determined using the odds ratio (OR) and 95 per cent confidence intervals (CI). Chi-square or Fisher's exact tests were used to calculate the *P* values. The Bonferroni correction was done in the correlates with $P<0.05$ to adjust the *P* value. The corrected *P* value was calculated by dividing the α value by the total number of tests, followed by the estimation of the family-wise error rate as $\alpha_{FW}=1-(1-\alpha_{PC})^c$ where α was the error rate, c was the number of comparisons performed and α_{PC} was equal to the specified per contrast error rate. The corrected *P* value (P_c) <0.05 was then considered significant.

All the patients had a clinical history of jaundice, acholic stools, dark urine and liver enlargement. The age ranged from 14 to 180 days in the patients and 1-18 yr in the controls, with the mean age being 2.5±1.1 months and 10.7±3.85 years in patients and controls, respectively. There was a slight male predominance in patients with a male-to-female ratio of 2.2:1. In controls, the ratio was 1.2:1. In the patient group, mean serum levels of total and conjugated bilirubin (13.5±6.2; reference range 0.1-1.2 mg/dl and 8.8±3.7 mg/dl; reference range <0.3 mg/dl, respectively), aspartate transaminase (324.9±221.1 U/l; reference range 8-33 U/l), alanine aminotransferase (220.3±169.8 U/l; reference range 4-36 U/l) and alkaline phosphatase (837.3±344.1 U/l; reference range 44-147 U/l) were elevated.

The allele frequency of HLA-B*51 was found to be significantly high in the patients (P_c=0.023, OR: 0.236, CI: 0.102-0.574) when compared with healthy controls. The allele frequency of HLA-B*52 was more frequent in the control group, suggesting its protective association with BA, though, after the Bonferroni correction, the association lost its significance (P_c=0.598; OR: 2.860; CI=1.110-7.210). Similarly, the allele frequencies of HLA-A*03 and HLA-A*11 were less commonly detected in patients, but their P_c values were not significant (P_c=0.574; OR=2.380; CI=1.0-5.490 and P_c=0.28; OR=2.1; CI=1.12-4.17, respectively; Table I).

HLA-DRB1 distribution analysis revealed four alleles: HLA-DRB*15 (52.1%), HLA-DRB*07 (27.4%), HLA-DRB*11 (21.9%) and HLA-DRB*13 (19.2%) were predominant in patients; however, neither of these was associated or had any protective significance in BA (Table I).

Genetic association with HLA genes is well established in certain autoimmune disorders such as systemic lupus erythematosus, rheumatoid arthritis, ankylosing spondylitis and celiac disease[15,16]. Many studies propose an element of autoimmunity in BA also. HLA molecules play a crucial role in the induction and regulation of immune response. The HLA association suggests its role in the immune response against unknown foreign or self-antigens which may be responsible for causing an irreversible bile duct destructive process seen in BA. Furthermore,

Table II. Reports on *HLA* association with biliary atresia

Population, Year	Patients	Method used	↑Frequency	*P*	↓Frequency	*P*	HLA antigens analysed	Reference
Northern Europe, 1993	55	CDC	HLA-B12 Haplotype A9-B5 Haplotype A28-B35	0.009 0.04 0.02	None	-	HLA-A: 1, 2, 3, 9, 10, 11, 19, 28 HLA-B: 5, 7, 8, 12-18, 21, 22, 27, 35, 37, 40 HLA-DR: 1-7, 9	11
Japan, 1997	13	IHC MDC	HLA-A33 HLA-B44 HLA-DR6	<0.05	HLA-DR4	<0.05	HLA-A: 2, 11, 24, 26, 31, 33 HLA-B: 35, 39, 44, 51, 52, 54, 55, 61, 62 HLA-C: Cw1, 3, 7 HLA-DR: 2, 4, 6, 9, 52, 53; HLA-DQ: 1, 3, 4, 6, 7	12
Spain, 1997	48	CDC and reverse hybridization	DR6	0.05	HLA-B12 (B44 and B45)	-	HLA-A: 1-3, 11, 23-26, 28-34, 36 HLA-B: 7, 8, 13-18, 22, 27, 35, 37, 41, 42, 44, 45, 49-53, 60, 61 HLA-DR: 1-4, 6-12	24
Egypt, 2002	18	CDC	HLA-B8 DR3	0.006 0.003	None	-	HLA-A: 1-3, 9-11, 28, 29, Aw19 HLA-B: 5, 8, 12-14, 18, 21, 22 HLA-DR: 1, 3-5, 7	10
Northern Europe, 2002	101	PCR-SSP and SSO	None	-	None	-	HLA-A: 1, 2, 29 HLA-B: 35, 44, 51 HLA-DR: B1, B3-B5; HLA-DQ: A1, B1; HLA-DP: B1	25
China, 2004	14	IHC and IEM	HLA-DR	<0.005	None	-	HLA-DR	26
Japan, 2005	392	Micro SSP HLA typing	HLA-DR2 Haplotype HLA-A24-B52-DR2	0.029 0.001	None	-	HLA-A: 1-3, 11, 24, 26, 30, 31, 33 HLA-B: 7, 13, 35, 37-39, 44, 46, 48, 51, 52, 54-56, 58-62, 67, 71, 75 HLA-DR: 1, 2, 4, 7-14	13
USA, 2013	180	PCR-SSP	None	-	None	-	HLA-A: 1-3, 11, 23-26, 29-33, 36, 66, 68, 74 HLA-B: 7, 8, 13-15, 18, 27, 35, 37-42, 44-46, 48-53, 55, 57, 58 HLA-C: 1-8, 12, 14, 15-17 HLA-DRB1: 1, 3, 4, 7-16; HLA-DPB1: 1-6, 9-11, 13, 17, 20, 104; HLA-DQB1: 2-6	14
India, 2021	73	PCR-SSP and SSO	HLA-B*51		None	-	-	Present study

↑, increased; ↓, decreased. CDC, complement-dependent cytotoxicity; IEM, immunoelectron microscopy; IHC, immunohistochemistry; MDC, microdroplet lymphocytotoxicity; PCR-SSP, Polymerase chain reaction-sequence-specific primer; SSO, sequence-specific oligonucleotide

its association with BA may suggest susceptibility to develop BA, such as celiac disease or ankylosing spondylitis[17].

Individuals with HLA-B51 are at risk of developing a rare disorder called Behcet's disease (BD). It is an immune-mediated vasculitis, in which patients present with uveitis, oral aphthous and genital ulcers and skin lesions[18,19]. Its association with HLA-B51 is 40-80 per cent in Turkish, European and Asian populations and 13 per cent among whites in Western countries[20,21]. Other conditions associated with HLA-B51 include mucocutaneous lymph node syndrome and susceptibility to rubella virus infection[22,23]. In the present study, the distribution analysis of HLA-B alleles showed a significantly higher frequency of HLA-B51 in BA. Although a detailed ethnic and geographic background of our study participants, especially controls, was unavailable, most of them came from north Indian Hindu families, mainly from Punjab, Himachal Pradesh, Haryana, Uttar Pradesh and Bihar States. A few reports from other countries on HLA association with BA are compared in Table II[13,14,24-26].

HLA-B51 is one of the two split antigens of the HLA-B5 serotype. HLA-B51 heterotrimer presents endogenous peptides to $CD8^+$ T cells and γδT cells and promotes their activation. Since HLA-B51 is a known risk factor for the development of autoimmune BD, it has been proposed that HLA-B51-restricted cytotoxic cells may target self-antigens expressed on specific tissues. Alternatively, microbial or injury-related peptides could be cross-reactive and responsible for tolerance breakdown and autoimmune manifestations[27-31]. Furthermore, it has been noticed that hyperfunction of HLA-B51-positive neutrophils promotes a Th1 immune response, thereby leading to tissue injury in BD[32,33].

The low prevalence of HLA-A*03, HLA-A*11 and HLA-B*52 alleles in the patient group suggests a protective association; however, their P_c values could not achieve significance. No other HLA-A and DRB*01 allele associations were noted in our patients. Lack of association with HLA-types, *i.e*. HLA-A33, HLA-B8, HLA-B12, HLA-B44, HLA-DR2, HLA-DR3 and HLA-DR6, has been reported in the Western population and may be due to geographic, racial and ethnic variations in this region[11,14]. Other possible reasons could be limited sample size due to the rarity of the disorder, the inclusion of non-age-matched controls and intermediate resolution techniques used for the molecular analysis. The latter also limits the suballele detection for HLA-B*51, which should be kept in mind while designing future studies.

To conclude, our study showed a higher frequency of HLA-B51 antigen in infants with BA, thus reinforcing the hypothesis that genetic factors may contribute to the pathogenesis of BA. Since an early diagnosis and intervention is the key for BA, further associations concerning genetic make-up could be explored in future studies.

***Financial support & sponsorship*:** The study received funding from the Indian Council of Medical Research, New Delhi, India [RFC. No. (P-31)BMS/Adhoc/58/2014-15].

***Conflicts of Interest*:** None.

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Received April 19, 2021

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