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Research Article

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The association between HLA B-27 and antinuclear antibody through IIFT: A retrospective study

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Abstract

The human leukocyte antigen (HLA) B27, is a Class 1 surface antigen encoded by the B locus in the major histocompatibility complex (MHC) that presents microbial antigens to T lymphocytes. The antinuclear antibody (ANA) is a class of antibodies that bind to cellular components in the nucleus including proteins, DNA, RNA, and nucleic acid-protein complexes, and defines the feature of connective tissue autoimmune disorders. The indirect immunofluorescence assay (IIFT) uses human epithelial cells and primate liver to identify antinuclear antibodies due to its excellent sensitivity and specificity and thus is the gold standard test.

Aims and Objectives

The main aim of the study is to assess the association between HLA B27 and antinuclear antibodies detected by IIFT. Along with it we also tried to find out the association of HLAB27 positive with other factors such as hemoglobin, rheumatoid arthritis factor (RA), and erythrocyte sedimentation rate (ESR).

Materials and methods:

Design: It is a retrospective observational study.

Study Period

This study was carried out at All India Institute of Medical Sciences, Patna from May 2022 to November 2022.

Study Setting

This is a retrospective study involving samples for all HLA B27 and ANA Screening sent to Biochemistry central lab a period from 2019 till the study period. A total of 225 samples were processed for HLA B27 by Flowcytometry (Beckman and Coulter) and ANA screening in Biochemistry central lab was included in the study. Detailed demographic details, Hb Red blood cell count, ESR, and RA were taken through electronic medical records available on Hospital Information System.

Results: *Of 225 study participants, 54.6% were ≤ 26 years old, and 22.2% were positive for HLA B27. It was also observed that 58.2 % were positive for ANA Screening. An association between the HLA B27 status ANA Screening was found statistically insignificant, p =0.20.*

Conclusions: *No significant association was found between HLA B27 positive cases with ANA screening.*

Keywords: Leukocyte, Sjogren's syndrome, Antibodies, Histocompatibility, Ankylosing Spondylitis

1. Introduction

Major histocompatibility complex (MHC) class I and class II proteins play a pivotal role in the adaptive branch of the immune system. The human Major Histocompatibility Complex (MHC) is situated on chromosome 6 and encompasses over 200 genes. These genes are commonly referred to as Human Leukocyte Antigen (HLA) genes, owing to their initial identification based on variations in antigens found on white blood cells among different individuals [1]. In humans, three class I genes exist HLA-A, HLA-B, and HLA-C. Additionally, three pairs of MHC class II genes are named HLA-DR, HLA-DP, and HLA-DQ [2]. The primary role of the Major Histocompatibility Complex (MHC) is to facilitate the presentation of antigens to T cells, allowing the immune system to distinguish between self (our cells and tissues) and nonself (invading pathogens or modified self-cells). The human leukocyte antigen (HLA) B27, is a Class 1 surface antigen, a major histocompatibility complex (MHC) gene variant, and is remarkably associated with ankylosing Spondylitis [3].

Antinuclear antibodies (ANAs) comprise a wide array of autoantibodies, with the ability to bind and potentially disrupt specific cellular structures located within the nucleus of the cells [4]. They play a crucial role in the assessment of individuals with a wide spectrum of Connective tissue diseases (CTD) [5]. The association between ANAs and certain disease suggests that these antibodies can be used as important biomarkers for screening and diagnosis which would help in a better understanding of the disease.

First described in 1948, ANA identification has been the foundation of diagnosis for connective tissue autoimmune disorders including systemic lupus erythematosus (SLE), Sjogren's syndrome, and polymyositis/dermatomyositis. The first to be identified in this category was the anti-Smith antibody. Others include anti-SSA/Ro, anti-SSB/La, anti-U3-RNP, anticentromere, Scl-70, and Jo-1(1). Even though detectable levels of ANAs are found in 20 to 30% of the average population elevated titres of ANA's are characteristic of individuals with connective tissue disorders, still challenges [6].

ANAs are typically classified into two groups. The first group consists of antibodies that recognize DNA, histones and nucleosomes. The second group includes ANAs that bind to complexes of RNA with RNA-binding proteins (RBPs); however, these antibodies are directed to the protein components of complexes such as the small nuclear ribonucleoproteins (snRNPs) [5]. Examples of ANAs recognizing RBPs include anti-Sm, anti-RNP, anti-Ro and anti-La antibodies [7].

Previously ANA positivity was seen as a characteristic feature of systemic lupus erythematosus (SLE), significant enough to become a criterion for classification of the disease. Currently a positive result may not be very useful for diagnosing SLE because ANA positivity is widely positive in patients with musculoskeletal symptoms and ambiguous symptomatology; in fact, ANA positivity may complicate an otherwise rational diagnostic work-up [8]. Detection of these antibodies with high sensitivity and specificity is very important. This study aimed to see if there is any association between HLA-B27 and antinuclear antibodies detected by IIFT and identify any correlation between specific antinuclear antibodies and HLA-B27. Also, the secondary objective of the study was to see the association between HLA-B27 positive cases with the concentrations of hemoglobin, rheumatoid arthritis factor (RA), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP).

ANA assays

Since ANA testing was included as a standard test, several immunochemical techniques have been used for the assessment of antibody.

Immunofluorescence assay

For the past 50 years the primary method for ANA testing has been the fluorescent ANA assay, or indirect Immunofluorescences Assay (IIFA). IFA was created to increase the sensitivity and repeatability of ANA testing as compared to the previously used method of LE cell assay in various autoimmune disease particularly SLE [9].

The basic principle of IFA is to incubate serum or plasma samples with a source of cells, which could be a cell line or a tissue piece mounted on a glass plate. The basic idea of IFA is to incubate serum or plasma samples with a source of cells, which could be a cell line or a tissue piece mounted on a glass plate. Presently HEp-2 cell line is used because it exhibits a large range of antigens. A fluoresceinated anti-IgG reagent is used to detect the presence of antibodies, and visual inspection is used to determine positivity [10].

Generally, majority of SLE patients have high (95–99%), blood ANA positive rates. However, a significant frequency of positive results has also been observed in the blood of individuals with various rheumatic disorders, including systemic sclerosis, myositis, and RA [11], [12].

The fluorescence patterns observed in IFA are antibodies to cytoplasmic, mitotic and nuclear molecules can also be detected. Common patterns detected by IFA include homogeneous, speckled, rim and nucleolar patterns. Technical expertise is required for the recognition of these patterns due to the abundance of topological features [13].

A variety of multiplex assays for the detection of ANAs are available that recognizes nuclear autoantigens linked to various rheumatic diseases. Among these tests, line immunoassays are a useful method to find antibodies against particular autoantigens. ANA profile is basically an indirect membrane-based enzyme immunoassay for the qualitative measurement of IgG Class

antibodies against 17 antigens in human serum or plasma. The detection of ANA (Anti-nuclear antibodies) and ENA (Extractable nuclear antigens) antibodies is important for diagnosing connective tissue disease [14].

It has been seen that more commonly male patients with HLA-B27 positive arthritis or sacroiliitis are characterised by repeated attacks of acute anterior uveitis [15]. However, not much is known and studied about any possible association between HLA B27 and antinuclear antibodies present or not. The Primary objective of the study was to see if there is any association between HLA B27 and antinuclear antibody detected by IIFT. Also, to see if there is any association between HLA B27+ with the haemoglobin, rheumatoid arthritis factor (RA), erythrocyte sedimentation rate (ESR).

2. Material and Methods

This is a retrospective study conducted on the patients reporting to the, Orthopaedics, General Medicine, and General Surgery Out Patient Department at the All-India Institute of Medicine and Sciences, Patna, India. The study was carried out between April 2022 to October 2022. The study protocol was duly approved by the Institutional Ethical Committee in AIIMS Patna and Research (Ethics Committee no: AIIMS/Pat/IRC/2022/856). A written informed consent was taken from all the patients participating in the study. Consecutive sampling method was used. The study population comprised 225 patients among which 123 patients (54.6%) were ≤ 26 years of age and rest 102(45.3%) were > 26 years of age. Out of 225 study participants 74 (32.9 %) were females and 151 (67.1%) were males. A detailed medical history was obtained from each patient, and all study participants were thoroughly taken for a physical examination. After obtaining their demographic data (name, age, gender, and address), all the selected patients were examined by the physicians of the concerned departments for evaluating the necessary investigations, and clinical tests were performed to confirm the clinical status. All patients were also tested for complete blood count (CBC) by a automated hematology counter. Serum CRP levels were analysed by the immunoturbidimetric method (Beckman Coulter 680) according to the manufacturer's instructions, and results were expressed as mg/L. ESR was measured by capillary photometry, and results were expressed as mm/hr.

HLA B-27 typing

HLA B27 typing was done on NAVIOS flow cytometer (Beckman and Coulter) and ANA screening was done by IIFT using ANA Hep2 antibodies and ANA profile by Line ImmunoAssay (HumaBlot 44 FA). The Duraclone B27 reagent is used on a flow cytometer with a 488nm laser, capable of detecting forward and side scatter. The Duraclone B27 reagent is comprised of a two-color, immunofluorescence stain, suitable for detecting the HLA B27 antigen expressed in human whole blood samples using a lyse. The Navios flow cytometer is equipped with an argon laser excitable at 488 nm emitting Blue light emitted at 488 nm, Red light at 638 nm, and Violet light at 405 nm. Three detectors are present Forward Scatter Detector, providing up to 3 measurements of forward angle, Side Scatter Detector, and High-performance photodiode with electronic attenuation Fluorescence Detectors FL1 - FL10 Fluorescent Detectors. When the fluorochrome-conjugated cells intercept the laser beam in the flow cell, scattered and fluorescent light provide information about particle size, shape, granularity, and fluorescence intensity.

Blood samples: Peripheral blood (3 ml) was collected aseptically by venipuncture, using ethylenediaminetetraacetic acid (EDTA) vacutainers from patients enrolled for the study.

HLA-B27 determination Peripheral blood was used to analyze the expression of HLA-B27 antigen on the T cell surface using a monoclonal antibody specific for HLA-B27 (Duraclone B27 Kit) by flow cytometry technique. Blood samples were stained with anti-HLA-B27 antibody conjugated with fluorescein and with anti-CD3 antibody conjugated with phycoerythrin for 20 min in the dark. When anti-HLA-B27 FITC/CD3 PE monoclonal antibody reagent was added to human whole blood, the fluorochrome-labelled antibodies are bound specifically to the leucocyte surface antigens. This test is based on the ability of specific monoclonal antibodies to bind to the antigenic determinants expressed by T-lymphocytes on the surface. Specific staining of the T-lymphocytes is performed by incubating the human whole blood sample with Duraclone B27 reagent that contains an anti-CD3 antibody. Stained samples are treated with Duralyse to lyse red blood cells and fix before analysis on flow cytometer.

DuraClone B27 reagent also contains fluorescent reference beads that are used to perform an initial calibration of the assay by setting their Median fluorescent intensities (MdFI) at defined target regions. Using these settings as the basis, T-lymphocytes selected through gating of the CD3+ specific population are analyzed for staining by HLA-B 27 conjugates. The test result is a direct comparison of the HLA B27 MdFI of the T lymphocyte population with the 'Determinant' value. Determinant value is the cut-off value to type the sample as HLA B27 positive or negative. The Indeterminant Zone around the Determinant is defined to account for process-associated variability and is calculated as the variance of Operators. The Indeterminant zone of Navios lies between 11.4 to 18.0 [16].

Sample analysis

The acquired samples were then be analyzed by HLA-B27 acquisition software. When around 3000 events were acquired, a dot plot of PE (FL2 detector) versus side scatter (SSC) was used by the software algorithm to gate the CD3-positive fluorescent cells. The MdFI intensity of the HLA-B27 FITC signal (FL1) was calculated for the CD3 T lymphocytes and compared to the predetermined decision marker. The T-lymphocyte population was displayed in a FITC/FL1 histogram, where the MdFI was

calculated.

ANA screening

The indirect immunofluorescence test using Euroimmun (Medizinische Labordiagnostika AG) Mosaic Hep-20-10 / Liver (Monkey) kit is used for detection of Antinuclear Antibody. This test kit provides qualitative or semiquantitative invitro determination of human antibodies of immunoglobulin class IgG against cell nuclei to support the diagnosis of autoimmune diseases particularly Connective tissue disorders. The substrate used is Hep 20-10 primate (human, monkey) liver cells (2 BIOCHIPs per field). The fluorescence is evaluated using the fluorescence microscope. The principle of the test involves the incubation of patient's diluted sample in the test field. If the reaction is positive specific antibodies of classes IgA, IgG and IgM attach to antigens. In a second step, the attached antibodies are stained with FITC labelled anti-human antibodies and made visible with a fluorescence microscope.

3. Results

The study included 225(N) participants out of which 123(54.6%) were ≤ 26 years of age and 102(45.3%) were > 26 years of age. The mean age of the participants was 28 years with a standard deviation of 12. The minimum age was 4 years and the maximum age was 67 years. Out of 225 study participants, 74 (32.9 %) were females and 151 (67.1%) were males (Table1). HLA B27 positive patients were 22.2% and rest 77.8% were Negative. It was also observed, 58.2 % were Positive for ANA Screening and 41.8 % were Negative.

Variable	Category	Frequency	Percentage (95% CI)
Age	≤26 years	123	54.6 (48.1-61.0)
	>26 years	102	45.3 (38.9-51.8)
Gender	Female	74	32.9 (26-9 -39.2)
	Male	151	67.1 (60.7 – 73.0)

Table 1: Socio-demographic distribution of study participants. (N=225)

CI - Confidence Interval

The chi-square test was used to find an association between the gender of study participants, HLA B27, and ANA Screening. The results indicated a significant association between the males with positive HLA B27 status, $\chi2 = 6.46$, $p = 0.011$. However, there was a statistically insignificant association found between the gender and the ANA Screening categories, $\chi2 = 1.38$, $p = 0.24$ Table (2).

Biochemical markers	Gender	Negative Frequency (%)	Positive Frequency (%)	Pearson's Chi-square (p-value)	Odds ratio
a) HLA B27	F	65 (87.8)	9 (12.2)	6.46 (0.011)	2.69
	M	110 (72.8)	41 (27.2)		
b) ANA Screening	F	35 (47.3)	39 (52.7)	1.38 (0.24)	1.40
	M	59 (39.1)	92 (60.9)		

Table 2: Association of HLA B27 and ANA Screening with gender of the study participants.

An association between the HLA B27 status of study participants and ANA Screening status categories was examined using a chi-square test. The results showed a statistically insignificant association between the HLA B27 status of study participants and ANA Screening status categories, $\chi2 (1) = 1.6$, $p = 0.20$ (Table 3).

ANA Screening outcomes	HLA B27 status	Negative Frequency (%)	Pearson chi square (p-value)	Crude Odds ratio (95% CI)
	Negative n(%)	Positive n(%)		
Negative	77 (81.9)	17 (18.1)	1.6 (0.20)	1.53 (0.79 – 2.94)
Positive	98 (74.8)	33 (25.2)		

Table3: Association of HLA B27 with ANA Screening outcomes of the study participants

CI = Confidence Interval

An independent sample t-test was applied to test whether mean Hb and RBC of HLA B 27 positive study participants was different from HLA B27 negative study participants. The values of Hb and RBC across HLA B27 was normally distributed as reported by visual inspection of Q-Q plot to test the normality. It was observed that there is statistically significant difference in mean Hb and RBC with HLA positive (11.76 ± 2.0) p value = <0.001and (4.33 ± 0.75) p value = 0.009 (Table 4). Nevertheless, another biochemical parameter such as R factor and ESR were not normally distributed. The Median ± IQR 9.6 ± 3.6 IU/ml and 28 ± 40.0 mm/hr with p value 0.94 and <0.001respectively (Table 5).

Parameters	HLA B 27		Mean difference	t(df)	p - value
	Negative	Positive			
	Mean ± SD	Mean ± SD			
Hb	13.02 ± 2.0	11.76 ± 2.0	1.253	3.9 (223)	<0.001
RBC	4.7 ± 0.9	4.33 ± 0.75	0.374	2.65 (223)	0.009

Table 4: Association of parameters in study participants with HLA B27 status

Parameters	HLA B 27		Mann-Whitney U value	p - value
	Negative	Positive		
	Mean ± SD	Mean ± SD		
Hb	13.02 ± 2.0	11.76 ± 2.0	1.253	3.9 (223)
RBC	4.7 ± 0.9	4.33 ± 0.75	0.374	2.65 (223)

Table 5: Association of parameters in study participants with HLA B27 status

Discussion

This study was carried out to evaluate any possible association that exists between HLA B27 and antinuclear antibodies detected by IIFT in a tertiary care center. Besides, different clinical parameters such as Hb, RBC, R factor, and ESR were assessed in males and females at various age groups (4-67 years).

In the present study, a total of 225 participants were enrolled, out of which 123(54.6%) were ≤ 26 years of age and 102(45.3%) were > 26 years of age. The minimum age was 4 years and the maximum age was 67 years. Out of 225 study participants, 74 (32.9 %) were females and 151 (67.1%) were males. The present study demonstrated a significant association between males with HLA B27 positive i;e 72.8 % of males as compared to females. Several studies have also shown a higher preponderance of HLA b27 in males as compared to females [17]. A recent study conducted by Haridas et al also aligns with the same finding [18]. which has shown that males were more frequently affected than females (male: female ratio of 3.4:1) in the Indian population.

The flow cytometry analysis of HLA-B27 undertaken in the present study shows that there is a statistically insignificant association between the HLA-B27 status of study participants and ANA Screening status by IIFT. In contrast, other studies have shown a higher frequency of HLA-B27 positivity in the Indian population with Ankylosing Spondylitis [19]. In another study, when patients with negative RA factor were tested, HLA-B27 frequency was found to be more prevalent [20] whereas in this study it was not associated significantly.

Tests for CRP and ESR are frequently performed on patients with inflammatory diseases. One of the acute phase reactants that rises in the blood during inflammation is CRP. Generally, elevated CRP levels indicate underlying inflammation.

The normal reference ranges for the RA factor in our laboratory is < 10 and for ESR <15 mm/hr. In the present study, the clinical evaluation of the inflammatory markers concerning HLA-B27 was found to be clinically significant. The majority of HLA B27 positive phenotypes were negative for RA, indicating no specific relationship between RA value and HLA B27 positivity. This finding is consistent with a previous report, which has also shown the same results [21]. In the present study, ESR and hemoglobin were positively correlated with HLA B27 positive cases whereas in other studies no positive correlation was found between them [19].

HLA-B27 detection is also useful for screening relatives who may have HLA-B27 antigen and are at risk of developing autoimmune diseases. Even though the current study can reflect the HLA-B27 status in patients with autoimmune diseases in the Indian population, it has a limitation of a small sample size. Therefore, further extensive studies with a higher number of suspected Autoimmune patients are required to confirm the prevalence of HLA-B27 in the Indian population.

Conclusions

Taken together, the present study is established to observe if there is any association between HLA-B27 and antinuclear antibodies detected by IIFT and identify any correlation between specific antinuclear antibodies and HLA-B27 in a tertiary care center. The detection of HLA-B27 is by flow cytometry technique, which proves to be a reliable, inexpensive screening test with high specificity and sensitivity and can be safely used by clinicians. The sensitivity of the HLA-B27 screening test by flow cytometry was 99%, while the specificity was 100%. It was found that the most common pattern observed in ANA was a speckled pattern detected by IIFT. However, in this study, no association was found between HLA B27 positive cases with ANA screening by IIFT.

Additional Information

4. Disclosures

Human subjects: Consent was obtained by all participants in this study. Institutional Ethics Committee, All India Institute of Medical Sciences, Patna issued an approval letter. Animal subjects: All authors have confirmed that this study did not involve **animal subjects** or tissue. Conflicts of interest: In compliance with the ICMJE uniform disclosure form, all authors declare the following: Payment/services info: All authors have declared that no financial support was received from any organization for the submitted work. Financial relationships: All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. Other relationships: All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work. Acknowledgments The authors express their gratitude to the Central Research Facility, and Central Laboratory, Department of Biochemistry, AIIMS Patna for carrying out this study.

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Competing interests: Nil

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