https://doi.org/ 10.33472/AFJBS.6.4.2024.332-350

African Journal of Biological Sciences

Research Paper Open Access

Association of genetic polymorphism of gene PTPN 22 with Rheumatoid arthritis in central Indian population

Dr.Jyotsna Mishra

Department of Zoology, Government College, Devetalab, Mauganj, India

Email: Jyotsna.mishra05@gmail.com

Article History

Volume 6,Issue 4, Feb 2024

Received:17 Feb 2024

Accepted : 01 Mar 2024

doi: 10.33472/AFJBS.6.4.2024.332-350

Abstract:

Study of the genetics of rheumatoid arthritis (RA) began about four decades ago with the discovery of HLA-DRB1. Since the beginning of this century, a number of non- HLA risk loci have been identified through genome-wide association studies (GWAS). We now know that over

100 loci are associated with RA risk. Because genetic information implies a clear causal relationship to the disease, research into the pathogenesis of RA should be promoted. However, only 20% of GWAS loci contain coding variants, with the remaining variants occurring in non-coding regions, and therefore, the majority of causal genes and causal variants remain to be identified. The use of epigenetic studies, high-resolution mapping of open chromatin, chromosomal conformation technologies and other approaches could identify many of the missing links between genetic risk variants and causal genetic components, thus expanding our understanding of RA genetics.

Keywords: rheumatoid arthritis, TNF, IL-1, cytokines, gene polymorphisms

INTRODUCTION:

Rheumatoid arthritis (RA) is an inflammatory rheumatic disease that causes chronic synovial inflammation, eventually leading to disabling joint destruction as well as systemic complications. Most epidemiologic studies indicate that the prevalence of RA is 0.5%–1.0%. Between 70% and 80% of patients with RA have autoantibodies such as rheumatoid factor and anti-citrullinated protein antibodies (ACPA), suggesting that RA is an autoimmune disease. The majority of

rheumatic diseases involve complex traits in which multiple genetic and environmental factors interact. Twin studies have estimated that the heritability of RA is ~60%. This applies primarily to patients with RA who are positive for ACPAs, whereas the heritability of seronegative RA appears to be lower. Since 2007, rapid advances in genome-wide association study (GWAS) technologies have facilitated the identification of hundreds of genetic risk factors for many complex diseases. To date, more than 100 genetic loci have been associated with RA **[1].**

However, the relationship of these loci to the disease remains to be elucidated. As a genetic factor has a clear causal relationship to RA, it is important to understand the pathologic process from a genomic standpoint. Recent studies of complex trait diseases have indicated that many disease susceptibility variants regulate the expression levels of a number of genes that function in a cell-specific manner. Furthermore, the epigenome is thought to play an important role in this phenomenon. Obtaining a more thorough understanding of this complex regulatory network is vital to determining which genes and cell types play pivotal roles in RA, thus helping to identify key pathways that drive RA and enable stratification of patients into groups based on the causative pathways. Here, we describe the state of genetic research to date, envisaging a better understanding of the pathogenesis of RA. The immune system defends us against microorganisms that can cause infection **[3].** To protect the individual effectively the immune system must fulfil four main tasks. First immunological recognition, the presence of an infection must be detected. This task is carried out by the leukocytes in the innate immune system, which provide an immediate response, and by the lymphocytes of the adaptive immune system. The second task is to contain the infection and if possible eliminate it completely. To do that we need immune effector functions such as the complement system of blood proteins, antibodies and the destructive capacities of leukocytes among them lymphocytes. At the same time the immune response must be kept under control so that it does not do damage to itself. Immune regulation, the ability of the immune system to self-regulate, is an important function of the immune response and failure of such regulation can cause autoimmune diseases like RA. The fourth task is to protect the individual against recurring infections due to the same pathogen and therefore the adaptive immunity has evolved the capacity of generating an immunological memory **[2-4].**

Innate immunity serves as a first line of defense. It works as an early barrier to pathogens that acts immediately but does not generate a lasting protective immunity. Key components of the innate immunity are anatomical barriers such as the epithelia of the skin, the gastro-intestinal and respiratory tracts and professional phagocytes like neutrophils, macrophages and dendritic cells. Phagocytes can engulf microbes and destroy and eliminate them by degrading enzymes and cytotoxic mediators like antimicrobial peptides (AMPs) and reactive oxygen species (ROS). AMPs are naturally occurring peptides considered to be among the earliest developed molecular effectors of innate immunity **[2].** The immune system is triggered by detection of pathogen (non-self) associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) from invading organisms or injured structures. The macrophages, neutrophils and dendritic cells have receptors to recognize these patterns, so called pattern recognition receptors (PRRs). The dendritic cells work as a bridge between innate and adaptive immunity. They capture the microbial antigen,

migrate to peripheral lymphoid organs and use their major histocompatibility complex (MHC) 12 class II molecules to display the antigen, in conjunction with co-stimulatory signals, to T cells **[5].**

This initiates the early events in adaptive immunity where antigen specific T and B cells are activated After activation, they undergo clonal proliferation and maturation. These features allow the adaptive immune response to be directed towards specific pathogens. Subpopulations of these cells later become memory cells that can quickly mount an immune response to the same microbe when reactivated. Upon encounter with the antigen, clonal expansion of antigen-specific T and B cells occurs **[7].** The specificity of T and B cell receptors is acquired through gene translocation and mutations that allow an increase in affinity and specificity. Innate immune cells do recognize molecular motifs that are shared by various classes of microbes through genetically encoded PRRs, of which toll-like receptors (TLR) are a major family. TLRs are highly conserved across evolutionary time and serve to activate host defense through a signalling pathway that operates in most multicellular organisms. In vertebrates TLRs have a key role in enabling the initiation of adaptive immunity. TLR- 4 for example detects the presence of Gram-negative bacteria through its association with the peripheral membrane protein CD14, which is a receptor for bacterial lipopolysaccharide (LPS) **[8].** Other TLRs respond to other molecular patterns found on or in pathogens. TLRs activate the transcription factor NFκβ, which then induces the transcription of a variety of genes, including cytokines, chemokines and co-stimulatory molecules that have essential roles in directing the course of the adaptive immune response **[6-9].**

Review of literature

Lymphoid tyrosine phosphatase (LYP) encoded by PTPN22 is an intracellular enzyme that dephosphorylates Src family kinases Lck and Fyn as well as components of the TCR/CD3 complex and other key signaling molecules. Therefore, LYP appears to be an important negative regulator of T-cell responses. PTPN22 encodes for an 807 amino acid residue protein called LYP (lymphoid tyrosine phosphatase), which has been shown to negatively regulate T-cell signaling. A single-nucleotide polymorphism in the PTPN22 gene at nucleotide position 1858 C4T (codon 620), resulting in an arginine-to-tryptophan (CGG to TGG) transition, has been shown to be a gain-of-function mutation, with a more potent negative regulation of T-cell signaling through reduced Lck (leukocyte-specific protein tyrosine kinase)-mediated phosphorylation of the TCRx chain, reduced tyrosine phosphorylation of LAT (linker for activation of T cells), and reduced activation of Erk2 (Senapati S, et. al. 2014). The mutant, LYP-Trp620, has been associated with several autoimmune diseases. Rheumatoid arthritis (RA) is a destructive inflammatory condition affecting about 1% of the UK population. A genetic component of susceptibility to RA is well established and around 30% of it has been attributed to the HLA-DRB1 locus. Association between a functional single-nucleotide polymorphism (SNP) in the coding region of the gene PTPN22 and RA. PTPN22 encodes the lymphocyte-specific phosphatase (Lyp), which is an important potential factor in modifying signalling through the T-cell receptor (TCR) .The presence of the PTPN22 1858 T/T genotype increases the risk of RA more than 2-fold. However, little is known about its effects on

severity and co-association with other autoimmune diseases (Michoua L, et. al. 2007; Massey J, et. al. 2018).

Recently, Bottini et al described a single nucleotide polymorphism (SNP) in PTPN22 gene, 1858C>T, changing arginine to tryptophan residue in position 620 of mature protein. The 1858T (620W) variant was found associated with insulin-dependent diabetes mellitus. Subsequently, association with 1858T was reproducibly described for other autoimmune diseases, including rheumatoid arthritis (RA. In a recent case–control association study, Begovich et al found a strong association of the PTPN22 1858C/T (rs2476601) single-nucleotide polymorphism (SNP) with RA. The presence of the minor 1858T allele increased the relative risk for RA almost two-fold. The carrier frequency of the 1858T risk allele in Caucasians with RA was 28% while this allele was present in approximately 17% of Caucasian controls. PTPN22 has been shown to bind to an intracellular tyrosine kinase, Csk. This binding occurs by virtue of a proline-rich SH3 binding site on PTPN22, interacting with the SH3 domain of Csk. As shown in Figure 5, these molecules act in concert to inactivate Lck, an Src family kinase that is involved in early T cell signaling events. Csk acts to phosphorylate tyrosine 505 (an inhibitory phosphate for Lck), while PTPN22 acts to remove the activating phosphate at tyrosine 394. The combined effect of these activities is to convert Lck to an inactive configuration **(Guan Y, et. al. 2019; Murdaca G, et. al. 2014).**

Regulation of the Lck activation state. Lck (green shading) is an Src family kinase involved in early T cell signaling events and is maintained in an inactive state in resting T cells by phosphorylation (P) of a C-terminal tyrosine 505 (Y505), as shown on the left side of the figure.The literature suggests that dephosphorylation of Y505 (possibly mediated by CD45) causes a conformational change in Lck, resulting in phosphorylation of an activating tyrosine residue Y394, leading to Lck activation, as shown at the right side of the figure. PTPN22 binds the tyrosine kinase Csk via a proline-rich SH3 binding site (P1). This binding is thought to enable colocalization of PTPN22 to Lck, dephosphorylation of Lck Y394, and return of Lck to an inactive state, with concomitant rephosphorylation of Y505 by Csk. TCR-T cell receptor; PAG phosphoprotein associated with glycosphingolipid-enriched microdomains. The frequency of the 1858T allele was significantly higher in both lepromatous and tuberculoid leprosy patients than in normal healthy controls. Although homozygous 1858TT was absent from both groups of patients, as well as from the control samples studied, heterozygous CT was significantly increased in both groups of patients **(Joo Y, et. al. 2017).** All genotype frequencies in patients as well as in controls were in Hardy–Weinberg equi-librium. The allele frequency of the minor T allele varies in different ethnic groups. Contrary to reports of an absence of the T allele in Asia. we observed a very low frequency (1.9%) of this allele in normal healthy individuals from North India as compared with that reported for the populations of European ancestry. Earlier reports of an absence of the 1858T allele in individuals from Asia have been from studies conducted in Japan, Korea, and China; these populations are Mongoloid in origin and differ ethnically from Indians. North Indians have been described as being basically Caucasoids, with a racial admixture of Mongoloid **(Murdaca G, et. al. 2014; Frisell T, et. al. 2016).**

MATERIALS AND METHODS

Patient recruitment:

Rheumatoid arthritis patients were recruited from sanjay Gandhi hospital, Rewa, Hamidia hospital, Bhopal; Bombay hospital Indore, (M.P.) during the year 2020-2021. 112 patients were enrolled in the study. All the patients were of Central Indian origin. The dignosis of RA was based on various laboratory tests (Rheumatoid Factor, Sed rate, Hemocrit, Synovial fluid analysis, Citrulline antibody, Antinuclear antibodies (ANA), C-Reactive Protein (CRP), Anti-CCP antibodies) and radiological criteria. All patients participating in study provided informed consent. Institutional ethics committee of Shyam Shah medical college, Rewa (M.P.), India, approved the experimental protocol.

Healthy controls:

112 randomly selected healthy controls (HC) were enrolled in the study. They consisted of medical staff and healthy volunteers from Rewa, Jabalpur, Bhopal, Indore as well as individuals residing in central region of India. Hence, control group was drawn from same area assuming similar environmental and social factors.

Sample collection:

Approximately 5 ml. of blood sample was collected in 0.5 M EDTA tubes from each RA patients as well as from healthy controls. These samples were stored frozen at -80˚C until DNA was extracted from them.

Method for DNA isolation:

Genomic DNA was extracted from whole blood by the modification of salting out procedure described by Miller and coworkers (Miller et al. 1988). Frogen blood sample was thawed at room temperature. 0.5 ml. of whole blood sample was suspended in 1.0 ml. of lysis buffer (0.32 M Sucrose, 1 mM Mgcl2, 12 mM Tris and 1% Triton-X-100) in a 1.5 ml. microcentrifuge tubes. This mixture was mixed gently by inverting the tube upside down for 1 min. The mixture was then allowed to stand for 10 min. at room temperature to ensure proper lysis of cells. The mixture was centrifuged at 11,000 rpm for 5 min. at 4˚C to pellet the nuclei. The supernatant was discarded carefully in a jar containing disinfectant, as pellet formed is loosely adhered to the bottom of centrifuge tube. The pellet was resuspended in 0.2 ml. of lysis buffer and recentrifuge at 11,000 rpm for 5 min. The pellet was than dissolved in 0.2 ml. of deionized autoclaved water and mixed thoroughly on vortexer. The mixture was centrifuged at 14,000 rpm for 1 min. at 4˚C. Supernatant was discarded to gain an intact pellet. To the above pellet, 80 µl. of proteinase K buffer (0.375 M Nacl, 0.12 M EDTA, pH 8.0) and 10 µl. of 10% SDS (10% w/v SDS, pH 7.2) was added. Mixture was well frothed with the help of micro tip to allow proper lysis of pelleted nuclei. After digestion was complete 100 µl. of saturated cold 5M Nacl was added and

shaken vigorously for 15 sec. To the above mixture 0.2 ml. of deionized, autoclaved water and 0.4 ml. of phenol-chloroform (4:1 v/v) was added to remove most of the non nuceic acid organic molecules. Microcentrifuge tube was inverted upside down until the solution turned milky. Phases were separated by centrifuging the above mixture at 12,000 rpm for 10 min. at 4˚C. Aqueous (top) layer was saved and transferred in another microcentrifuge tube. Transfering of any interface layer was avoided. To the aqueous layer, 1 ml. chilled absolute ethanol was added and the tube was inverted several times until the DNA precipitated. DNA precipitates like thread. This was centrifuged at 14,000 rpm for 4 min. at 4˚C to pellet the DNA thread. Supernatant was discarded. The pellet was washed twice with 1 ml. of 70% alcohol. The mixture was again centrifuged at 14,000 rpm for 1 min. 4˚C. Supernatant was discarded and pellet was air dried for 10-20 min. The pelleted DNA was rehydrated in 100-200 µl. of TE buffer pH 7.4 (10 mM Tris-HCL pH 7.4, 1mM EDTA, pH 8.0). DNA was allowed to dissolve overnight at 37˚C before quantitating.

Determination of quality and quantity of isolated DNA:

The isolated DNA is to be used for PCR based study. Therefore its suitability for PCR along with its size heterogeneity is among the most important criterion for purity. As a matter of general practice all DNA preparations were tested for quality and quantity measures, as described in the following paragraphs.

Quantitation by UV spectrophotometry:

The isolated genomic DNAs were then tested for purity by measuring their absorbance values at 230 nm, 260 nm, 280 nm and 300 nm using a UV visible spectrophotometer (Systronic, India). A DNA prepration was considered to be good if it had A 260 nm / A 280 nm ratio as approximately 1.8 and A 300 nm was 0.1 or lesser. The absorbance at 260 nm was used to calculate the amount of DNA, using the relationship that double stranded DNA at 50µg/ml concentration has an absorbance= 1.0 at 260 nm.

Agarose Gel Electrophoresis:

Gel electrophoresis of the genomic DNAs was carried out for qualitative estimation of samples prepared. A good DNA prepration appears as single band. A horizontal agarose slab gel electrophoresis apparatus (Banglore Genei, Bangaore, India) was used. In brief, 2 µl of each genomic DNA was loaded on 0.8 agarose (0.8 % w/v, Sigma) containing ethidium bromide solutions (0.5 µg/ml) and electrophoresis was done at 80 V in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Lambda DNA *Eco* RI / *Hind* III double digest (Banglore Genei, Bangaore, India) was used as molecular weight marker after completion of electrophoresis, the DNA bands were visualized and photographed using an UV transilluminator (312 nm) and gel documentation system (Vilber Lourmate, Cedex 1, France) respectively.

Polymorphism screening:

In general, the genomic DNA extracted from peripheral blood of healthy individuals and diseased individuals was subjected to PCR followed by restriction digestion and electrophoresis to genotype both the groups for relevant gene of interest. All the PCRs were carried out in a PTC 200 thermocycler (MJ Research Inc. USA). PCR is a rapid, inexpensive and simple mean of producing relatively large copy number of DNA molecules from the small amounts of source DNA material, even when the source DNA is of relatively poor quality. Due to the extreme sensitivity, precautions were taken against contamination of the reaction mixture with the trace amounts of DNA, which could serve as an unwanted template. Appropriate negative controle was included in each PCR run carried out for each gene, to monitor this contamination of PCR mix to avoid any false positive results. The negative controle used for PCR contained whole PCR reaction mix except target DNA which was replased by HPLC purified water free of RNase, DNase, and any contamination from any other source resembling the gene sequence.

Subsequently restriction enzyme digestion was performed by incubating the double stranded DNA with appropriate amount of restriction enzyme, in its respective buffer as recommended by the supplier and at optimal temperature for that specific enzyme. A typical digestion includes one unit of enzyme per microgram of starting DNA. One enzyme unit is usually defined as the amount of enzyme needed to completely digest one microgram of double stranded DNA in one hour at the appropriate temperature. Their biochemical activity of the restriction enzyme is the hydrolysis of phosphodiester backbone at specific sites in a DNA sequence. Precaution was taken to avoid star activity of restriction enzymes. When DNA is digested with certain restriction enzymes under non-standard conditions, cleavage can occur at sites different from the normal recognition sequence. Such aberrant cutting is called “star activity” which can be due to high pH (>8.0) or low ionic strength, extremely high concentration of enzyme (>100 U/µg of DNA) and presence of organic solvents in the reaction (e.g. ethanol, DMSO).

The PCR and restriction digestion conditions were optimized for specific locus of relevant segment of the gene to be studied. The PCR products as well as the digested products were separated on either agarose gel or polyacrylamide gel depending on their size. Gels were stained with ethidium bromide solution (0.5µg/ml) and subsequently visualized and photographed under UV transilluminator.

Detection of PTPN22 -1858 SNP via PCR-RFLP:

The nucleotide positon -1858 (at codon 620) gene has a single nucleotide polymorphism that results in change of nucleotide from cytosine (C) to thiamine (T). The oligonucleotides sequences (primers) were designed to create a recognition site for the restriction enzyme XcmI in the T allele.

Primer sequences:

Sense oligo 5’ –TCA CCA GCT TCC TCA ACC ACA-3’

Antisense oligo 5’-GAT AAT GTT GCT TCA ACG GAA TTTA-3

PCR mix:

The PCR reaction was carried out in a total volume of 25 µl containing 100 ng of genomic DNA, 10 pM of each primer, 2mM MgCl2, 0.2 mM deoxy-nucleotides (dNTPs), 1X buffer and 2U of Taq polymerase.

Thermal profile:

Thermal profile used for the amplification of desired segment of gene was as follows: Initial denaturation at ….and 35 cycles of denaturation at 94^0C, annealing at 60^0C for 1 min and extension at 72^0C for 1 min, followed by final extention at 74^0C for 5 min. PCR products were separated on 3% agarose gel using a 100 bp molecular weight (MW) marker to confirm the PCR product size.

Restriction digestion by *X*cmI:

The C to T transition at codon 620 creates in the 1858T allele a restriction site for *X*cmI. When gene was amplified by PCR than incubated with *X*cmI restriction enzyme (New England Biolabs, USA).

Recognition site for *X*cmI restriction enzyme is:

5’…CCANNNNNNNNTGG…3’

3’…GGTNNNNNNNNACC…5’

The PCR product was digested using 0.4U XcmI restriction enzyme

(New England BioLabs) for 4 h at 37^0C

Genotyping:

Digetion of the amplified 215 bp PCR product gave two fragments of 174 bp and 41 bp respectively if the product was excisable by *X*cmI. Depending on the digestion pattern, individuals were scored as genotype CC when homozygous for presence of *X*cmI site, genotype TT when homozygous for absence of *X*cmI site and genotype CT in case of heterozygosity.

RESULTS:

This study based on comparative statistical analysis between Rheumatoid arthritis (RA) patients group and healthy control (HC) group. Comparative Statistical analysis of anthropometric and clinical parameter may give information about association with Rheumatoid arthritis. Thus

genetic association of gene PTPN22 would reveal significant differences between the groups. This population based study revealed genetic association in central India.

Anthropometric results:

The descriptive data and comparison of anthropometric and biochemical parameters of Rheumatoid arthritis (RA) patients versus controls are presented in Table no. 4.1. The age, sex, BMI, WHR were the parameters. As expected the Rheumatoid arthritis (RA) patients had markedly higher levels of high age group (P<0.0001***), weight of women (P<0.0001***) then men (P=0.5045ns) and BMI of Women (P<0.0001***) then Men (P=0.1913ns). The parameter Waist circumference (cm) and Hip (cm) determine the WHR. Our statistical data from Waist circumference (cm) and Hip (cm) was not significant different between Rheumatoid arthritis (RA) patients group and healthy control (HC) group. WHR was not significantly different between patient and healthy population. Thus WHR in Women (P=0.2904ns) and Men (P=0.1912ns) were not found were not found association with Rheumatoid arthritis (RA) **(See Table No. 1).**

TABLE No-1

Comparison studies of anthropometric parameters between Rheumatoid arthritis (RA) patients and healthy controls.

Characteristics	*Cases (96)*	*Controls(112)*	*P-value*
n(Men/Women)	96(54/42)	112(62/50)	
Age(years)	59.6±12.4	52.5±12.5	P<0.0001***
Height(m)	162.50±11.3	161.2±12.4	0.4333,ns
Weight (Kg)			
Women	69.5 ±4.7	60.6 ± 4.5	P<0.0001***
Men	68.4±5.6	67.8±7.1	0.5045,ns
BMI (kg/m^2)			
Women	25.6±3.1	22.1 ± 4.3	P<0.0001***
Men	24.6±4.7	23.8± 4.1	0.1913,ns
Waist circumference (cm)			
Women	92.5±6.2	93.6±6.7	0.2233,ns
Men	90.0±7.0	89.0±6.0	0.2685,ns
Hip (cm)			
Women	95.9±2.4	96.1±2.2	0.5315,ns
Men	90.8±4.3	91.2±1.5	0.3579,ns
WHR			
Women	0.97±0.05	0.98±0.08	0.2904,ns

Men	0.98±0.08	0.99±0.01	0.1912,ns

(* denotes level of significant change between case and control)

Biochemical and clinical findings:

Biochemical test performed in the blood sample for following clinical parameters and the findings were tabulated. Statistical analysis was done by using student's t-test and p value suggests the level of significant were changed here. The descriptive data and comparison of biochemical parameters of Rheumatoid arthritis (RA) patients versus healthy controls are presented in Table no. 4.2. As expected the Rheumatoid arthritis (RA) patients had markedly higher levels of ESR (P<0.0001***), α $_1$ACT (P<0.0001***) and Serum CRP (P<0.0001***) compared to that of control subject. Thus P-Value of ESR (Erythrocyte sedimentation rate) , α $_1$ACT (Alpha 1 antichymotrypsin) and CRP (C-Reactive Protein) concentration revealed association significantly with Rheumatoid arthritis (RA) patients. Whereas rest of all parameters such as Post-Prandial Glucose (mg/Dl), HbA1C(%), HDL-C(mmol/L), LDL-C (mg/dL), TG(mg/dL), Systolic BP (mmHg), Diastolic BP (mmHg) and Blood Urea(mg/dL) were not significantly different between patient and healthy population **(See Table No.2).**

TABLE No-2

Comparison of Biochemical and clinical findings of Rheumatoid arthritis (RA) patients and Healthy controls.

Characteristics	*Cases (96)*	*Controls(112)*	*P-value*
Post-Prandial Glucose (mg/Dl)	117.7±12.4	118.4±11.6	0.6747,ns
HbA1C(%)	5.8±0.7	5.7±0.8	0.3424,ns
HDL-C(mmol/L)	108.8±12.2	109.3±11.6	0.7625,ns
LDL-C (mg/dL)	42.1±2.6	41.8±3.7	0.5063,ns
TG(mg/dL)	125.9±13.2	126.2±12.2	0.8650,ns
Systolic BP (mmHg)	125.4±8.1	124.8±5.7	0.5332,ns
Diastolic BP (mmHg)	87.1±5.8	86.5±6.2	0.4744,ns
Blood Urea(mg/dL)	18.1±1.7	17.8±1.8	0.2204,ns
ESR, mm/h	63.58±15.34	45.62±18.23	P<0.0001***
α $_1$ACT (mg/dL)	1.9±0.41	1.2±0.35	P<0.0001***
Serum CRP, (mg/dL)	14.5±3.7	12.7±2.6	P<0.0001***

(* denotes the level of significant change between case and control)

Hardy Weinberg Equilibrium Test:

The genotype frequencies of each gene in each study group were tested to be in accordance with Hardy Weinberg equilibrium using chi squre ($\chi 2$) test for independence. When the calculated

value of $\chi 2$ was less than tabulated value of $\chi 2$ at degree of freedom 1 (d.f. = 1) and level of significance (P = 0.05), the population is at equilibrium for the gene and vice versa. The standard tabulated value of $\chi 2$ at degree of freedom 1 and level of significance 0.05 is 3.84. All the tabulated $\chi 2$ values for the genes were compared to this value. The genotype frequencies of all the study groups included in the study were in accordance with Hardy Weinberg equilibrium **(See Table No. 4.3).**

TABLE No-4.3

Hardy Weinberg Equilibrium Test for both Rheumatoid arthritis (RA) patients and Healthy control population.

Gene	*X^2 value for Case*	*X^2 value for control*
PTPN22	**2.975**	**1.679**

The X^2 value indicates the difference between expected and observed values for genotype counts.

Tabulated Value = 3.84

Detection of Genetic Polymorphism in PTPN22- C1855T gene:

Protein tyrosine phosphate, non-receptor type 22 (Lymphoid tyrosine phosphatase) is located in chromosome 1. The 1858C-T (Arg620Trp) single nucleotide polymorphism (rs2476601) was found associated with autoimmune diseases, including rheumatoid arthritis (RA). The PCR products 265bp was digested by restriction enzyme *XCm1*, generated two fragments of 169 bp and 96 bp fragments. CC a wild type genotype generate CT heterozygous and TT mutant genotype (depicted in figure no 1).

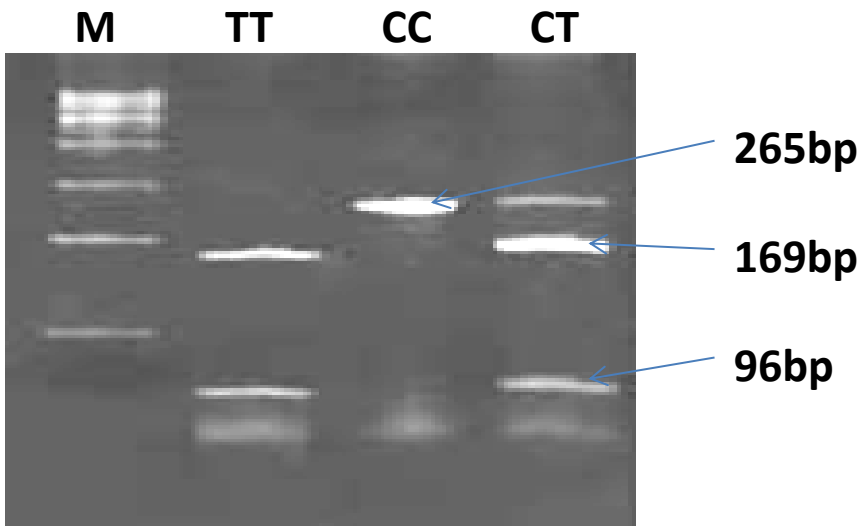


Figure No.-1: Representative gel picture of PTPN22 polymorphism. Lane M represents 50 bp molecular marker, Lane CC Wild type genotype, Lane CT heterozygous genotype and Lane TT variant genotype.

Overall distribution of PTPN22 genotypes was significantly different in Rheumatoid Arthritis and Healthy group (χ^2=13.09, P=0.0014**). Here significant differences showing between frequency of Healthy control group and disease group of 'CC' genotype (32.29% vs 55.35%). Similarly, heterozygous 'CT' and mutant homozygous '*TT*' genotype was also showing significant difference. An odds ratio of CC genotype (0.3846) indicates protective effect to disease. Distribution of Allele frequency between Rheumatoid arthritis (RA) group and healthy control was significantly different (χ^2=11.53, P=0.0007***) and associated with Arthritis. Thus carriage rate was also significant different (χ^2=4.493, P=0.0340*) between case and control group. Odds ratio of C allele (0.4848) indicate protective effect and found in high frequency at healthy group in compare to disease group (75.89% vs 60.41%). Thus carriage rate of C allele was also high in healthy group but T allele in low frequency **(See Table No. 4.8 and 4.9).**

TABLE No-4.8

Frequency distribution and association of Genotype, allele frequency and carriage rate of PTPN22 gene polymorphism in population of Vindhyan region using **Chi Square Test**

PTPN22 GENE	CASE N= 90		CONTROL N=112		CHI SQUARE VALUE χ^2 (P Value)
	N	%	N	%	
Genotype					
CC	31	32.29	62	55.35	
CT	54	56.25	46	41.07	13.09 (0.0014 **)
TT	11	11.45	04	03.57	
Allele					
C	116	60.41	170	75.89	
T	76	39.58	54	24.10	11.53 (0.0007***)
Carriage Rate					
C	85	56.66	108	68.35	
T	65	43.33	50	31.64	4.493 (0.0340*)

(- denotes the level of significant association between case and control.)*

(N – Number of individuals in study group.)

(% - Genotype allele frequency and carriage rate expressed in percentage.)

TABLE No-4.9

Fisher Exact Test values of PTPN22 gene polymorphism

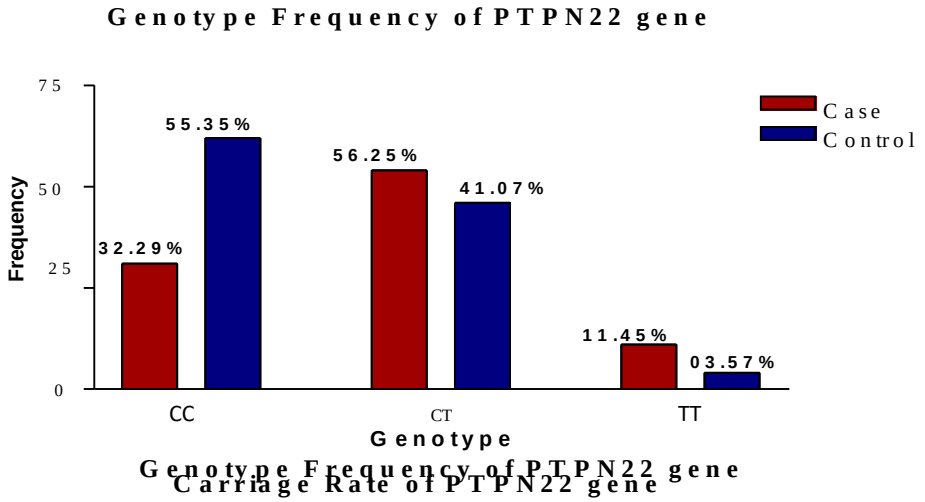
PTPN22 GENE	CASE N= 90		CONTROL N=112		P Value	ODDS RATIO AND CI
	N	%	N	%		

Genotype						
CC	31	32.29	62	55.35	0.0012**	0.3846 (0.2181 to 0.6784)
CT	54	56.25	46	41.07	0.0367*	1.845 (1.062 to 3.203)
TT	11	11.45	04	03.57	0.0334*	3.494 (1.074 to 11.36)
Allele						
C	116	60.41	170	75.89		0.4848 (0.3181 to 0.7388)
T	76	39.58	54	24.10	0.0010***	2.063 (1.353 to 3.143)
Carriage Rate						
C	85	56.66	108	68.35		0.6054 (0.3800 to 0.9644)
T	65	43.33	50	31.64	0.0449*	1.652 (1.037 to 2.631)

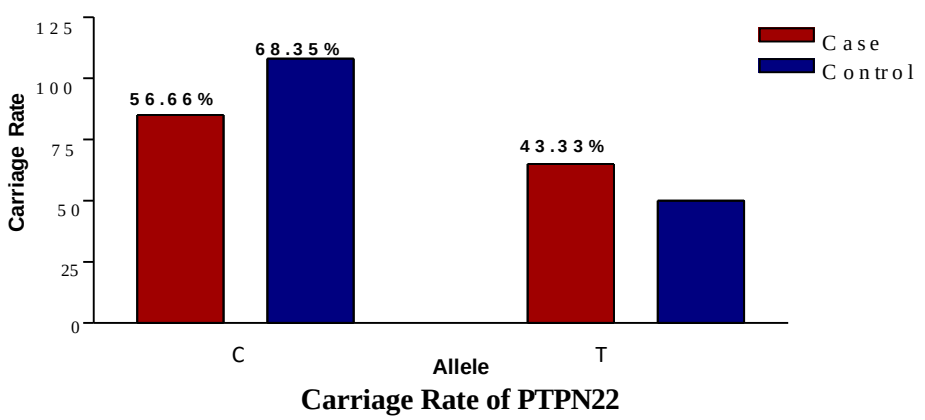
(- denotes the level of significant association between case and control.)*

(N – Number of individuals in study group.)

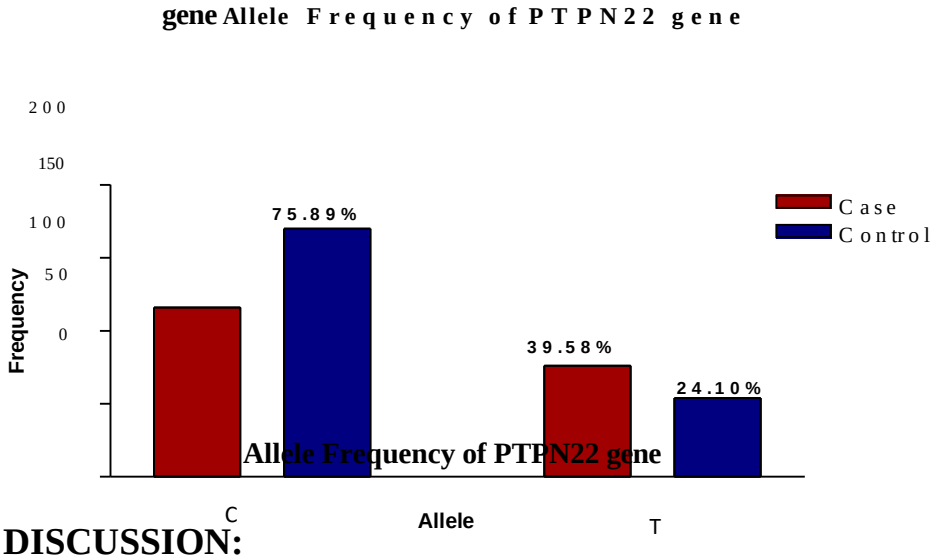
(% - Genotype allele frequency and carriage rate expressed in percentage.)



Genotype Frequency of PTPN22 gene



Carriage Rate of PTPN22 gene



DISCUSSION:

Rheumatoid arthritis (RA) is a chronic auto-inflammatory disease affecting connective tissue, characterized by progressive joint damage and specific systemic disorders. To date, the number of patients with autoimmune arthritis is almost 1% of the world's population, establishing an urgent problem for healthcare systems worldwide. An evident feature of RA is clinical polymorphism, represented by a wide variability of symptoms, clinical forms, and progression rates. RA is considered to be a multi-factorial disease triggered by a genetic predisposition and environmental factors. Both clinical and genealogical studies have shown that the disease can accumulate in families (**Lebre M, et. al. 2009).** This relationship has been confirmed by modern molecular genetics. The opportunity to identify RA risk groups in different populations, as well as the possible prognostic value of some genetic variants for disease development, progression, and treatment, including a personalized anti-rheumatic therapy response, has promoted new studies of germ line genetic variants in RA patients. Rheumatoid arthritis (RA) is the most common inflammatory arthropathy worldwide (**Eektimmerman, F.; et. al. 2020).**

Our data from clinical parameters revealed Biochemical test performed in the blood sample for following clinical parameters and the findings were tabulated. Statistical analysis was done by using student's t-test and p value suggests the level of significant were changed here. The descriptive data and comparison of biochemical parameters of Rheumatoid arthritis (RA) patients versus healthy controls are presented in Table no. 4.2. As expected the Rheumatoid arthritis (RA) patients had markedly higher levels of ESR (P<0.0001***), α $_1$ACT (P<0.0001***) and Serum CRP (P<0.0001***) compared to that of control subject. Thus P-Value of ESR (Erythrocyte sedimentation rate) , α $_1$ACT (Alpha 1 antichymotrypsin) and CRP (C-Reactive Protein) concentration revealed association significantly with Rheumatoid arthritis (RA) patients. Whereas rest of all parameters such as Post-Prandial Glucose (mg/Dl), HbA1C(%), HDL-C(mmol/L), LDL-C (mg/dL), TG(mg/dL), Systolic BP (mmHg), Diastolic BP (mmHg) and Blood Urea(mg/dL) were not significantly different between patient and healthy population. C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) can be considered as inflammation indicators in RA patients.

In addition, most of adipokines are involved in modulation of inflammation and thus might hypothetically play a role in RA pathogenesis8. Some studies have shown that adipokines including leptin, resistin, and visfatin may contribute to RA pathophysiology. Obesity and

increased body fat mass are associated with higher production of adipokines with pro-inflammatory characteristics. In this study, two other controversial adipokines, namely nesfatin-1 and asymmetric dimethylarginine (ADMA), were evaluated to reveal the possible relationships between them and RA activity.

Our statistical data from **PTPN22 gene polymorphism revealed that** Protein tyrosine phosphate, non-receptor type 22 (Lymphoid tyrosine phosphatase) is located in chromosome 1. The 1858C-T (Arg620Trp) single nucleotide polymorphism (rs2476601) was found associated with autoimmune diseases, including rheumatoid arthritis (RA). The PCR products 265bp was digested by restriction enzyme *XCm1* generated two fragments of 169 bp and 96 bp fragments. CC a wild type genotype generates CT heterozygous and TT mutant genotype. Overall distribution of PTPN22 genotypes was significantly different in Rheumatoid Arthritis (RA) and Healthy group (χ^2=13.09, P=0.0014**). Here significant differences showing between frequency of Healthy control group and disease group of 'CC' genotype (32.29% vs 55.35%). Similarly, heterozygous 'CT' and mutant homozygous '*TT*' genotype was also showing significant difference. An odds ratio of CC genotype (0.3846) indicates protective effect to disease. Distribution of Allele frequency between Rheumatoid arthritis (RA) group and healthy control was significantly different (χ^2=11.53, P=0.0007***) and associated with Arthitis. Thus carriage rate was also significant different (χ^2=4.493, P=0.0340*) between case and control group. Odds ratio of C allele (0.4848) indicate protective effect and found in high frequency at healthy group in compare to disease group (75.89% vs 60.41%). Thus carriage rate of C allele was also high in healthy group but T allele in low frequency.

CONCLUSIONS:

Though there are many immunological and clinical studies in India in Rheumatoid arthritis disease have been reported. undertook this study to evaluate the possible genetic polymorphism in the PTPN22 genes and to determine whether these polymorphisms can act as genetic marker for susceptibility to Rheumatoid arthritis from Central region of India.

The response to therapy is probably partially determined by the genetic makeup of the individual. As stated previously, RA is a disease defined by well accepted criteria [105], but its clinical features and the molecular pathways involved are heterogeneous [106]. Therefore, the response to different treatments varies considerably among individual patients. With the development of a variety of costly new drugs and a lack of complete information on side effects, such as susceptibility to infection, the need for genetic markers prognostic for treatment response is increasing. These genetic markers may lie in some of the genes described above, genes encoding the proteins involved in the drug target, drug metabolism or disease pathogenesis.

REFERENCES:

1. Smolen JS, Aletaha D, Barton A, et al. Rheumatoid arthritis. Nat Rev Dis Primers 2018;4:18001.

2. MacGregor AJ, Snieder H, Rigby AS, et al. Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. Arthritis Rheum 2000;43:30–7.
3. Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 2007;447:661–78.
4. Okada Y, Wu D, Trynka G, et al. Genetics of rheumatoid arthritis contributes to biology and drug discovery. Nature 2014;506:376–81.
5. Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis. an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 1987;30:1205–13.
6. International HapMap Consortium. The International HapMap Project. Nature 2003;426:789–96.
7. Ozaki K, Ohnishi Y, Iida A, et al. Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction. Nat Genet 2002;32:650–4.
8. Suzuki A, Yamada R, Chang X, et al. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nat Genet 2003;34:395–402.
9. Kochi Y, Yamada R, Suzuki A, et al. A functional variant in FCRL3, encoding Fc receptor-like 3, is associated with rheumatoid arthritis and several autoimmunities. Nat Genet 2005;37:478–85.
10. Plenge RM, Cotsapas C, Davies L, et al. Two independent alleles at 6q23 associated with risk of rheumatoid arthritis. Nat Genet 2007;39:1477–82.
11. Plenge RM, Seielstad M, Padyukov L, et al. TRAF1-C5 as a risk locus for rheumatoid arthritis--a genomewide study. N Engl J Med 2007;357:1199–209.
12. Remmers EF, Plenge RM, Lee AT, et al. STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. N Engl J Med 2007;357:977–86.
13. Plenge RM, Padyukov L, Remmers EF, et al. Replication of putative candidate-gene associations with rheumatoid arthritis in >4,000 samples from North America and Sweden: association of susceptibility with PTPN22, CTLA4, and PADI4. Am J Hum Genet 2005;77:1044–60.
14. Ikari K, Kuwahara M, Nakamura T, et al. Association between PADI4 and rheumatoid arthritis: a replication study. Arthritis Rheum 2005;52:3054–7.
15. Abecasis GR, Auton A, BrooksLD, et al. An integrated map of genetic variation from 1,092 human genomes. Nature 2012;491:56–65.
16. Li Y, Willer C, Sanna S, et al. Genotype imputation. Annu Rev Genomics Hum Genet 2009;10:387–406.
17. Stahl EA, Raychaudhuri S, Remmers EF, et al. Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. Nat Genet 2010;42:508–14.
18. Okada Y, Terao C, Ikari K, et al. Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population. Nat Genet 2012;44:511–6.
19. Eyre S, Bowes J, Diogo D, et al. High-density genetic mapping identifies new susceptibility loci for rheumatoid arthritis. Nat Genet 2012;44:1336–40.

20. Kochi Y, Thabet MM, Suzuki A, et al. PADI4 polymorphism predisposes male smokers to rheumatoid arthritis. Ann Rheum Dis 2011;70:512–5.
21. Kurreeman FA, Stahl EA, Okada Y, et al. Use of a multiethnic approach to identify rheumatoid- arthritis-susceptibility loci, 1p36 and 17q12. Am J Hum Genet 2012;90:524–32.
22. Kim K, Bang SY, Ikari K, et al. Association-heterogeneity mapping identifies an Asian-specific association of the GTF2I locus with rheumatoid arthritis. Sci Rep 2016;6:27563.
23. Okada Y, Muramatsu T, Suita N, et al. Significant impact of miRNA-target gene networks on genetics of human complex traits. Sci Rep 2016;6:22223.
24. Sakaue S, Hirata J, Maeda Y, et al. Integration of genetics and miRNA-target gene network identified disease biology implicated in tissue specificity. Nucleic Acids Res 2018.
25. Zhang Y, Qi G, Park JH, et al. Estimation of complex effect-size distributions using summary-level statistics from genome-wide association studies across 32 complex traits. Nat Genet 2018;50:1318–26.
26. Saxena R, Plenge RM, Bjonnes AC, et al. A multinational arab genome-wide association study identifies new genetic associations for rheumatoid arthritis. Arthritis Rheumatol 2017;69:976–85.
27. Danila MI, Laufer VA, Reynolds RJ. Dense Genotyping of Immune-Related Regions Identifies Loci for Rheumatoid Arthritis Risk and Damage in African Americans. Mol Med 2017;23:1–187.
28. Traylor M, Curtis C, Patel H, et al. Genetic and environmental risk factors for rheumatoid arthritis in a UK African ancestry population: the GENRA case-control study. Rheumatology 2017;56:1282–92.
29. López Herráez D, Martínez-Bueno M, Riba L, et al. Rheumatoid arthritis in Latin Americans enriched for Amerindian ancestry is associated with loci in chromosomes 1, 12, and 13, and the HLA class II region. Arthritis Rheum 2013;65:1457–67.
30. Pickrell JK, Berisa T, Liu JZ, et al. Detection and interpretation of shared genetic influences on 42 human traits. Nat Genet 2016;48:709–17.
31. Zhernakova A, Stahl EA, Trynka G, et al. Meta-analysis of genome-wide association studies in celiac disease and rheumatoid arthritis identifies fourteen non-HLA shared loci. PLoS Genet 2011;7:e1002004
32. Chatterjee N, Shi J, García-Closas M. Developing and evaluating polygenic risk prediction models for stratified disease prevention. Nat Rev Genet 2016;17:392–406.
33. Stahl EA, Wegmann D, Trynka G, et al. Bayesian inference analyses of the polygenic architecture of rheumatoid arthritis. Nat Genet 2012;44:483–9.
34. Finucane HK, Bulik-Sullivan B, Gusev A, et al. Partitioning heritability by functional annotation using genome-wide association summary statistics. Nat Genet 2015;47:1228–35.
35. Lee SH, Byrne EM, Hultman CM, et al. New data and an old puzzle: the negative association between schizophrenia and rheumatoid arthritis. Int J Epidemiol 2015;44:1706–21.
36. Bulik-Sullivan B, Finucane HK, Anttila V, et al. An atlas of genetic correlations across human diseases and traits. Nat Genet 2015;47:1236–41.

37. Oken RJ, Schulzer M. At issue: schizophrenia and rheumatoid arthritis: the negative association revisited. Schizophr Bull 1999;25:625–38.
38. van der Woude D, Houwing-Duistermaat JJ, Toes RE, et al. Quantitative heritability of anti-citrullinated protein antibody-positive and anti-citrullinated protein antibodynegative rheumatoid arthritis. Arthritis Rheum 2009;60:916–23.
39. Kochi Y, Yamada R, Kobayashi K, et al. Analysis of single-nucleotide polymorphisms in Japanese rheumatoid arthritis patients shows additional susceptibility markers besides the classic shared epitope susceptibility sequences. Arthritis Rheum 2004;50:63–71.
40. Padyukov L, Seielstad M, Ong RT, et al. A genome-wide association study suggests contrasting associations in ACPA-positive versus ACPA-negative rheumatoid arthritis. Ann Rheum Dis 2011;70:259–65.
41. The International HIV Controllers Study. The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. Science 2010;330:1551–7.
42. Jia X, Han B, Onengut-Gumuscu S, et al. Imputing amino acid polymorphisms in human leukocyte antigens. PLoS One 2013;8:e64683.
43. Raychaudhuri S, Sandor C, Stahl EA, et al. Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis. Nat Genet 2012;44:291–6.
44. Okada Y, Kim K, Han B, et al. Risk for ACPA-positive rheumatoid arthritis is driven by shared HLA amino acid polymorphisms in Asian and European populations. Hum Mol Genet 2014;23:6916–26.
45. Han B, Diogo D, Eyre S, et al. Fine mapping seronegative and seropositive rheumatoid arthritis to shared and distinct HLA alleles by adjusting for the effects of heterogeneity. Am J Hum Genet 2014;94:522–32.
46. Reynolds RJ, Ahmed AF, Danila MI, et al. HLA-DRB1-associated rheumatoid arthritis risk at multiple levels in African Americans: hierarchical classification systems, amino acid positions, and residues. Arthritis Rheumatol 2014;66:3274–82.
47. Okada Y, Suzuki A, Ikari K, et al. Contribution of a Non-classical HLA Gene, HLA-DOA, to the Risk of Rheumatoid Arthritis. Am J Hum Genet 2016;99:366–74.
48. Hirata J, Hosomichi K, Sakaue S. Genetic and phenotypic landscape of the MHC region in the Japanese population. Nat Genet In Press.
49. Bottini N, Musumeci L, Alonso A, et al. A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. Nat Genet 2004;36:337–8.
50. Bottini N, Vang T, Cucca F, et al. Role of PTPN22 in type 1 diabetes and other autoimmune diseases. Semin Immunol 2006;18:207–13.
51. Rieck M, Arechiga A, Onengut-Gumuscu S, et al. Genetic variation in PTPN22 corresponds to altered function of T and B lymphocytes. J Immunol 2007;179:4704–10.
52. Vang T, Congia M, Macis MD, et al. Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. Nat Genet 2005;37:1317–9.
53. Gregersen PK, Lee HS, Batliwalla F, et al. PTPN22: setting thresholds for autoimmunity. Semin Immunol 2006;18:214–23.

54. Vang T, Landskron J, Viken MK, et al. The autoimmune-predisposing variant of lymphoid tyrosine phosphatase favors T helper 1 responses. Hum Immunol 2013;74:574–85.
55. Wang Y, Ewart D, Crabtree JN, et al. PTPN22 Variant R620W Is Associated With Reduced Toll-like Receptor 7-Induced Type I Interferon in Systemic Lupus Erythematosus. Arthritis Rheumatol 2015;67:2403–14.
56. Chang HH, Dwivedi N, Nicholas AP, et al. The W620 polymorphism in PTPN22 disrupts its interaction with peptidylarginine deiminase type 4 and enhances citrullination and NETosis. Arthritis Rheumatol 2015;67:2323–34.
57. Dai X, James RG, Habib T, et al. A disease-associated PTPN22 variant promotes systemic autoimmunity in murine models. J Clin Invest 2013;123:2024–36.
58. Zhang J, Zahir N, Jiang Q, et al. The autoimmune disease-associated PTPN22 variant promotes calpain-mediated Lyp/Pep degradation associated with lymphocyte and dendritic cell hyperresponsiveness. Nat Genet 2011;43:902–7.
59. Hagberg N, Joelsson M, Leonard D, et al. The STAT4 SLE risk allele rs7574865[T] is associated with increased IL-12-induced IFN-γ production in T cells from patients with SLE. Ann Rheum Dis 2018;77:1070–7.
60. Kochi Y, Okada Y, Suzuki A, et al. A regulatory variant in CCR6 is associated with rheumatoid arthritis susceptibility. Nat Genet 2010;42:515–9.
61. Ishigaki K, Kochi Y, Suzuki A, et al. Polygenic burdens on cell-specific pathways underlie the risk of rheumatoid arthritis. Nat Genet 2017;49:1120–5.