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HLA-A*02 is a Protective Allele for Influenza Virus Infection in the Population of Northeast India: A Case-Control Study

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Abstract**—***HLA-A*02 allele has been considered as a protective allele for disease progression in different population worldwide. However, its protective effect on influenza virus infection in Northeast Indian population remains unknown. To investigate the association, influenza-like illness (ILI) patients were enrolled in the present study. HLA-A*02 frequency was considerably higher in non-influenza control group (35/80, 43.75%) compared to influenza infected cases (17/80, 21.25%) with P-value = 0.003. The allele frequency of HLA-A*02 haplotypes were also higher in controls (25/80, 31.25%) compared to cases (16/80, 20%) with P-value = 0.146. Conclusively, the present study reveals that HLA-A*02 may be a protective allele against influenza virus infection in Northeast Indian population. Further studies on HLA-A*02 in a larger study population size might be of potential interests.*

Keywords**—***Influenza virus, human leukocyte antigen, PCR-SSP, Northeast India.*

I. INTRODUCTION

Influenza virus infection is responsible for considerable disease burden with substantial morbidity and mortality annually [1,2]. Globally, around 290,000-650,000 influenza related respiratory deaths have been recorded annually with death rate of approximately 0.1% every year [3]. The emergence of novel pandemic strain influenza A(H1N1)pdm09 during 2009, has strengthened the awareness of influenza virus infection among the population and disease potentiality [4]. Therefore, it has been recognized that population-based studies remains an important tool to study the disease pattern from person to person and to compare the disease severity [5]. The impact of influenza virus infection on disease progression is well understood across different locations and among different populations [6]. However, the overall burden of influenza virus infection among the population always remains important. Various populations with ethnic diversity seem to have differential frequencies of influenza virus infections with unidentified risk factors for the viral transmission [6]. As previously reported, about two-third of influenza H1N1 infected hospitalized cases and 40% of fatal cases globally, did not have any chronic illness. However, this study could not assess the other risk factors (such as, pregnancy, obesity, alcohol and smoking misuse) related with the cases [7]. This may be associated with the interplay of host genetic component and disease susceptibility [8]. Human leukocyte antigen (HLA), with a diverse allelic polymorphism and differential haplotype frequencies plays an important role in the regulation of disease outcome in host organism [9]. HLA plays a crucial role in the presentation of antigen and serves as a leading candidate for genetic susceptibility for various infectious diseases [10]. Many studies have provided evidence that different combinations of HLA molecules are associated with different impact on disease progression [11]. It has been studied that HLA class I molecule participate in both innate and cell mediated immunity and plays an active role in viral clearance, reducing the disease severity caused by influenza virus [11]. Among HLA class I alleles, HLA-A*02, which is widely distributed in the world population, is highly heterogenic and represents the most predominant allele, and are also associated with many diseases [12]. However, its association with influenza virus infection still remains unclear. Our previous study identified HLA allele polymorphism in influenza virus infected population of Assam, Northeast India [13]. However, currently there are no studies in the population of Northeast India on HLA-A*02 allele frequency and its role to influenza virus infection. Thus, the aim of the present study was to identify the HLA-A*02 allele frequency in influenza virus infected population of Assam, Northeast India, compared to non-infected control group.

II. MATERIALS AND METHODS

Ethical Approval

This study was approved by the Institutional Human Ethics Committee of ICMR-Regional Medical Research Centre, N.E. Region, Dibrugarh, Assam, India. The study protocol was explained to all participants and signed informed consent was obtained from all the patients prior to sample collection. All the information was collected regarding illness variables, social and demographic factors, pharmacological and co-morbid conditions.

Study Subjects

Subjects with influenza-like illness (ILI), attending out-patients department (OPD) of primary health centres in Dibrugarh (Assam) were enrolled in the prospective case-control study during January 2018 to December 2019. The study subjects were classified into two categories: influenza

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positive patients were considered as cases and influenza negative patients were referred to as control group.

Collection of Clinical Specimens

Nasopharyngeal and throat swabs were collected from patients in viral transport medium maintaining the cold chain (+4˚C)and transported to the laboratory of ICMR-Regional Medical Research Centre, Dibrugarh, Assam for diagnosis. Three millilitre whole blood samples were collected in EDTA vials from the same patients and carried to the laboratory for further analysis.

Sample Processing and Nucleic Acid Isolation

The clinical specimens of nasopharyngeal/Throat swabs were mixed thoroughly in vortex mixer followed by centrifugation at 1000g for 10 minutes at +4˚C. The collected supernatant of the clinical specimens were further used for viral RNA extraction. 140 µl of the supernatant was taken for viral RNA extraction using QIAamp Viral RNA mini kit (Qiagen, GmbH, Hilden, Germany), according to instructed protocol with proper biosafety measures.

Peripheral Blood Mononuclear Cells (PBMC) Isolation

The collected whole blood samples were used for isolation of peripheral blood mononuclear cells (PBMC) using equal amount of Phosphate buffer saline (PBS) and lymphoprep (Alere Technologies AS, Oslo, Norway). Genomic DNA was extracted from PBMC using QIAamp DNA Blood mini kit (Qiagen, GmbH, Hilden, Germany) according to instructed protocol.

Detection of Influenza Virus and Its Subtypes

Influenza viruses and its subtypes (Influenza A- influenza A(H1N1)pdm09, H3N2 and Influenza-B) were detected from the extracted RNA by Real-time RT-PCR utilizing TaqMan chemistry and WHO recommended primers and probes (H1N1 primer forward (F): 5’ GTGCTATAAACACCAGCCTYCCA 3’, H1N1 primer reverse (R): 5’ CGGGATATTCCTTAATCCTGTRGC 3’, H1N1 probe: **FAM**-CAGAATATACA”T”CCRGTCACAATTGGARAA-**BHQ;** H3N2 primer (F): 5’ AAGCATTCCYAATGACAAACC 3’, H3N2 primer (R): 5’ ATTGCRCCRAATATGCCTCTAGT 3’, H3N2 probe: **FAM**-CAGGATCACATATGGGSCCTGTCCCAG-**MGBNFQ** and Influenza B primer (F): 5’ TCCTCAAYTCACTCTTCGAGCG 3’, Influenza B primer (R): 5’ CGGTGCTCTTGACCAAATTGG 3’, Influenza B probe: **NED**-CCAATTCGAGCAGCTGAAACTGCGGTG-**MGBNFQ**). The PCR reaction was carried out using Superscript III Platinum One step quantitative RT-PCR system with Rox (Invitrogen, Carlsbad, CA) according to manufacturer’s instruction.

Influenza Virus Isolation

Clinical specimens that were tested positive for influenza viruses with cycle threshold (CT) value ≤30 were subjected to Madin-Darby canine kidney cell (MDCK) for virus isolation. The cells were preserved in minimum essential medium that were supplemented with FBS 10%, Penicillin 100 U/ml, Streptomycin 100 µg/mL and Amphotericin B 0.25 µg/mL followed by incubation at 37˚C and 5% CO_2. One mL of each clinical specimen was inoculated into MDCK cell lines maintained with viral growth medium. The inoculated cells were incubated for 1 hour followed by addition of viral growth medium. Infected cells were further monitored for few days and harvested when cytopathic effect was observed. Later, supernatants were collected from all the infected cells and using guinea pig RBC, hemagglutination (HA) test was performed. The HA positive isolates were further treated to hemagglutination inhibition (HI) test to identify its subtypes.

HLA Typing

Genomic DNA isolated from the clinical specimens were subjected to PCR assay for detection of HLA class I (HLA-A*02) allele for both cases and controls using sequence specific primer (PCR-SSP) sets (HLA-Ready Gene ABDR Low resolution typing kit, Inno-Train Diagnostik GmbH, Kronberg, Germany). Allele specific bands were further detected in the PCR products at 2% agarose by gel electrophoresis.

Statistical Analysis

Allele and haplotype frequencies of HLA-A*02 were enumerated by direct counting method, which was calculated as the ratio of the number of times different alleles appeared in the sample to the total number of alleles. Odds ratio and confidence interval (CI) at 95% was calculated using Chi-square test. The P-value was calculated using two-tailed Fisher’s exact test and $P \leq 0.05$ were considered significant.

III. Results

We analyzed a total of 542 patients of >12 years of age (mean age = 30 years) with ILI symptoms that were recruited during January 2018 to December 2019. The clinical characteristics of the patients that included both influenza infected cases and non-influenza controls are described in Table I. Clinical symptoms between the two groups were statistically significant that included nasal discharge (88.23%/66.08%, P-value = 0.0001), cough (97.64%/91.46%, P-value = 0.045), sore throat (82.35%/47.04%, P-value = 0.0001), breathlessness (41.17%/5.47%, P-value = 0.0001), vomiting (18.82%/7%, P-value = 0.001) and oxygen requirement (21.17%/1.53%, P-value = 0.0001), respectively. However, overt co-morbid conditions were not observed in the study population. Influenza A and B viruses were detected in 15.68% (85/542) of the ILI patients. Among the detected influenza viruses, 71.76% (61/85) were influenza A positive (Influenza A(H1N1)pdm09 57.37%, 35/61 and H3N2 42.62%, 26/61) and 28.23% (24/85) were positive for influenza-B viruses. In this study, influenza virus detection was significantly higher (P-value = 0.0002) in females with 64.70% (55/85) than that of males with 35.29% (30/85).

TABLE I. Clinical characteristics and demographic details of influenza infected cases and controls.

Characteristics of patients	Influenza positive cases (n=85)	Influenza negative Controls (n=457)	p-value
Age (years)	40.68±19.95	30.21±13.30	0.0042*
Sex ratio (male/female)	30/55	134/323	0.3037
Clinical symptoms			
Fever	85 (100%)	447 (100%)	-
Rhinorrhea	75 (88.23%)	302 (66.08%)	0.0001*
Cough	83 (97.64%)	418 (91.46%)	0.0456*
Sore Throat	70 (82.35%)	215 (47.04%)	0.0001*
Breathlessness	35 (41.17%)	25 (5.47%)	0.0001*
Vomiting	16 (18.82%)	32 (7.00%)	0.0013*
Oxygen requirement	18 (21.17%)	7 (1.53%)	0.0001*

Note: p-values are calculated between Influenza infected cases and Controls. *p-value ≤0.05 are significant.

The virus infected almost all age groups in the population. The total 85 influenza positive sample were considered as cases and an equal number of samples that were negative for the virus were considered as the control group. Among the 85 influenza positive samples, 30 representative samples were used for virus isolation, of which virus isolation could be done in 20 (66.66%, 20/30) clinical samples. The HA titre of the samples range between 1:4 and 1:128. Analysis of HLA-A*02 allele was performed in 80 influenza infected cases and 80 non-influenza controls. HLA-A*02 allele typing revealed a difference in the allele frequencies between influenza infected cases and controls as shown in Figure I (A). The overall HLA-A*02 allele frequency in non-influenza control group (35/80, 43.75%) was relatively higher compared to influenza infected cases (17/80, 21.25%) with P-value = 0.003, Odds ratio = 2.882 and 95% CI = 1.439-5.772. The heterozygous combinations of HLA-A*02 allele observed in the study population also revealed a higher frequency in the control group (25/80, 31.25%) compared to influenza infected cases (16/80, 20%) with P-value = 0.146, Odds ratio = 1.818 and 95% CI = 0.881-3.750. Figure I (B) represents the allele frequencies of different heterozygous combinations of HLA-A*02 allele in influenza infected cases compared to controls. However, there were no homozygous combinations of HLA-A*02 allele in the present study population.

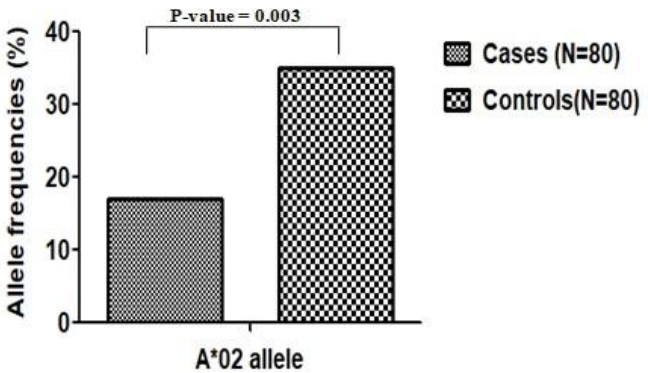

Figure I (A). Representation of HLA-A*02 allele frequencies in influenza infected cases and controls.

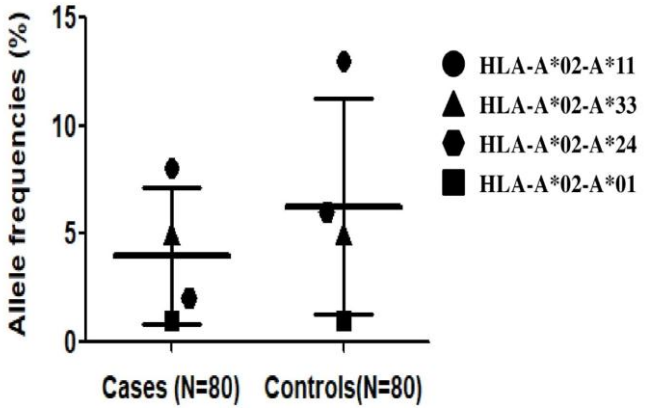

Figure I (B). Allele frequencies of HLA-A*02 heterozygous combinations in influenza infected cases and controls. Figure represents mean and standard deviation of the two groups.

IV. Discussion

HLA-A*02 allele has been observed as the most predominant HLA-A allele in many population [12]. Allele frequency of HLA-A*02 is significantly higher in various tribal population, including Northeast India, as previously studied [12]. The frequency of HLA-A*02 allele in Munda tribe of Northeast India is found to be 21%. This allele was considered to be associated with increased risk for dengue hemorrhagic fever and reduced risk for classical Hodgkin lymphoma [14]. The present study mainly focused on HLA-A*02 allele frequencies in influenza infected cases and non-influenza control group. This study identified for the first time the association of HLA-A*02 allele with influenza virus infection in the population of Northeast India. We analyzed the differences of HLA-A*02 allele frequency in 80 influenza infected cases and equal number of non-influenza controls. Simultaneously, the low-resolution analysis of HLA-A*02 allele showed a considerable difference in HLA-A*02 allele frequency in both the groups. Allele frequency of non-influenza control group was higher (43.75%) compared to influenza infected cases (21.25%), which may serve as a protective allele for influenza virus infection. It has been found that HLA-A*02 allele serve as a protective factor for the development of Epstein-Barr virus and classical Hodgkin lymphoma in many population [15]. Another study reported that immunization of influenza A derived peptides that binds HLA class I and class II molecules results in HLA-A*02 restricted response to the peptide. Furthermore, the study observed the protective effect of HLA-A*02 allele by peptide immunization in mice challenged with influenza A virus [16]. However, some studies did not find any relationship between HLA-A*02 allele and influenza virus infection [7]. In our study, it has been found that the control group (31.25%) has relatively higher frequency of HLA-A*02 allele compared to influenza infected cases (20%) with P-value = 0.146. Although combined with other alleles, presence of HLA-A*02 allele may act as a dominant factor and decrease the disease susceptibility.

V. CONCLUSION

In conclusion, our study reports for the first time the association of HLA-A*02 allele with influenza virus infection in the population of Northeast India. The present study identified that HLA-A*02 allele and its haplotypes may serve as a protective allele against influenza virus infection. However, further studies in a larger population size and different ethnic groups may convincingly elucidate the role of HLA-A*02 allele against influenza virus infection.

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